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born in Istanbul, Turkey

oral examination: ……

Specifying molecular determinants of the subcellular targeting of synaptic and extrasynaptic $GABA_A$ receptors

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SUMMARY

How $GABA_A$ receptor subtypes segregate so precisely to different subcellular locations is not understood. In this study, I have expressed the recombinant □2 and □ $GABA_A$ receptor subunits in cultured hippocampal neurons to analyze the differential cell surface expression and sub-membrane segregation of synaptic and extrasynaptic $GABA_A$ receptors. My data demonstrate that the synaptic targeting of □2-containing $GABA_A$ receptors does not depend on the cytoplasmic TM3-TM4 domain of the □2 subunit. This result was actually surprising, until Alldred et al published their work last year (2005) showing that the synaptic localization of receptors with the □2 subunit requires the TM4 domain rather than the large TM3-TM4 cytoplasmic loop. The premise of our work, when we started, was that the loop region would be responsible. On the other hand, I showed here that the TM3-TM4 cytoplasmic domain of the □ subunit is either a factor for the extra-synaptic clustering of the □ containing $GABA_A$ receptors (this would be an active mechanism) or alternatively that this loop region may not contain any information at all on receptor targeting, and subunits with this domain may simply lack sufficient "information" to be placed in synapses (passive exclusion). I cannot currently distinguish between these two possibilities. Nevertheless, by comparing the □ subunit TM3-TM4 loop amino acid sequences across the whole span of vertebrate evolution, I discovered that the loop was remarkably conserved. This would suggest that the loop's tertiary structure and specific proteins that bind to it are important for some function(s) of □-containing receptors. A passive exclusion mechanism might have lead to a degeneration of the loop sequence.

ZUSAMMENFASSUNG

Wie $GABA_A$-Rezeptor-Untereinheiten so präzise an verschiedene subzelluläre Orte verbracht werden, ist nicht vollständig verstanden. In der vorliegenden Arbeit habe ich rekombinante □2- bzw. □-Untereinheiten des $GABA_A$-Rezeptors in kultivierten hippokampalen Neuronen exprimiert, um die unterschiedliche Expression an der Zelloberfläche und die Verteilung über verschiedene Membrankompartimente von synaptischen und extrasynaptischen $GABA_A$-Rezeptoren zu analysieren. Meine Ergebnisse zeigen, dass die synaptische Lokalisierung von $GABA_A$-Rezeptoren, die □2-Untereinheit enthalten, nicht von der zytoplasmatischen TM3-TM4 Domäne der □2-Untereinheit abhängt. Dieser Befund war in der Tat überraschend, bis Alldred et al. letztes Jahr (2005) publizierten, dass die synaptische Lokalisation des Rezeptors mit einer □2-Untereinheit eher von der TM4 Domäne abhängt als von der zytoplasmatischen TM3-TM4 Domäne. Unsere anfängliche Arbeitshypothese hingegen war, dass die zytoplasmatische Schleifenregion für die Lokalisation verantwortlich ist. Auf der anderen Seite konnte ich zeigen, dass die zytoplasmatische TM3-TM4 Domäne der □□Untereinheit eine Rolle für die extrasynaptische Lokalisation von $GABA_A$-Rezeptoren mit □-Untereinheit spielt (dies bedeutete einen aktiven Mechanismus) oder alternativ, dass diese Schleifenregion keine Information bezüglich der Lokalisation des Rezeptors enthält und Untereinheiten mit dieser Domäne mangels fehlender Information nicht in Synapsen lokalisiert werden (passiver Ausschluss). Welcher Mechanismus vorliegt, kann auf Grundlage der vorliegenden Daten nicht entschieden werden. Eine Analyse der Aminosäuresequenz der zytoplasmatischen TM3-TM4 Domäne der □□Untereinheit zeigt, dass diese über verschiedene Spezies hinweg hochgradig konserviert ist. Dies legt nahe, dass die Tertiärstruktur und spezifische Proteine, die mit dieser Schleifenregion interagieren, eine wichtige Rolle für die Funktion von $GABA_A$-Rezeptoren mit □-Untereinheit spielen. Ein passiver Ausschluss hingegen hätte wohl eine Degeneration der Schleifensequenz erlaubt.

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1 INTRODUCTION AND OVERVIEW

Brain information processing depends on appropriately integrating excitatory and inhibitory neural inputs as circuits (Fried et al., 2002; Schummers et al., 2002; Koch et al., 1983). Synapses are the specialized subcellular structures by which the majority of these signals between central neurons are transmitted via the "fast" transmitters glutamate, GABA, and glycine. For glutamatergic synapses, the postsynaptic neuron harbors an electron-dense meshwork of proteins that form a complex apparatus of sub-synaptic scaffold, which, based on its appearance in electronmicrographs (em), is called a postsynaptic density (PSD). The PSD provides the framework for the clusters of specific glutamate-gated neurotransmitter receptors, as well as for the voltage-gated ion channels and diverse second messenger signalling molecules concentrated at postsynaptic membranes precisely matching with the active zone of the presynaptic bouton, which contains the specialized assembly for the neurotransmitter release upon a depolarising action potential (reviewed by Waites et al., 2005). Unlike glutamatergic synapses, the potsynaptic areas of GABAergic synapses are not marked by any electron dense zones in em; thus GABAergic synapses are termed "symmetrical".

The formation, maturation and elimination of both GABAergic and glutamatergic synapses (synaptogenesis) is essential for the integrity of neural networks. Any alterations during this process can cause cognitive disorders (Zoghbi, 2003). Moreover, the molecular basis of learning and memory is believed to be synaptic plasticity, which requires bi-directional regulation of pre- and postsynaptic boutons both structurally and functionally (Chklovskii, 2004). A defining event during synaptogenesis is the formation of neurotransmitter receptor clusters in distinct domains of the postsynaptic membrane. Therefore clustering of neurotransmitter receptors on the postsynaptic membrnane arises as a theme in order to understand the molecular mechanisms governing the neural networks and synaptic plasticity. On the other hand, neurotransmitter receptors are not only clustered in the postsynaptic membrane. They also form nonsynaptic clusters

with diverse structural and functional properties in specific sub-membrane domains of dendritic tree, somatic and axonal segments, providing distinct signalling gateways. Moreover, diversity and dynamics of these gateways is a prominent factor that provides single neurons great computational power (Hausser et al., 2000). With this way, even simple types of input signals can processed for further refinement. For example, cerebellar granule cells, at their distal dendritic arbors, are innervated by Golgi cells, which are the only inhibitory input source. Yet these cells express at least two GABA receptor subtypes located in specific domains of the plasma membrane (Jones et al., 1997; Brickley et al., 2001). One of these receptor subtypes, the synaptic $GABA_A$ receptors, are massively clustered at the postsynaptic membrane, besides to other sub-membrane regions and responsible for the phasic inhibition which is the classical fast response to pre-synaptic GABA release. On the other hand another receptor subtype is only located at extrasynaptic sites. These extrasynaptic receptors have higher affinity for GABA and mediate a different form of GABAergic inhibition called tonic inhibition. Tonic inhibition sets the threshold for the action potential generation (Hausser and Clark, 1997) and shunts the excitatory synaptic inputs (Hamann, et al., 2002). To perform these tasks correctly, the synaptic and extrasynaptic receptors must be sorted, targeted and clustered precisely on the required membrane compartments. Therefore the formation and maintenance of these distinct clusters must be mediated by regulated trafficking of these receptors, a phenomenon not well understood. My thesis study concerns the clustering of $GABA_A$ receptors on the distinct subdomains of plasma membrane by focusing on two groups of $GABA_A$ receptor subtypes: γ2-containing $GABA_A$ receptors (γ2-$GABA_AR$) and δ-containing $GABA_A$ receptors (δ-$GABA_AR$).

1.1 $GABA_A$ receptors

$GABA_A$ receptors are GABA-gated anion channels responsible (together with ligand-gated glycine receptors) for most fast inhibitory synaptic transmission in the vertebrate central nervous system. $GABA_A$ receptors are permeable to HCO_3^-

and Cl^- ions; the permeability ratio of HCO_3^-/Cl^- is approximately 0.2 to 0.4 (reviewed in Kaila et al., 1997). HCO_3^- moves out of the cell causing a mild depolarization (the reversal potential for HCO_3^- is –12 mV). In mature neurons Cl^- usually moves into the cell overriding this mild depolarization, causing a strong inhibitory hyperpolarization as the Cl^- reversal potential is 15-20 mV more negative than the resting membrane potential. The Cl^- gradient is maintained by K-Cl co-transporters (Rivera et al., 2005). Depending on the intracellular Cl^- concentration, $GABA_A$ receptor activation can also lead to Cl^- efflux and depolarization. This is the case, for example, during embryonic and early postnatal development when K-Cl cotransporters are not expressed at sufficient levels to efficiently transport Cl^- out of the cell (Rivera et al., 2005). There are interesting caveats: adult dopaminergic neurons in the substantia nigra pars compacta have little KCC2 expression (Gulacsi et al., 2003), possibly explaining the relatively low efficacy of $GABA_A$ receptor-mediated inhibition in nigral dopaminergic neurons (Gulasci et al., 2003). Further, KCC2 expression can vary in subdomains of neurons, thus affecting local Cl^- gradients. KCC2 is absent from the axon initial segments of neocortical pyramidal cells (Szabadics et al., 2006). Thus GABAergic terminals arriving at this location may produce depolarization via $GABA_A$ receptors in this context. In hippocampal neurons the dendritic KCC2 channels can also be transiently inhibited by Ca^{2+} entry through voltage-gated Ca^{2+} channels, thus producing local changes in the dendritic Cl^- gradient, and affecting the efficacy of $GABA_A$ receptor inhibition and possibly inducing plasticity at GABAergic synapses (Fiumelli et al., 2005). This is a potential mechanism to bear in mind when considering GABAergic function for example in the basal ganglia.

1.1.1 $GABA_A$ receptors: genes

In mammals, $GABA_A$ receptors form as heteropentameric assemblies from a family of 19 subunits encoded by distinct genes (α1-α6, β1-β3, γ1-γ3, δ, ε, π, θ and ρ1-ρ3) (Korpi et al., 2002a; Rudolph and Mohler, 2006; Whiting, 2006). Depending on the subunit composition $GABA_A$ receptors differ in their

biophysical properties and affinity for GABA (see section 1.1.7 $GABA_A$ receptors: how subunit combinations affect synaptic and extrasynaptic transmission"), their pharmacology (see section 1.1.8 $GABA_A$ receptor agonists, antagonists and allosteric modulators) and location on the cell (see section 1.1.6 Extrasynaptic $GABA_A$ receptors: δ4$\beta\delta$ subtype). Along with the closely related glycine receptors, $GABA_A$ receptors were originally cloned by the classical *tour-de-force* method: peptide sequences obtained from purified (bovine brain) receptors were used to construct synthetic DNA probes to screen brain cDNA libraries (Schofield et al., 1987; Grenningloh et al., 1987). This was the starting point. Within a few years, this now historical technique of screening cDNA libraries had revealed most of the gene family, all the α1-6, β1-3, γ1-3 subunits and one δ subunit (Seeburg et al., 1990); over the remaining decade, a few more subunits, such as ε, θ and π were characterized (Davies et al., 1997; Hedblom and Kirkness, 1997; Bonnert et al., 1999; Sinkkonen et al., 2000). With the completion of the human genome database, Simon and colleagues did an *in silico* hybridization screen, searching for further undescribed mammalian $GABA_A$ receptor genes but found none. Most of the subunit gene family members are in clusters (Simon et al., 2004), suggesting gene and then cluster duplication during the evolutionary origin of vertebrates: α2, α6, α1, α2form a cluster in that order on human chromosome 5q34; the γ3, γ5, γ3 genes cluster in that order on human chromosome 15q13; the α1, α2, α4, α1 genes cluster in that order on chromosome 4p12; the ε, θ3, π genes in that order on X q28; the β1 and β2 genes, 40 Kb apart on 6 q15; the δ, β3 and ρ subunit genes are isolated on human chromosomes 5q35.1, 3q12.1 and 1p36.3 respectively (Simon et al., 2004). The complete genome data makes it an easy task to see the gene cluster organizations at a few keyboard strokes (http://www.ensembl.org/Homo_sapiens/index.html).

As determined by both *in situ* hybridization (mRNA localization) with gene-specific probes and immunocytochemistry (protein localization) with subunit-specific antibodies, the expression of the individual subunit genes is age- and region-specific (Wisden et al., 1992; Laurie et al., 1992a, b; Fritschy and Mohler, 1995; Schwarzer et al., 2001). Some $GABA_A$ receptor subunit genes have

extremely restricted expression patterns; the ρ6 subunit gene expresses only in cerebellar and cochlear nucleus granule cells (Lueddens et al., 1990) the ρ subunit genes are mainly expressed in retina, with low transcript levels in the hippocampus and colliculi – these receptors, because of their unique pharmacology, used to be termed "$GABA_C$"; the θ gene is expressed in non-neural tissues (Hedblom and Kirkness, 1997). The ε and π subunit genes are mainly transcribed in the locus ceruleus (the adrenergic nucleus in the brainstem), dorsal raphe (serotinergic cells) and cholinergic cells (Sinkkonen et al., 2000; Moragues et al., 2002).

The clustering of the $GABA_A$ receptor subunit genes raises the question of whether the clustered genes are co-regulated. The α1 and α2 genes do indeed share identical transcription patterns, nucleus for nucleus and even have the same RNA levels in each area (Wisden et al., 1992; Duncan et al., 1995); thus these two genes may share regulatory elements. All the other subunit genes have sometimes common, sometimes divergent expression patterns, with no correlation with which gene is in which cluster (Wisden et al 1992).

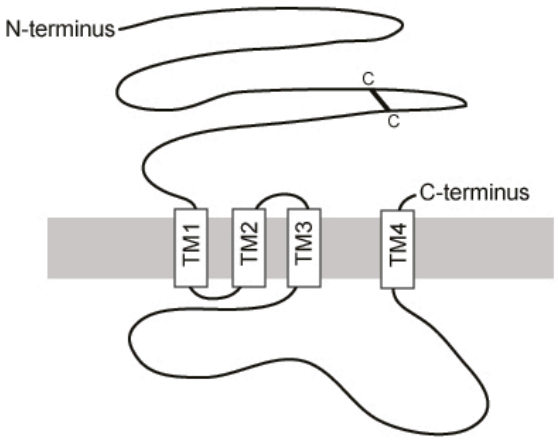


Figure 1.1: Predicted topology of a $GABA_A$ receptor subunit. The cysteine disulfide bridge in the N-terminus is indicated by a black bar. Transmembrane domains are shown as open boxes labelled TM1-4.

1.1.2 $GABA_A$ receptor structure

The $GABA_A$ receptor belongs to a superfamily of ligand-gated ion channels ("Cys-loop receptors") that in vertebrates includes the nicotinic acetylcholine receptors (nAChR), the 5-hydroxytryptamine type 3 (5-HT_3) receptors, the zinc-activated ion channel (ZAC) and the glycine receptors (GlyR) (reviewed in Cromer et al., 2002; Lester et al., 2004; Peters et al., 2005; Unwin, 2005; Sine & Engel, 2006) (see Figure 1.1 for a schematic showing one individual subunit situated in the membrane). In imagining how the $GABA_A$ receptor must look, I can do no better than quote Unwin 2005 for his empirical observations on the *Torpedo* nicotinic acetylcholine receptor: "The receptor (a large 290 kDa glycoprotein) is composed of elongated subunits, which associate with their long axes approximately normal to the membrane, creating a continuous wall around the central ion-conducting path. The whole assembly presents a rounded, nearly 5-fold symmetric assembly when viewed from the synaptic cleft, but is wedge-shaped when viewed parallel with the membrane plane. The subunits of the receptor all have a similar size 30 Å x 40Å

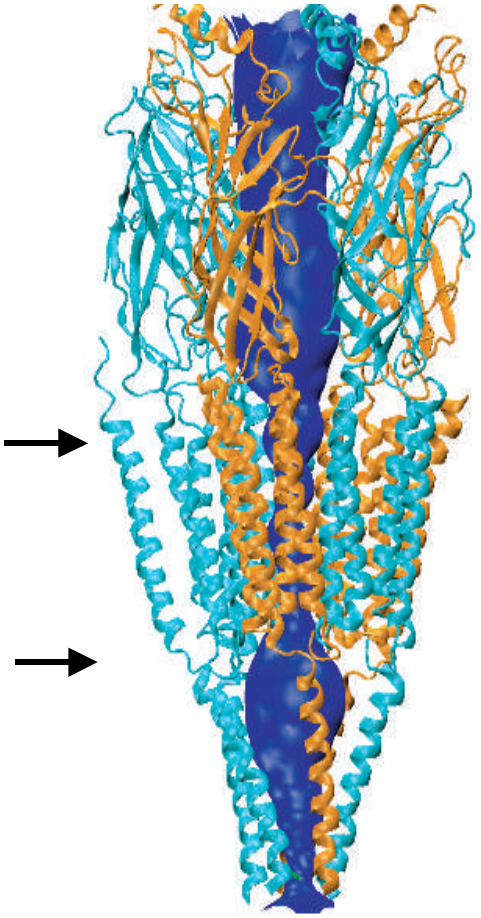


Figure 1.2: Structural model of the acetylcholine receptor from the Torpedo electric organ. Ribbon representation of the receptor was generated using coordinates deposited in the Protein Data Bank (code 2BG9), with the _-subunits in orange and non-_-subunits in pale blue. The outer surface of the central vestibule is shown in dark blue. Arrows indicate the limits of the cell membrane. In the membrane region, the radius of the vestibule is defined mainly by the M2 helices (re-produced from Sine and Engel, 2006).

x 160 Å and the same three-dimensional fold. Each subunit is a three-domain protein and so portions the channel naturally into its ligand-binding, membrane-spanning and intracellular parts" (Unwin, 2005; reviewed by Sine & Engel, 2006; Figure 1.2).

In $GABA_A$ receptors, the arrangement of subunits around the channel is probably □□□□□ counterclockwise when viewed from the extracellular space (Baumann et al., 2002). Current thinking is that for those cells in which they are expressed, □ and □ subunits can replace the □ and □ subunit within the pentamer, whereas the □ subunit might replace a □ subunit (Sieghart and Sperk, 2002). As for all members of the nicotinic receptor superfamily, all $GABA_A$ receptor subunits contain a large extracellular N-terminal domain of approximately 200 amino acids shaped by a cysteine disulfide bridge (the so-called "Cys-loop"). For $GABA_A$ subunits, the amino acid consensus sequence of the Cys-loop is C******F/YP*D***C*****S (where * is a degenerate residue; Simon et al 2004). Each subunit contains four predicted transmembrane spanning domains (TM1 to TM4) of about 20 amino acids and a large intracellular loop between TM3 and TM4 (TM3-TM4 loop) (Figure 1) (Macdonald and Haas, 2000). Many $GABA_A$ receptor subunits have the amino acid sequence (TTVLTMTT) in the TM2 domain (Seeburg et al., 1990). Five of these eight amino acids have been proposed to line the ion channel. TM1, TM3 and TM4 segregate TM2 from membrane lipid (Unwin, 2005). The amino acids specifying that the nicotinic receptors gate cations have been identified. The selectivity filter and gate lies at the intracellular end of the TM2 domains and includes part of the TM1–TM2 loop. Mutating these amino acids in the □7 subunit of homomeric nicotinic receptors produced acetycholine-gated anion channels (Galzi et al., 1992). The converse can also be done: mutation of five amino acids in the TM1–TM2 loop of the $GABA_A$ receptor □3 subunit to the corresponding amino acids of the □7 nicotinic acetylcholine subunit produces cation-selective $GABA_A$ receptors (Jensen et al., 2002). Similar mutations in the □2 or □2 subunits did not change ion selectivity. Thus the □ subunits predominantly determine the ion selectivity of the $GABA_A$ receptor (Jensen et al., 2002).

In the 5-HT_3 and nAChRs, residues in the TM3-TM4 loop region influence single channel conductance (Peters et al., 2005; Hales et al., 2006). The TM3-TM4 loop, which may be relatively unstructured in the nAChR (Kukhtina et al., 2005), contributes key sites for attaching anchor and regulatory proteins involved in locating the receptor at synapses and in governing the activity of $GABA_A$ receptors (Kittler and Moss, 2003) (see sections 1.1.5, "Synaptic $GABA_A$ receptors: □□□ subunit combinations and anchoring role of the □2 subunit and gephyrin" and 1.1.8.7 "Regulation of $GABA_A$ receptor function by neuromodulators: the role of kinases and phosphatases"). But the TM4 region of the □2 subunit is necessary and sufficient to confer a synaptic localization on the receptor (Alldred et al., 2005).

The atomic structure of a $GABA_A$ receptor subunit complex has not so far been solved directly. Instead, realistic models have used the empirically determined structural coordinates of the muscle nicotinic acetylcholine receptor from the electric organ of the *Torpedo* ray fish and a related snail acetylcholine receptor binding protein (AChBP) (Brejc et al., 2001; Cromer et al., 2002; Ernst et al., 2003; Unwin, 2003, 2005; Sine and Engel, 2006). The AChBP shows sequence similarity with the N-terminus of the nAChR at regions that build the agonist binding sites; the AChBP contains a Cys-loop but lacks the transmembrane domains. It assembles as soluble homopentamers (Brejc et al., 2001). The crystal structure of the AChBP, with bound ligand, provided a template for comparative modelling of the N-terminal extracellular domain of $GABA_A$ receptors (Ernst et al., 2003), whereas Unwin's most recent structure of the *Torpedo* nAChR at 4 Å resolution obtained by cryo-em, and incorporating insights from the AChBP, has given a full scale atomic model (Protein Data Bank Code 2BG9; see Figure 1.2). According to Xiu and colleagues, 2BG9 represents a substantial advance for the field, and all modern attempts to obtain molecular scale information on the structure and function of Cys-loop receptors must consider this as a starting point (Xiu et al., 2005; Unwin, 2005; Sine & Engel, 2006). 2BG9 provides us with a view of how the entire $GABA_A$ receptor must look, including the transmembrane and large cytoplasmic loops (Unwin, 2003, 2005).

Before 2BG9, modellers used family conservation patterns and fold predictions to estimated that 60-75% of the amino acid residues of the $GABA_A$ receptor subunits have structural equivalents in the AChBP template (Ernst et al., 2003). The accuracy of the $GABA_A$ receptor model will be limited in regions where alignment is unclear (e.g due to low sequence identity) or in regions where the AChBP differs from other family members due to its soluble, non-membrane-bound nature (Ernst et al., 2003). A model of the extracellular domain of a pentameric $GABA_A$ receptor consisting of two α, two β and one γ2 subunit is shown in (Figure 1.3). In this model the amino acids known to contribute to ligand binding sites and interfaces are correctly positioned and the interface-forming segments and the solvent accessibility of individual residues correlate well with experimental data (Ernst et al., 2003). Six "loops" (loop A, B, C for the plus side and D, E, F for the minus side) at the interface between neighbouring subunits form the ligand binding sites (Sigel and Buhr 1997; Olsen et al 2004). The binding pocket for GABA forms at the interface between the β and the α subunit (Figure 1.3a, c and d), the binding pocket for benzodiazepines lies at the interface of the α and the γsubunit (Figure 1.3a and b). The predicted space for agonist binding is formed by loops A, B, C, D and E (blue volume in Figure 1.3d) and correlates with experimental data from photolabeling of α1F64 by [^{3}H]muscimol and substituted cysteine accessibility mapping (Ernst et al., 2003; Olsen et al., 2004).Amino-acid residues on loops A, B, C, D and E at the interface of the α and the γsubunit influence binding, potency and efficacy of benzodiazepines (see section 1.1.8.1 $GABA_A$ receptors: allosteric modulation by benzodiazepines and related ligands) (Figure 1.3b) (Ernst et al., 2003). The predicted benzodiazepine pocket is larger than the GABA pocket. It communicates with the Cys-loop of the α subunit and extends down to the membrane-near part, which possibly contains side chains from the linker between transmembrane region 2 and 3 of the α subunit (Ernst et al., 2003).

1.1.3 $GABA_A$ receptor gating by agonist

As for all other ligand-gated channels, $GABA_A$ receptors convert chemical messages into electrical signals. In less than a millisecond, the binding of two (tiny) molecules of GABA between the β and α subunits induces a conformational change in the (giant) receptor oligomer that opens the central ion channel (see Baumann et al., 2003). This remarkable process is called "gating". In the opinion of Xiu and colleagues "the gating mechanism for the Cys-loop superfamily is one of the most challenging questions in molecular neuroscience". The full 2BG9 model of the nAChR suggests ways in which the agonist-binding site couples to the transmembrane region and initiates gating; principles applying to the nAChR are likely to apply, with minor variations, to other members of the superfamily (Unwin, 2005; Sine & Engel, 2006). The basis for a model of how gating works, is that specific ion pairs exert precise control over gating (Kash et al., 2003; Xiu et al., 2005). The membrane-near location of two flexible loops, loop 2 and loop 7 (the Cys-loop) in the crystal structure of the AChBP suggested an involvement in gating of the Cys-loop in the $GABA_A$ receptor. Indeed, Kash and colleges using an "ion pair model", found by site-directed mutagenesis in the β1 subunit that optimal gating needs electrostatic interactions between negatively charged residues in loops 2 and 7 (Asp57 and Asp 149) and a positively charged residue in the region linking transmembrane domains 2 and 3 (Lys 279) (Kash et al., 2003). For the β2 subunit of the $GABA_A$ receptor the interaction between an acidic residue in loop 7 (Asp 146) and a basic residue in pre-transmembrane domain-1 (Lys 215) helps couple agonist binding to channel gating (Kash et al., 2004). Studies on other members of the "Cys-loop" family found residues at corresponding regions in the nicotinic acetylcholine receptor and the serotonin 5-HT_3 receptor as critical coupling elements for gating (Lee and Sine, 2005: Lummis et al., 2005). Nevertheless, building on the results of Kash and colleagues in a detailed and broad examination of electrostatic interactions in the subunits, Xiu and colleagues concluded that no specific ion pair interaction in

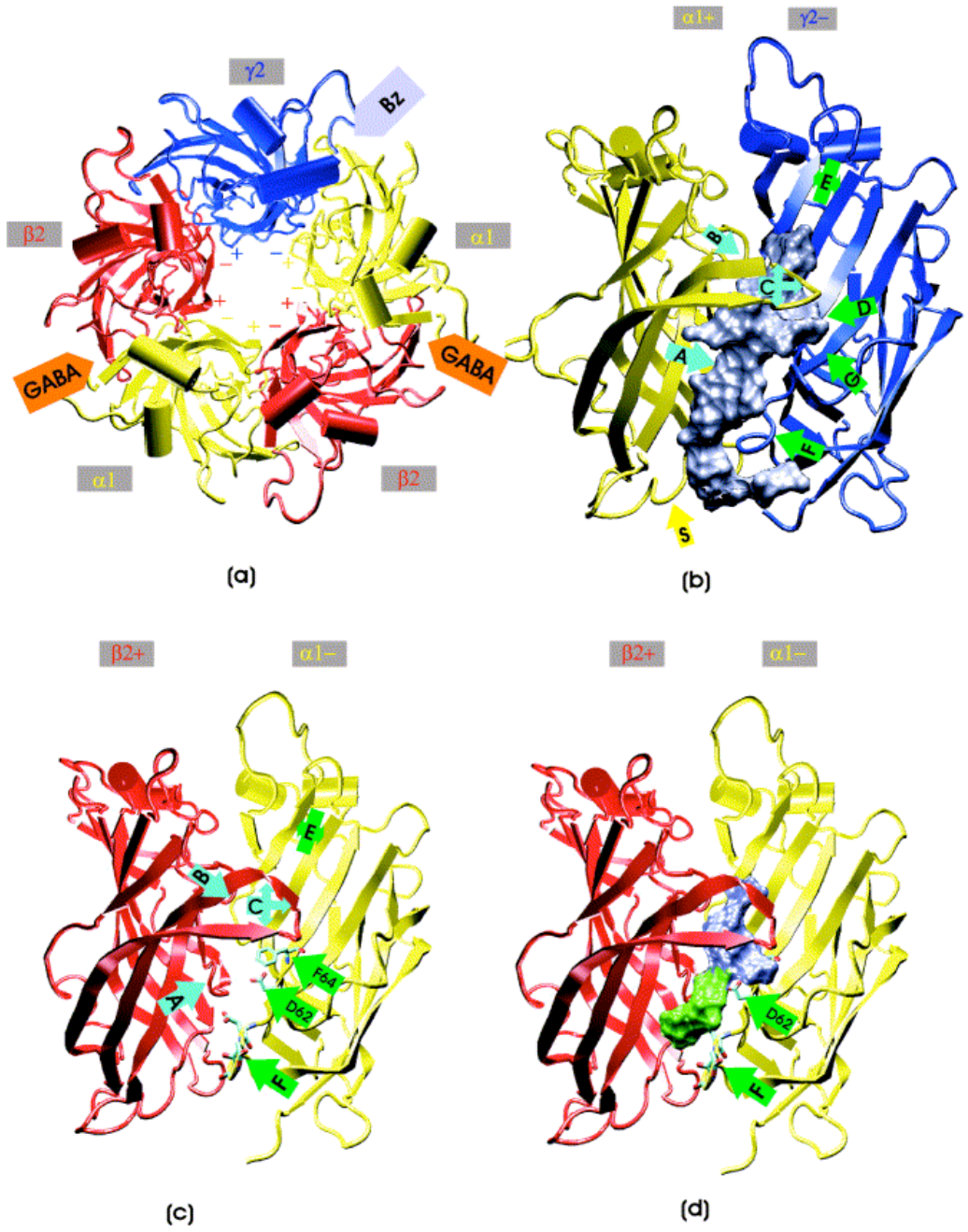


Figure 1.3: Model of the extracellular domains of a pentameric $GABA_A$ receptor consisting of 2 □, 2 □ and 1 □2 subunit. (a) View from the extracellular space. GABA binds to the interface between the □ and the □ subunit, benzodiazepines bind to the interface between the □ and the □2 subunit. (b) Predicted benzodiazepine-binding pocket between the □ and the □2 subunit, viewed from the side. The binding site loops are labelled A to G. (c) and (d) The □ and □ subunit viewed from the side. Loops A, B, C, D and E form the predicted GABA-binding pocket (blue volume in (d). (reproduced from Ernst et al., 2003).

fact influences gating, but instead a cluster of charges is important; specific ion pair interactions are nonessential, and it is misleading to focus only on specific residues: “Receptors have evolved to create a compatible collection of charged residues that allows the receptor to assemble and also facilitates the existence of and interconversions among multiple states” (Xiu et al., 2005).

1.1.4 Subunit assembly rules for $GABA_A$ receptors

The $GABA_A$ receptor subunit combinations found in brain are partly governed by which cell types express which genes (e.g. Wisden et al., 1992) and partly by preferential partnering of subunits within a given cell (e.g. Jones et al., 1997); for example, the α4 and α6 subunits assemble preferentially with the δ subunit (Jones et al., 1997; Peng et al., 2002).

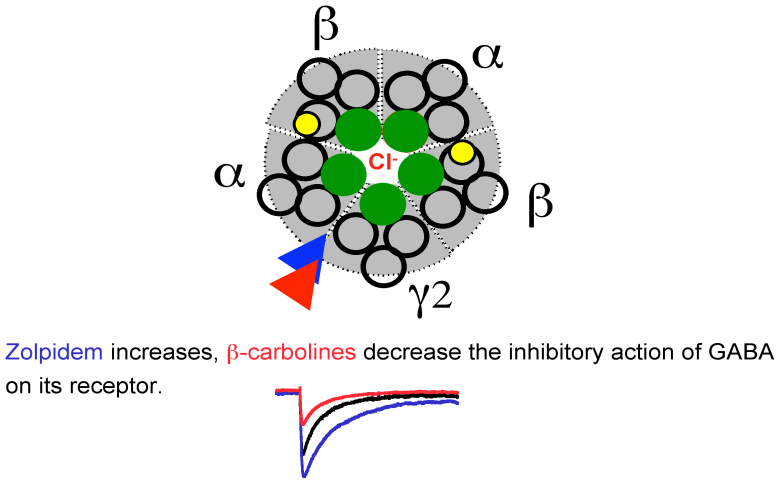


Figure 1.4: Molecular composition of most abundant $GABA_A$ receptors and some of pharmacologically active zones. In the brain most of the $GABA_A$ receptors are composed of 2α, 2β, and γ2 subunit. Each subunit has 4 transmembrane domains, where the internal channel pore for the passage of Cl^- ions is represented by green. 2 GABA molecules (yellow) bind on the β subunits at the interface with the a subunit and causes the channel pore to open. Zolpidem binding site (blue arrow) and β-carboline binding site (red arrow) is located in between the α and γ2 subunit. On the bottom of the figure, schematic representation for the effect of zolpidem (red line) and β-carboline (blue line) on the inhibitory action of GABA (black line) is shown.

The majority of mammalian brain $GABA_A$ receptors are probably $\alpha\beta\gamma$2 combinations. The subunit ratio is probably 2α/2β/1γ (Ernst et al., 2003) (Figure 1.4). Some receptors also contain different α and β subunits e.g. α1β2α2γ2 (Benke et al., 2004). According to Benke et al., 2004, who analysed whole mouse brain samples, the α1β1γ2 combination is the most abundant $GABA_A$ receptor subtype in the brain (61% of total). Other combinations were found in smaller quantities: α1β2 γ2 (13%), α1β3γ2 (15%), α2β2γ2 (12%), α2β3γ2 (2%), and α3β3γ2 (4%). Within the α1-containing receptor population most receptors are α1β1γ2, whereas in the α2- and α3-containing receptor populations, receptors with two different β subunit types predominate (Benke et al., 2004). Of course, these percentages are from homogenized brain; within particular cell types, some of these rare subtypes will be the most important receptor subtype.

1.1.4.1 Expression of $GABA_A$ receptor subunit genes in the vertebrate brain

$GABA_A$ receptor expression has been mapped by ligand autoradiography (e.g. Niddam et al., 1987; Faul and Villiger, 1988; Olsen et al., 1990; Duncan et al., 1995; Waldvogel et al., 1999; Korpi et al., 2002a), *in situ* hybridization with gene-specific probes (e.g. Wisden et al., 1992; Laurie et al., 1992; Persohn et al., 1992; Petri et al., 2002), single-cell PCR (e.g. Criswell et al., 1997; Guyon et al., 1999; Okada et al., 2004), and immunocytochemistry with subunit-specific antibodies at the light and electron microscopic level (e.g. Fritschy and Mohler, 1995; Somogyi et al., 1996; Pirker et al., 2000; Fujiyama et al., 2000, 2002; Schwarzer et al., 2001).

1.1.4.1.1 Autoradiography of $GABA_A$ receptors

Ligand-autoradiography, although usually lacking cellular resolution, serves as a highly useful indicator of which $\alpha\beta\gamma$2 subunit combination is present in a brain region (Korpi et al., 2002a), and is also a superbly quantitative technique (Korpi et al., 2002a). For example, the ligand [^{3}H]flunitrazepam is incubated with a

brain section in the absence or presence of the discriminating ligand CL218,872. If the binding signal is reduced or even completely vanishes by co-incubation with CL218, 872 (which has high affinity for □1□□2 receptors and so displaces [^{3}H]flunitrazepam), then this is a "BZI region". If CL218,872 fails to displace [^{3}H]flunitrazepam, then this is a "BZII region" (reviewed in Niddam et al., 1987). Thus BZI-type binding marks □1□□2 receptors; ^{3}H-zolpidem autoradiography on brain sections also selectively highlights □1□□2 receptors directly (Duncan et al., 1995; Korpi et al., 2002a). BZII-type binding marks □2, □3 and □5□□2 type receptors (the □4□□2 receptors are not picked up by this method, as they do not bind [^{3}H]flunitrazepam (see section 1.1.8.1 "$GABA_A$ receptors: allosteric modulation by benzodiazepines and related ligands"). The □□□□type receptors with □1 or □3 are not picked up by BZI and BZII screening, neither are □4□□-type receptors. However, many novel ligands are these days available, which could be used as autoradiographic probes.

Autoradiography with [^{3}H]muscimol (high-affinity site), commonly assumed to mark all $GABA_A$ receptors, selectively marks only □4□□ and □6□□ receptors; mouse brain sections with no □ subunit no longer give detectable [^{3}H]muscimol signals (Korpi et al., 2002b). Thus the [^{3}H]muscimol autoradiographic signals detected in many basal ganglia areas such as the striatum (Olsen et al., 1990; Jones et al., 1997; Korpi et al., 2002b;) will originate from □4□-containing receptors.

1.1.4.1.2 *In situ* hybridization and immunocytcohemistry of $GABA_A$ receptors

In situ hybridization is used to localize mRNA in brain regions or cell types. The radioactive version is a sensitive assay for which cell type expresses which gene; the use of digoxygenin-labelled probes is less sensitive but permits double labelling for multiple gene expression. There is good agreement between different labs on the results with $GABA_A$ receptor gene expression, probably because hybridization of nucleic acids is reasonably standardized between labs.

The disadvantage of *in situ* hybridization is that no information is obtained on protein localization on the cell, or even if the mRNA is translated. Thus immunocytochemistry with subunit-specific antibodies gives the ultimate biological information. However, antibodies can be problematic; Saper and Sawchenko (2003) point out that antibodies are biological agents, not standard chemical reagents: antibodies may bind to a wide variety of antigens other than the one that they were raised to recognize, and there is no way to be sure that the pattern they stain really represents that antigen. If investigating localization in the rodent nervous system, a knockout mouse for the particular antigen is the best control of antibody specificity (Saper and Sawchenko, 2003; Aller et al., 2005). This has been done for some (e.g. α1, α3, α6 and γ) (Jones et al., 1997; Tretter et al., 2001; Yee et al., 2005; Kralic et al., 2006), but not all $GABA_A$ receptor antibodies used in published papers.

1.1.5 Synaptic $GABA_A$ receptors: αβγ subunit combinations and anchoring role of the γ2 subunit and gephyrin

Placing $GABA_A$ receptors at synapses requires specific proteins that interact directly or indirectly with the γ subunits. For example, targeting some $GABA_A$ receptor subtypes to GABAergic terminals involves the widely expressed microtubule-binding protein gephyrin (Ramming et al., 2000). The best studied brain area for this has been the hippocampus, but splice forms of gephyrin are found throughout the basal ganglia (Ramming et al., 2000) and so the principles of $GABA_A$ receptor targeting would be expected to be similar there. In hippocampal neurons *in vitro* and *in vivo*, gephyrin either helps convey some $GABA_A$ receptor subtypes to the synapse or anchors them there – this requires the γ2 subunit (Kneussel et al., 1999; Brunig et al., 2002). Without the γ2 subunit no $GABA_A$ receptors are found in synapses in the developing or adult hippocampus (Gunther et al., 1995; Essrich et al., 1998; Schweizer et al., 2003), and without gephyrin much reduced numbers of some synaptic $GABA_A$ receptor subtypes, especially α2-containing, are found; some receptor clusters, especially those containing the α1 subunit, persist in hippocampal gephyrin knockout neurons

(Levi et al., 2004). Other γ subunits can replace synaptic targeting function of γ2; in γ2 knockout mice, $GABA_A$ receptors can be restored to hippocampal synapses by expressing the γ3 subunit by transgenic rescue (Baer et al., 1999; Luscher and Keller, 2004). Some conserved sequence identity in the large TM3-TM4 intracellular loops of the γ subunits may indicate binding sites for parts of the synapse-anchoring mechanism. Distributed cysteine residues are conserved in the γ subunit large intracellular loops, but are absent from the α, β and δ subunits. Palmitoylation of these cysteine residues via a thioester bond plays some role in targeting γ subunit-containing receptors to the synapse (Luscher and Keller, 2004); in cultured hippocampal neurons, cysteine-alanine substitutions in the γ2 subunit loop region interfere with expression and clustering of receptors (Rathenberg et al., 2004). A surprising finding is that the TM4 region of the γ2 subunit is also involved in synaptic targeting, possibly by interacting with lipid rafts occurring in the synapse or by other membrane proteins (Alldred et al., 2005). In transfected hippocampal cultures, analyses of chimeric γ2/α2 subunit constructs showed that γ2 TM4 is necessary and sufficient for postsynaptic clustering of $GABA_A$ receptors, whereas the cytoplasmic γ2 subunit domains are dispensable (Alldred et al., 2005). In contrast, both the TM3-TM4 loop and the TM4 domain of the γ2 subunit contribute to efficient recruitment of gephyrin to postsynaptic receptor clusters and are essential for restoration of miniature IPSCs (Alldred et al., 2005). Thus the γ2 subunit TM3-TM4 cytoplasmic loop might be needed for inserting receptors into the plasma membrane but is dispensable for delivery of receptors to subsynaptic dendritic sites (Alldred et al., 2005). Gephyrin does not bind the γ2 receptor subunit directly. The identity of the missing link(s) between gephyrin and $GABA_A$ receptor subunits is unknown. As mentioned above, the targeting of γ2-containing receptors to hippocampal synapses must depend on both the α subunit and the γ2 subunit. According to some investigators α5βγ2 receptors seem largely extrasynaptic and non-colocalized with gephyrin (Crestani et al., 2002), and when α6βγ2 receptors (normally only found in cerebellar granule cells) are ectopically expressed in pyramidal cells these receptors remain extrasynaptic (Wisden et al., 2002). So it

is not simply that a γ2 subunit (or even gephyrin) guarantees a stable synaptic placement of the $GABA_A$ receptor. In addition to gephyrin other clustering proteins must contribute to the synaptic localization of selected $GABA_A$ receptor subtypes (Kneussel et al., 2001).

1.1.5.1 $GABA_A$ receptor occupancy at synapses is dynamic

It is important to keep in mind that $GABA_A$ receptor expression on the surface of neurons is dynamic; receptors rapidly recycle, and leave from or insert into the synapse by rapid lateral diffusion and/or endo/exocytosis; a static crystalline scaffold of $GABA_A$ receptors anchored at the synapse would seem to be the

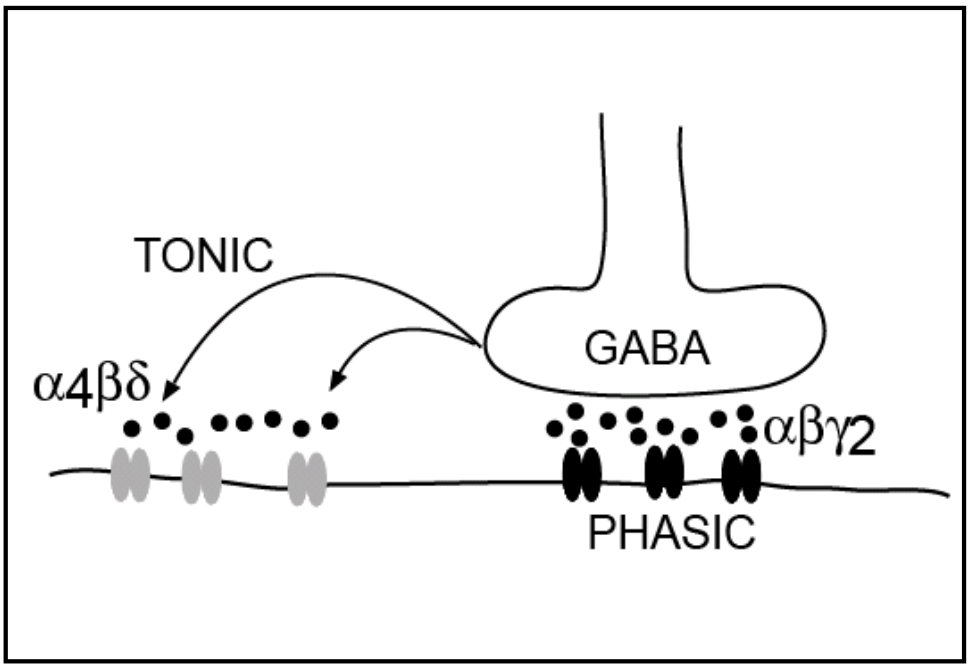


Figure 1.5: Phasic and tonic GABAergic inhibition. Fast synaptic (phasic) inhibition is mediated mainly via γ2 subunit-containing receptors (shown in black). δ subunit-containing receptors (shown in grey) are located peri- or extrasynaptically and are tonically activated by GABA diffusing out of the synaptic cleft.

wrong view (Kittler and Moss, 2003; Thomas et al., 2005); $GABA_A$ receptors diffuse into a synaptic zone and are transiently "captured" by the anchoring complex. However, for some inhibitory hippocampal synapses, a direct relationship exists between the number of synaptic $GABA_A$ receptors and the

strength of the synapse, but it is not clear what mechanisms maintain fixed numbers of $GABA_A$ receptors long-term at specific synapses (reviewed by Nusser, 1999).

As for glutamate receptors at excitatory synapses, neurons probably recycle $GABA_A$ receptors as a strategy for setting their degree of excitability (Kittler and Moss, 2003). $GABA_A$ receptors constitutively internalize by clathrin-dependent endocytosis; this requires interactions between the β and $\gamma 2$ subunits and the AP2 adaptin complex (Kittler and Moss, 2003).

1.1.6 Extrasynaptic $GABA_A$ receptors: $\alpha 4\beta\delta$ subtype

Besides mediating precisely timed synaptic point to point inhibition (phasic inhibition) via $\gamma 2$ subunit-containing receptors, $GABA_A$ receptors can also convey less time-locked signals. Low GABA concentrations in the extracellular space, resulting from GABA diffusing from the synapse, can tonically activate extrasynaptic $GABA_A$ receptors (Brickley et al., 2001; Staley and Scharfmann, 2005; Farrant and Nusser, 2005; see Figure 1.5).

This “tonic inhibition” is temporally uncoupled from the fast synaptic events, causing a continually present background inhibitory conductance. Such conductances alter the input resistance of the cell and thus influence synaptic efficacy and integration; tonic extrasynaptic conductances, by increasing the electrical leakiness of the dendritic membrane, substantially and indiscriminately diminish the size of excitatory signals in dendrites (reviewed by Farrant and Nusser, 2005; Staley and Scharfmann, 2005).

Receptors with the δ subunit, $\alpha 4\beta\delta$ in forebrain and $\alpha 6\beta\delta$ in cerebellar granule cells, are extrasynaptic; δ subunits are perisynaptic (annular), localized around the edge of synapses in hippocampal dentate granule cells, and totally extrasynaptic on cerebellar granule cells (Nusser et al., 1998; Wei et al., 2003; see Figures 1.6 and 1.7).

In all regions so far tested (cerebellar granule cells, hippocampal dentate granule

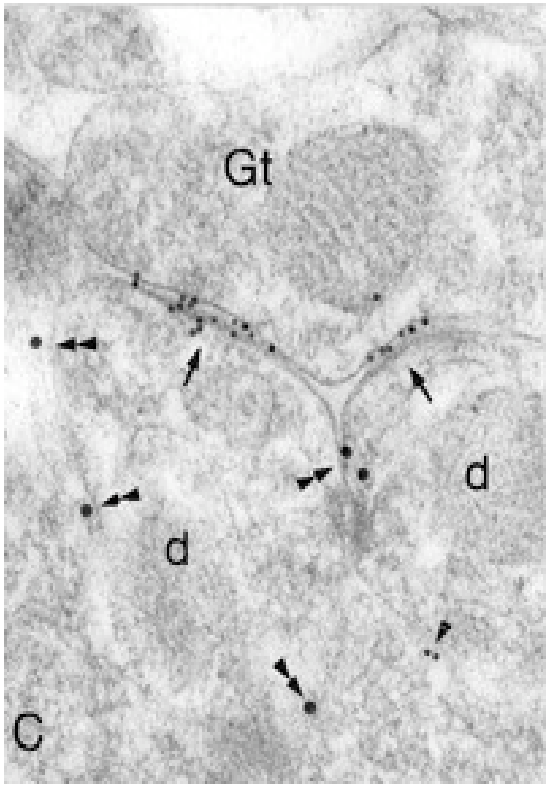


Figure 1.6: Immunogold electron microscopy of cerebellar granule cells to show extrasynaptic $GABA_AR$ subtypes (□-containing $GABA_ARs$). In the postsynaptic sites of these cells, where their dendrites (d) are innervated with Golgi cell terminals (Gt), □ subunit is located extrasynaptically as shown by large immunogold particles (double arrowheads). As a negative control □2 and □3 subunits are labeled by small immunogold particles located synaptically (black arrows). (Nusser et al., 1998).

cells, thalamic relay nuclei), □ subunits contribute to $GABA_A$ receptors that provide an extrasynaptic tonic conductance (Brickley et al., 2001; Stell et al., 2003; Cope et al., 2005). For $GABA_A$ receptors containing □4□□ subunits in the basal ganglia, for example in the striatum, it is predicted that the receptors are extrasynaptic and that their key properties are high affinity for neurotransmitter and limited desensitization, enabling them to contribute to tonic background conductances (see above) (Brickley et al., 2001; Semyanov et al., 2004).

1.1.7 $GABA_A$ receptors: how subunit combinations affect synaptic and extrasynaptic transmission

Many factors will influence the type of inhibitory postsynaptic currents (IPSCs) mediated by $GABA_A$ receptors: the number of $GABA_A$ receptors at the synapse; the subunit composition of the receptors which influences the kinetics; the

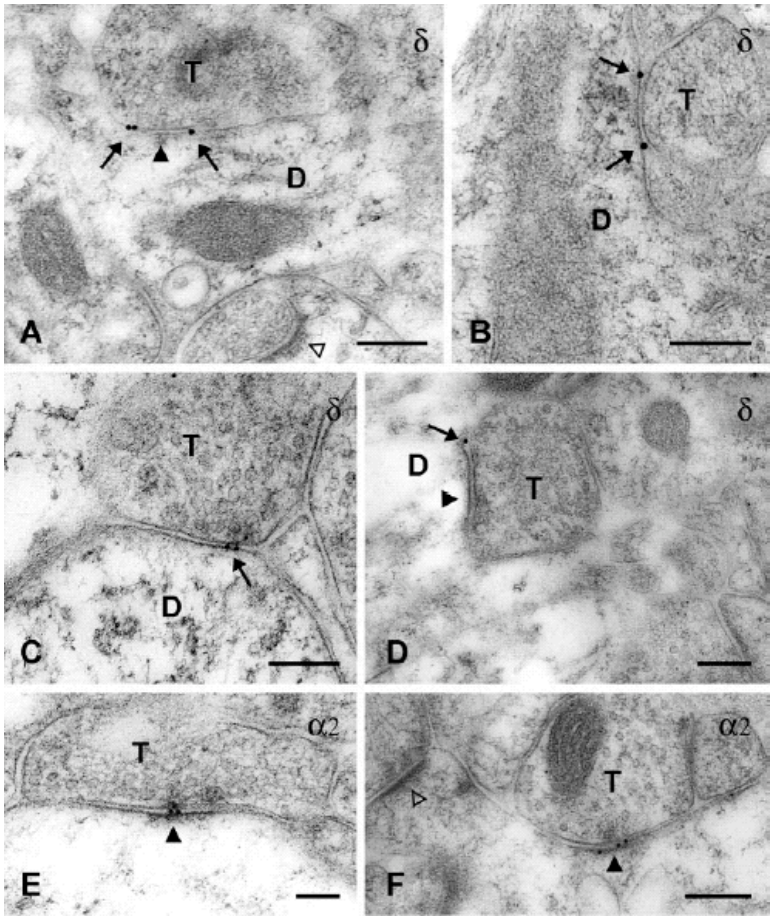


Figure 1.7: Perisynaptic localization of subunit containing $GABA_A$Rs shown by electron microscopy in the mouse dentate gyrus granule cells. Immunogold labeling of the and 2 subunits of the $GABA_A$ receptor. Gold particles indicate the location of the subunit (A–D, arrows) were present on or near the plasma membrane of dendrites (D) that were in contact with axon terminals (T). Gold particles were localized primarily at perisynaptic sites, just outside or at the outer edge of symmetric synapses (A, D, solid arrowheads). In contrast, immunogold labeling for the 2 subunit was observed directly at symmetric synapses (E, F, solid arrowheads). No labeling for either the or 2 subunit was observed at asymmetric synapses on spines (A, F, open arrowheads). Scale bars, 0.2 m. (reproduced from Wei, et al., 2003).

phosphorylation state of the receptor; a differential modulation of synaptic and non-synaptic receptors; the Cl^- reversal potential; the GABA transient in the synaptic cleft; a presynaptic regulation by neuromodulators (Mody and Pearce, 2004). A typical synaptic pulse of GABA is often cited as 0.3 to 1.0 mM lasting for less than 1 ms (Mody and Pearce, 2004). Under these conditions, all synaptic $GABA_A$ receptors will be saturated and give maximal responses. Nevertheless, receptor subunit composition affects the single channel conductance, how fast the receptors gate, how fast they switch off (deactivate and desensitize), how they respond to allosteric modulators. GABA also diffuses out of the synaptic cleft, where μM GABA concentrations are typically present (Nusser and Farrant, 2005); at these concentrations the GABA sensitivity of the receptor is critical. The sensitivity of α1β3 and α1β3γ containing receptors is significantly higher (mean EC_{50} of approximately 2 μM and 3.5 μM, respectively) than that of α1β3γ2 containing receptors (mean EC_{50} of approximately 13 μM) (Fisher and Macdonald, 1997). The single channel conductance of recombinant αβγ2 or αβγ receptors lies in the range of 25-30 pS. The single channel conductance of αβ-heterodimeric channels is 11-15 pS (Fisher and Macdonald, 1997).

The transient kinetic properties of $GABA_A$ receptors depend on the subunit composition. The time course of the $GABA_A$ receptor current is governed by three different kinetic processes: activation, desensitization and deactivation. During activation the current shows a rapid rise to the maximum. During this time the agonist binds to the receptor and the channel opens. Desensitization describes the unresponsiveness of the receptor and current decline in the continued presence of agonist. Deactivation describes the current decline after removal of the agonist. Current activation for recombinant α1β3 and α1β3δ receptors is slower than for α1β3γ2 receptors, with mean 10% to 90% rise times varying from 1.7 and 2.4 ms for α1β3 and α1β3δ receptors and only 0.5 ms for α1β3γ2 receptors (Haas and Macdonald, 1999). The γ subunit also influences the activation rate. Recombinant α2β1γ2 receptors have a more rapid activation (10% to 90% rise time of 0.5 ms) than α1β1γ2 receptors (10% to 90% rise time of 1 ms) (Lavoie et al., 1997). Desensitization is also influenced by the subunit

composition. Currents of recombinant $\alpha1\beta3$ and $\alpha1\beta3\gamma2$ receptors desensitize quicker and more completely than currents of $\alpha1\beta3\delta$ receptors (Haas and Macdonald, 1999; see.Figure 1.8).

Thus the δ subunit reduces speed and extent of receptor desensitization (Figure 1.8). As receptors with the δ subunit are primarily extrasynaptically and perisynaptically located (Nusser et al., 1998; Wei et al., 2003), their limited desensitization and high sensitivity to GABA might be important for their roles in tonic background conductance (see below). $\alpha1\beta3\gamma2$ currents deactivate more slowly than $\alpha1\beta3$ or $\alpha1\beta3\delta$ currents, mainly due to a significantly longer slow decay component (Haas and Macdonald, 1999). Again also the γ subunit composition influences the kinetics. Deactivation of $\alpha2\alpha1\gamma2$ containing receptors is six to seven times slower than deactivation of $\alpha1\alpha1\gamma2$ receptors (Lavoie et al., 1997).

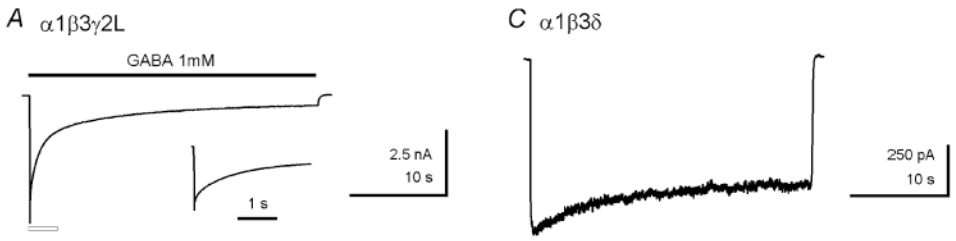


Figure 1.8: Electrophysiology of synaptic and extrasynaptic $GABA_A$Rs. δ containing receptors (C) do not desensitize in the presence of 1mM GABA fibroblast cells transfected and patch clamped, where as $\gamma2$ containing ones (A) respond with quickly desensitizing hyperpolarizing currents (reproduced from Haas and Macdonald, 1999).

1.1.8 $GABA_A$ receptor agonists, antagonists and allosteric modulators

$GABA_A$ receptors display a rich pharmacology (Korpi et al., 2002a; Sieghart and Sperk, 2002; Rudolph and Mohler, 2006; Whiting 2006). Generic $GABA_A$ receptors are selectively activated by the GABA agonist muscimol, and blocked competitively by the GABA antagonists bicuculline and SR95531 (receptors assembled with ρ subunits are bicuculline- and barbiturate-insensitive, having their own unique pharmacology). Picrotoxin blocks $GABA_A$ receptors non-competitively, probably by binding to a site in the channel (Korpi et al., 2002a). Many drugs bind at sites on the $GABA_A$ receptor distinct from the GABA binding site; these drugs change the shape of the receptor oligomer so that the efficacy of GABA at opening the channel is either increased (positive allosteric agonists, e.g. diazepam) or decreased (negative allosteric agonists, e.g. the β-carboline DMCM). A few allosteric modulators occur naturally in the brain (e.g. Zn^{2+}, neurosteroids). Generally, positive allosteric agonists are used widely in medicine (e.g. for the induction and maintenance of general anesthesia or to treat anxiety disorders, states of agitation, epilepsy or sleep disorders) and there is scope to develop further these drugs to produce receptor subtype-selective drugs with fewer side-effects (Whiting, 2006; Rudolph and Mohler, 2006); however, negative allosteric agonists also have potential clinical applications; for example, the drug L-655 708 works selectively at α5$\beta\gamma$2 receptors (a subtype mainly expressed in the hippocampus), and by decreasing GABA's action there it acts as a cognition enhancer (Rudolph and Mohler, 2006). A feature of all allosteric modulators is that they usually only work when GABA is at submaximal activating concentrations (below 1 mM), and they do not work in the absence of GABA (with the exception of some intravenous anesthetics). Nevertheless, some modulators (e.g. benzodiazepines) also strongly influence the deactivation rate of the receptors even at peak synaptic GABA concentrations, and this maybe how some of their in vivo effects originate (Mellor and Randall, 1997). In the following sections, I briefly consider the drugs that could act on $GABA_A$ receptor subunit combinations relevant for the basal ganglia (e.g. α1β2γ2, α2β2/3γ2, α4$\beta\gamma$2, α4$\beta\delta$)

1.1.8.1 $GABA_A$ receptors: allosteric modulation by benzodiazepines and related ligands

The main effects of benzodiazepines are sedation, anxiolysis, suppression of seizures and muscle relaxation. These drugs require □□□2-type receptors (e.g. □1□2□2 or □2□2□2 or □3□3□2) with the drug-binding site located between the □ and □2 subunits (Ernst et al., 2003). Note □4□□2-type receptors, which could potentially form in some nuclei of the basal ganglia, are insensitive to most BZ drugs, as are any receptors that contain the □ subunit. The substances that act at the benzodiazepine-binding site include the classical benzodiazepines like diazepam or flunitrazepam as well as chemically different substances like the imidazopyridine zolpidem (relatively selective for □1□□2-type receptors). Depending on the ligand the benzodiazepine site can mediate different effects. Benzodiazepine antagonists like flumazenil (Ro 15-1788) inhibit the effects of both agonists (positive allosteric modulators) and inverse agonists (negative allosteric modulators). In clinics, flumazenil is used in cases of benzodiazepine intoxication.

The most abundant receptor subtype in the brain, □1□2/□2 or □1□3□2, corresponds to the pharmacologically defined BZI site with high affinity for flumazenil (Ro 15-1788), Ro 15-4513 and flunitrazepam and selective affinity for ligands like zolpidem (Niddam et al., 1987; Pritchett et al., 1989). Receptor subtypes containing the □2 or □3 subunit along with □ and □2 correspond to the BZII site with high affinity for flumazenil, Ro 15-4513 and flunitrazepam but lower affinity to zolpidem (Pritchett et al., 1989; Hadingham et al., 1993). (see also the section 1.1.4.1.1 “Autoradiography of $GABA_A$ receptors”). The type of □ subunit has no effect on benzodiazepine pharmacology.

The BZ site is situated at the interface between the □ and the □ subunit (see above; Figures 1.3 and 1.4) (Ernst et al., 2003; Ogris et al., 2004). In the □1, □2, □3 and □5 subunits a mutation from histidine to arginine at position 101

abolishes binding of classic agonists like diazepam (Wieland et al., 1992; Korpi et al., 2002a). The diazepam-insensitive α4 (or α6) subunits naturally contain an arginine residue at the homologous position, and so α4β2γ2 or α6β2γ2 receptors are insensitive to most BZ ligands (Lueddens et al., 1990; Wisden et al., 1991; Korpi et al., 2002a). In the γ2 subunit a replacement of phenylalanine by isoleucine at position 77 abolishes binding of zolpidem, DMCM and flumazenil whereas flunitrazepam still shows high affinity binding (Buhr et al., 1997; Wingrove at al., 1997; Cope et al., 2004; Ogris et al., 2004). Methionine at position 130 in the γ2 subunit is required for high affinity binding of flunitrazepam but not flumazenil (Ro 15-1788) (Wingrove at al., 1997). The residues are distributed over the N-terminal domains of the α and γsubunits. In the assembled receptor the residues that form the BZ binding pocket are brought into close physical proximity by the so-called binding site "loops" (see above). It is not clear however, whether each of the above mentioned amino acid residues really participates in the lining of the BZ binding site or whether the inserted mutations have allosteric effects.

1.1.8.2 $GABA_A$ receptors: allosteric modulation by intravenous anesthetics

At clinically relevant concentrations general anesthetics modulate the activity of various ion channels (Krasowski and Harrison, 1999; Thompson and Wafford, 2001). Whereas volatile anesthetics (e.g. halothane, enflurane or isoflurane) are positive modulators of recombinant $GABA_A$ receptors, the main targets of these drugs *in vivo* are probably two pore domain (K2P) potassium channels (Franks and Honore, 2004). The intravenous anesthetics (e.g. barbiturates, steroidal anesthetics, propofol and etomidate) can modulate GABA's action at the receptor but can also activate the receptor directly in the absence of GABA at higher concentrations (Korpi et al., 2002a). Based on the analysis of knock-in mouse lines with propofol- and etomidate-insenstive β subunits (see below), propofol and etomidate exert nearly all of their anesthetic actions entirely through $GABA_A$ receptors (Rudolph and Mohler, 2004).

The action of etomidate and propofol absolutely requires residues in TM2 and TM3 in the β2 or β3 subunits (Jurd et al., 2003). A mutation of asparagine to methionine at position 265 (N265M) in the 2nd transmembrane domain of the β3 subunit abolishes the modulatory and direct effects of etomidate and propofol in recombinant receptors (Jurd et al., 2003). A mutation of aspargine at the same position in the β2 subunit also abolishes the action of etomidate on the $GABA_A$ receptor (Reynolds et al., 2003). In β3(N265M) mice propofol and etomidate do not suppress noxious-evoked movements and show a strongly decreased duration of the loss of righting reflex, two different endpoints of anesthesia. These results suggest that propofol and etomidate act mainly via the $GABA_A$ receptor and the β3 subunit in particular to induce deep anesthesia. The remaining effects of propofol and etomidate could be mediated by β2 subunit-containing receptors. Studies on β2(N265S) mice suggested that the β2 subunit mediates the sedative effects of etomidate whereas the β3 subunit is required for etomidate to induce a loss of consciousness (Reynolds et al., 2003). A highly interesting issue is the location in the brain where etomidate and propofol exert their anesthetic effects. Is the modulation of $GABA_A$ receptors in specific nuclei required to induce anesthesisa or do these drugs produce global effects at many $GABA_A$ receptors in all brain circuits? In any case, as $GABA_A$ receptors with both β2 and β3 are found throughout the basal ganglia, the operation of these nuclei will be profoundly affected by propofol and etomidate.

1.1.8.3 $GABA_A$ receptors: allosteric modulation by neurosteroids

Neuroactive steroids modulate $GABA_A$ receptor function in many brain regions (Belelli and Lambert, 2005; Farrant and Nusser, 2005). Naturally occurring steroid metabolites form locally in the brain: 5α-reductase transforms progesterone to 5αDPH, which in turn is reduced by 3α-hydroxysteroid oxidoreductase to allopregnanalone. Allopregnanalone potently activates $GABA_A$ receptors. No absolute specificity of neurosteroids for particular $GABA_A$ receptor subunit combinations exits. Many $GABA_A$ receptors are sensitive to the steroid THDOC, but receptors with the δ subunit are particularly sensitive: 30nM

THDOC enhances the peak currents of □1□3□ $GABA_A$ receptors (with 1 □M GABA) by up to 800%; other receptor isoform currents e.g. from □1□3□2 are enhanced to a smaller degree (5-50%) (Mihalek et al., 1999; Wohlfarth et al., 2002; Stell et al., 2003). Thus endogenous allopregnanolone may act on extrasynaptic □□□$GABA_A$ receptors to increase basal levels of inhibition. Mice without functional □ subunits have decreased sensitivity to the sedative/hypnotic, anxiolytic and pro-absence effects of neuroactive steroids (Mihalek et al., 1999).

1.1.8.4 $GABA_A$ receptors: allosteric modulation by Zn^{2+}

Zn^{2+} inhibits $GABA_A$ receptors (Hosie et al., 2003). In various brain regions, Zn^{2+} is synaptically released together with other neurotransmitters, both GABA and glutamate (reviewed by Mathie et al., 2006). Hippocampal mossy fibres have actually been the main area where synaptic Zn^{2+} actions have been investigated, but Zn^{2+} is worth bearing in mind as a *potential* modulator of GABAergic function in the basal ganglia. Zn^{2+} can reduce the amplitude, slow the rise time, and accelerate the decay of mIPSCs. On recombinant $GABA_A$ receptors, Zn^{2+} has an inhibitory potency 3400 times higher on □□ receptors than on □□□2 receptors (reviewed in Hosie et al., 2003). Thus the □2 subunit lowers the sensitivity of the $GABA_A$ receptor complex to Zn^{2+}. Hosie et al hypothesize that in addition to its role in promoting synaptic targeting and single channel conductance, the □2 subunit evolved to retain the fidelity of GABAergic inhibition in the presence of Zn^{2+} (Hosie et al., 2003). Nevertheless, Zn^{2+}'s potency is also □ subunit- and □ subunit-dependent. The IC_{50} for Zn^{2+} inhibition of □4□3□ and □4□3□2 receptors is similar (2 □M).

1.1.8.5 Extrasynaptic δ subunit-containing $GABA_A$ receptors: allosteric potentiation by ethanol

Blood alcohol levels of 1-3 mM can result from drinking half a glass of wine or less. Ethanol influences many channels, including the NMDA glutamate receptor (Hanchar et al., 2005). But amongst $GABA_A$ receptor subunit combinations, low concentrations of ethanol (about 3 mM, a concentration six times lower than the legal blood-alcohol limit for driving in most States in the USA) specifically potentiate GABA responses of cloned α4β δ and α6β δ receptors expressed in *Xenopus* oocytes (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003; Hanchar et al., 2004, 2005). This effect is δ subunit-dependent; δ3 subunits provide maximal sensitivity to ethanol (Wallner et al., 2003). Thus a glass of wine might, via α4β3δ and α6β δ receptors, enhance GABAergic tonic (extrasynaptic) inhibition in the striatum and cerebellum respectively (Hanchar et al., 2005). On the other hand, the ethanol sensitivity of δ6/δ KO mice is not different from wild-type mice (Korpi et al., 1999).

1.1.8.6 New subtype-selective drugs for $GABA_A$ receptors

$GABA_A$ receptors have always been fertile ground for drug companies. BZs, although for many years the main stay of clinical treatments for anxiety disorders, fell out of favour to selective serotonin reuptake inhibitors (SSRIs) due to side effects like sedation, cognitive impairment and abuse liability. But SSRIs are too slow acting for some situations, requiring several weeks to work. Thus there is a medical need for fast acting anxiolytics with few/no side effects (Whiting, 2006). To dissociate the wanted anxiolytic effects from the unwanted side effects of $GABA_A$ agonists, two different strategies were persued: The development of partial agonists and the development of receptor subtype specific compounds. Despite promising preclinical assays, partial agonists (e.g. bretazenil) so far did not meet the expectations in clinical trials. One compound (ocinaplon) though was reported to show anxiolysis and strongly reduced sedative side effects in Phase II clinical trials (Whiting et al., 2006). Since

different effects of benzodiazepines are mediated by different receptor subtypes the second strategy has focussed on the development of compounds with selectivity for those subtypes proposed to mediate the anxiolytic effects of benzodiazepines, that is α2 and α3 subunit containing receptors. Some of these compounds (e.g. L-838417, TP003, SL 651498) showed a promising separation of anxiolytic effects and side effects when tested in rodents and primates (McKernan et al., 2000; Griebel et al., 2001, 2003; Dias et al., 2005; Rowlett et al., 2005; Whiting, 2006). The usefulness of this second approach now has to be evaluated in clinical trials.

1.1.8.7 Regulation of $GABA_A$ receptor function by neuromodulators: the role of kinases and phosphatases

Many neuromodulators (e.g. dopamine) influence $GABA_A$ receptor function by coupling their receptors to G-protein linked second messenger systems to alter kinase and phosphatase activity (e.g. Chen et al., 2006). There has been one intriguing report that dopamine D5 receptors directly crosslink with $GABA_A$ receptors by using the TM3-TM4 loop of the γ2 subunit (Liu et al., 2000); so far this finding has not been followed up. More conventionally, phosphorylation is the common way to regulate ion channels (Kitler and Moss, 2003). This is underlined by studies on, for example, PKC γknockout mice; these mice show increased anxiety and have impaired $GABA_A$ receptor function (Hodge et al., 1999). For the $GABA_A$ receptor, the intracellular loops of the β and γ2 subunits in particular are phosphorylation targets. Studies of recombinant receptors have shown phosphorylation of these subunits by PKA, PKC, Src and PKB (Kittler and Moss, 2003; Wang et al., 2003). Depending on the subunit phosphorylation can have different functional effects, which might contribute to the diversity of $GABA_A$ receptor function. For example PKA mediated phosphorylation of the β1 subunit (serine 409) leads to negative modulation of the receptor, whereas phosphorylation of the β3 subunit (serine 408 and serine 409) enhances the activity of $GABA_A$ receptors (Kittler and Moss, 2003). Neuromodulators that influence $GABA_A$ receptor function via PKC include M1 muscarinic

acetylcholine receptors, serotonin (5-HT) type 4 receptors and TrKB receptor stimulation via BDNF (Kittler and Moss, 2003; Jovanovic et al., 2004). Modulation of $GABA_A$ receptor function after dopamine D4 and D3 receptor activation depends on PKA (Wang et al., 2002; Chen et al., 2006). Protein kinase B (Akt) can phosphorylate the γ2 subunit at serine 410, which promotes rapid insertion of the $GABA_A$ receptor into the membrane, resulting in increased sIPSC amplitudes after stimulation with insulin (Wang et al., 2003).

1.1.9 Function and physiological significance of $GABA_A$ receptor diversity

An important consequence of differences in subunit expression between cells or differential subcellular localization of subunits within a cell is that $GABA_A$ receptor kinetics might differ between different cells and different synapses (Thomson et al., 2000; Nyiri et al., 2001; Freund, 2003). In the hippocampus, for example, functionally distinct interneurons might signal via distinct $GABA_A$ receptor subtypes (Freund, 2003).

Knockout mouse lines have been generated for many of the $GABA_A$ receptor subunit genes (reviewed by Vicini and Ortinski, 2004). In the δ subunit knockout mice the amount of α4 subunit, which specifically co-assembles with δ subunit in the forebrain was decreased whereas the γ2 subunit was upregulated. Since the amount of the α4 subunit immunoprecipitated with γ2 antibody was increased, the remaining α4 subunit appears to more often co-assembles with γ2 subunit in the absence of δ subunit. Moreover these changes were largely confined to brain regions normally expressing the δ subunit. Therefore the δ subunit appears to interfere with the co-assembly of the α4 and γ2 subunits. The behavioural co-relation of δsubunit with specific functions have also been observed in the δ subunit KO mice. These include, attenuation of responses to neuroactive steroids, reduced ethanol consumption, attenuated withdrawal from chronic ethanol exposure and reduced anticonvulsant effect of ethanol (reviewed by Rudolph and Mohler, 2004). Similarly, extrasynaptic α5 containing receptors have some behavioural correlates: functional and behavioural analysis of mice

with targeted mutations of γ5 gene show that γ5 deficient mice have an enhanced learning of hippocampal dependent task (spatial learning) but no change in anxiety response (Colinson et al, 2002).

In a beautiful series of papers, mice with specific mutations in the key H101 coding position affecting BZ sensitivity were generated in the α1, α2, α3 and α5 subunit genes and the behavioural effects of diazepam were tested (Rudolph and Mohler, 2004). These mice have normal $GABA_A$ receptors, but in α1H101R mice for example, only the α2βγ2, α3βγ2 and α5βγ2-type $GABA_A$ receptors are diazepam-sensitive. Thus by a process of subtraction, it can be deduced how different α1βγ2, α2βγ2, α3βγ2, and α5βγ2 subtypes contribute to the diverse *in vivo* pharmacological effects of diazepam and other ligands requiring the H101 site. Thus α1H101R mice no longer become sleepy when given diazepam, and so the α1βγ2 receptors are required for the sedative effects of diazepam (Rudolph and Mohler, 2004), whereas the α2 (and α3) mediates diazepam's anxyiolytic effects (under the influence of diazepam α2α101R mice do not venture more into threatening areas, whereas their wild-type littermates do) (Rudolph and Mohler, 2004). A different set of studies using α3 selective inverse agonists and agonists also showed a significant contribution of the α3 subunit in anxyiogenesis and anxyiolysis (Atack et al., 2005; Dias et al., 2005). The muscle relaxant activity of diazepam is mediated by the α2 and α3 subunits, probably because these subunits are expressed in spinal motor neurons (Rudolph and Mohler, 2004; Wisden et al 1991)

1.2 Aim of the study

So far I presented the accumulating data in the literature suggesting that differentially targeted $GABA_A$ receptors are involved in specific functions during health and disease. Therefore the molecular dissection of the mechanism underlying the synaptic and extrasynaptic localization of $GABA_A$ receptors is becoming very important in order to map out the molecular components of specific behaviours mediated by synaptic and extrasynaptic $GABA_A$ receptor

signalling. The interesting question is: how does this specific segregation of receptor subtypes in to synaptic or extrasynaptic sites occur? More specifically, which molecular domains could contribute to mediate this kind of differential membrane targeting and clustering? With this present study I aim to identify some factors involved in differential targeting of $GABA_A$ receptors in to synaptic and extrasynaptic sites, that contribute answering this question.

2 METHODS

2.1 DNA Constructs

2.1.1 Generation of cDNA encoding a chimeric $GABA_A$ Receptor EGFP-tagged □2 subunit with the □ subunit TM3-TM4 loop (^{GFP}□2-TM3-TM4□)

As a starting point, I used the plasmid pRK5GFP□2, which contains the murine cDNA encoding □2L cDNA, tagged by EGFP as an N-terminal fusion protein in the mammalian CMV promoter-based expression vector pRK5 (Kittler et al 2000; gift from S. J. Moss). The unique Sal I site in the polylinker of the pRK5^{GFP}□2 vector was first deleted by digestion with Sal I, blunting with Klenow (see Appendix, section 5.3), and then religating to give plasmid pRK5^{GFP}□2□Sal. Two Sal I sites (GTCGAC) were then sequentially introduced into each end of the TM3-TM4 loop: The first one is introduced in between the first 6th and 7th aminoacids following the TM3, i.e., in the downstream of the HYFVSN and 83rd and 84th aminoacids, i.e., in the upstream of KMDS at the end of the TM3-TM4 loop of the □2L subunit by site-directed mutagenesis (Quick Change Site Directed Mutagenesis Kit, Stratagene). The Sal sites produced in-frame additions of two amino acids (VD) at each end of the □2 TM3-TM4 loop. For the SalI site near TM3 the primers were: 5'-TAT TTT GTC AGC AAC GTC GAC CGG AAG CCA AGC AAG-3' (sense) and 5'-CTT GCT TGG CTT CCG GTC GAC GTT GCT GAC AAA ATA-3' (antisense); for the SalI site at the 3' end of the loop the primers were 5'-CAT ATG CGC ATT GCC GTC GAC AAA ATG GAC TCC TAT-3' (sense) and 5'-ATA GGA GTC CAT TTT GTC GAC GGC AAT GCG CAT ATG-3' (antisense). The SalI sites are underlined. The mutations were verified by sequencing. This intermediate plasmid was termed pRK5^{GFP}□2(Sal)^2. The TM3 - TM4 loop of the ^{GFP}□2L subunit was then removed from the pRK5^{GFP}□2(Sal)^2 plasmid by Sal I digestion and religation to give pRK5^{GFP}□2(□-loop); this plasmid contains a unique Sal I site permitting subsequent in-frame insertions of other $GABA_A$ receptor subunit TM3-TM4 loops. The cDNA region encoding

the corresponding TM3 -TM4 loop (255bp) of the mouse $GABA_A$ □ subunit (see below) was amplified by PCR with primers, 5'-TTC AAT GCC GAC TAC GTC GAC AGG AAG AAA CGG AAA-3' and 5'-ATA GAT GTC GAT GGT GTC GAC GTC TGC ATC GAT GGG-3' flanked by Sal I sites (underlined), cloned into pPCRsript (Stratagene), sequenced and then inserted as a Sal I fragment into the $pRK5^{GFP}$□2(□-loop) plasmid to give the final plasmid $pRK5^{GFP}$□2TM3-TM4□.

2.1.2 Generation of the cDNA encoding EGFP-tagged $GABA_A$ receptor □ subunit (^{GFP}□)

The full-length murine $GABA_A$ □ subunit cDNA was amplified by PCR (with *Pfu* turbo DNA polymerase (Stratagene)) from mouse whole-brain cDNA (provided by C. Sandu) with the following primers, flanked by EcoRI sites (underlined): 5'-TGC CAT GAA TTC GCA ACT TTG CTT GCG CTG GGG CTA -3'; 5'-TGC CTA GAA TTC GAG GGC CTC CAG GAG TTT GTG GCA-3', and then cloned into pPCRscript (Stratagene), in which the Xho I site and Sal I sites in the vector's multiple cloning site had been deleted in advance, to give plasmid pScript-□. After complete sequencing to confirm that the sequence matched the database (NCBI database accession number NM008072), the □ subunit cDNA was then sub-cloned into the CMV promoter-based pRK5 mammalian expression vector (Aller et al, 2005) using EcoRI, to give plasmid pRK5□.

To generate an N-terminally tagged fusion protein of the □ subunit with EGFP ($pRK5^{GFP}$□), I copied exactly the strategy used by Kittler et al 2000 who produced the $pRK5^{GFP}$□2 subunit vector: the EGFP reading frame was inserted between amino acids 4 and 5 of the predicted mature N-terminus of the □ subunit; during processing in the secretary route, the signal peptide should be cleaved off, leaving the EGFP-tagged □ subunit.

An in-frame Xho I site (CTC GAG) encoding the dipeptide residues LE (underlined) was first inserted between the fourth and fifth amino acids of the

predicted mature □ subunit reading frame (downstream of QPHH) using plasmid pScript□ and the primers 5'-ACG CAG CCG CAC CAT CTC GAG GGC GCC AGG GCA ATG-3' and 5'-CAT TGC CCT GGC GCC CTC GAG ATG GTG CGG CTG CGT-3' (Stratagene Quickchange mutagenesis kit) to give plasmid pScript□Xho.

The EGFP reading frame was amplified by PCR from plasmid pEGFP-N1 (Clontech) with primers flanked by Xho I sites (underlined), with the stop codon of the EGFP excluded at the 5' end of the anti-sense primer: 5'-ACG TGG CTC GAG ATG GTG AGC AAG GGC GAG GAG CTG -3' and 5'- ATT GGA CTC GAG CTT GTA CAG CTC GTC CAT GCC GAG-3'. The Xho 1 coding sequences are underlined in the primers. The Xho-cut EGFP fragment was then placed into the XhoI site of pScript□Xho to give plasmid pScriptGFP□. The EGFP□ insert from pScript^{GFP}□ was then subcloned into pRK5 using EcoRI to give plasmid pRK5^{GFP}□.

2.1.3 Generation of cDNA encoding EGFP tagged □ subunit with an exchanged TM3-TM4 loop from the □2 subunit (^{GFP}□TM3-TM4□2)

Two in-frame Sal I sites (each encoding VD dipeptides) were sequentially introduced into plasmid pRK5^{GFP}□ at the end of the TM3 region (downstream of YAFAH, 6th and 7th) and the beginning of the TM4 region (upstream of TIDI, 91 and 92) of the native □ subunit reading frame by site-directed mutagenesis. The 5' Sal I site the primers were: 5'-TTC AAT GCC GAC TAC GTC GAC AGG AAG AAA CGG AAA-3'; and 5'-TTT CCG TTT CTT CCT GTC GAC GTA GTC GGC ATT GAA-3'. For the second Sal I site the primers were: 5'-CCC ATC GAT GCA GAC GTC GAC ACC ATC GAC ATC TAT-3' and 5'-ATA GAT GTC GAT GGT GTC GAC GTC TGC ATC GAT GGG-3' (SalI sites are underlined). The mutations were verified by sequencing. This intermediate plasmid was termed pRK5^{GFP}□(Sal)^2. The TM3-TM4 loop of the ^{GFP}□ subunit was then removed from the pRK5^{GFP}□(Sal)^2 plasmid by Sal I digestion and relegation to give pRK5^{GFP}□(□-loop); this plasmid contains a unique Sal I site permitting

subsequent in-frame insertions of other $GABA_A$ receptor subunit TM3-TM4 loops. The corresponding TM3-TM4 loop of $GABA_A$ □2 subunit was obtained by Sal I digestion of pRK5^{GFP}□2(Sal)^2 (see above) and gel purification (Qiagen gel purification kit) of the 230 bp fragment; this fragment was then ligated into pRK5^{GFP}□(□-loop) to give the final pRK5^{GFP}□TM3-TM4□2 plasmid. The construct was verified by complete sequencing.

2.1.4 Thy1 expression plasmids

The ^{GFP}□2, ^{GFP}□2TM-3TM4□, ^{GFP}□ and ^{GFP}□TM3-TM4□2 cDNAs were also all cloned, as EcoRI fragments, into the XhoI site of the Thy1.2 expression plasmid (Caroni, 1997) by blunting the Xho site and adding EcoRI adaptors. See Appendix, section 5.6, for full details of Thy1 plasmid construction.

2.2 Transfection of neurons in primary culture

Hippocampal neurons at DIV7 (see Appendix, section 5.13 for details of neuronal cell culture) in 24 well plates were transfected with the Effectene Transfection Kit (Qiagen) using equimolar ratios of each $GABA_A$ receptor subunit expression plasmid. The transfection mix (containing 0.4 □g DNA mix (200 ng/□l), 60 □l EC buffer and 3.2 □l enhancer) was incubated for 10 minutes at RT, then 6 □l Effectene reagent was added and the mixture was kept again at RT for another 10 minutes. After this incubation, 400□l fresh neurobasal medium was added to the transfection mix. 600 □l of the medium was removed from the each well containing neurons DIV7, and kept aside at 37 °C for later use, and the 220 □l of transfection mix were dispersed to each well, and incubated at 37° C 5□% CO_2 incubator for about 1 _ or 2 hours, after which the medium was exchanged by a mixture of conditioned medium (60 % competent medium, previouslly taken from each well and kept aside at 37 °C + 40 % fresh Neurobasal medium (Gibco), -prewarmed at 37°C). Neurons were maintained in the 37 °C, 5□% CO_2 incubator until DIV19, then processed for immunocythochemistry.

2.3 Immunocytochemistry on cultured neurons

Cultures were kept at 4 °C for 10 minutes, then the medium was exchanged to PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.4) containing 5 % NGS, and 1:10000 diluted GFP antibody (Molecular Probes), and left for incubation for 1 hour at 4 °C without shaking. After incubation the antibody solution was washed out by 2 times PBS for 5 minutes each. Afterwards, the cells were fixed with 4 % Paraformaldehyde/4 % sucrose in PBS for 10 minutes and washed for 3 times with PBS at room temperature for 5 minutes each. For membrane staining of chimeric $GABA_A$ receptors, the Alexa-488 secondary antibody (anti-rabbit, Molecular Probes) in PBS with 5% BSA and 5% NGS was used with a concentration of 1:1000, and cells were incubated for 2 hours at room temperature. Then the cells were washed for 5 minutes three times with PBS, and premeabilized with 0.2 % Triton PBS, for 5 minutes at room temperature before blocking the cells with 5%BSA, 5%NGS and 0.1 % Triton. After the blocking step, Synaptophysin, (Sigma) or GAD-67 (Chemicon) antibodies were used as a dilution of 1:1000 for 45 minutes, 1 hour at room temperature. Before applying the secondary antibody Cy-3 anti-mouse (Jackson Laboratories) with a dilution of 1:750, in the presence of 5 % BSA and 5 % NGS for 45 munites at room temperature, cells were washed 3 times at room temperature for 5 minutes with PBS. Finally, after rinsing the coverslips with distilled water, the cells were mounted with Mowiol on the slides and after overnight incubation at dark they were visualized with a laser scanning confocal microscope.

2.4 Image acqusition and analysis

For quantitative analysis and colocalization (with synaptic markers) of chimeric $GABA_A$ receptor clusters formed in the plasma membrane, digital microscopic images were recorded with a 40x oil immersion objective on a Leica confocal microscope (the confocal system is Leica TCS SP2, the microscope is a DM IRE2 and the software is called LCS-Leica Confocal Software) and the images

were captured with an associated CCD camera and transferred to a computer workstation. The filter sets of the confocal microscope was, Alexa (488 nm): 500nm-535nm, Cy-3 (543 nm): 555nm-700nm. Image files were acquired with a resolution of 1025x1025 pixels and when necessary appropriate parts of cells were zoomed digitally under 40x objective and saved separately as tiff files. The images recorded as tiff format were then transferred to Photoshop 4.01 (Adobe) for the preparation of pictures of cells.

For quantification of clusters and colocalization analysis, the original data files were directly analyzed with the Metamorph 6.01 software (Universal Imaging Corporation). The tiff files were first background substracted by the selection of the background area with the 'oval' tool, and subsequently executing the "use region for background' command of the software and then the files were saved separately into another folder. The saved files were reactivated and thresholded until the dendritic tree of neurons becomes sharpened. In order to determine the average cluster number per 100 μm dendrite, each separately discrete object in the images of the dendritic segments were highlighted with the "create regions around object" tool of the software. The objects then zoomed in for a better resolution and quantified with the "count objects manually" tool and the data were transfered into an excel worksheet. The length of dentrite (varying in between 50 μm to 250 μm) where the clusters were counted from the 4, 5 cells expressing each construct, were determined by the "measure xyz axis" tool under Apps Menu of the Metamorph software. Values were then normalized to 100 μm and reported as (mean) ± (standard error of the mean) (SEM). The cluster sizes were determined with the use of "integrated morphometry analysis" tool of the same program which gives relative values. In order to quantify the colocalization frequency of the chimeric receptors with synaptic markers, Metamorph 6.1 software was used to interactively define the chimeric $GABA_A$ receptor and GAD or synaptophysin colocalization. The images (previously background substracted and thresholded as described above) were analysed by 'Measure colocalization" tool to verify the percentage of colocalization per each condition. Data collected with Metamorph were transferred to a Microsoft Excel worksheet for further

evaluation. All statistical evaluations were done according to unpaired Student's t- test.

2.5 Determination of evolutionary conserved domains

In order to access the homologous sequences of mouse □2 and □ subunits in vertebrates, protein sequence databases (NCBI, ENSEMBLE, Expasy) were searched for pairwise similarity using the BLAST tool. In order to identify non-annotated peptide sequences, genomic databases translated in six reading frames were searched using the TBLASTN program. The BLAST programs determine the statistical significance of local alignments produced by database searches using the extreme value distribution statistics for a single alignment segment and the sum statistics for two or more compatible segments. The values of the probability (*P*) of obtaining an alignment with a given score by chance computed by BLAST programs are reliable as long as regions for low complexity do not contribute to the alignment scores (reviewed by Altschul, et al., 1994). Therefore, a *P* value of 0.001 or less was considered a strong indication of homology. The sequences were downloaded and saved as EditSeq protein files, before being exported to MacAlign software for alignment by Clustal method (See appendix for the accesssion number of peptide sequences used in the alignments). The conserved domain database, provided by ENSEMBL, was searched to identify any potentially significant motif in the intracellular domain of □ subunit.

3 RESULTS

3.1 Construction of $GABA_A$ receptor □2 and □ subunit chimeras tagged with EGFP

I first assembled the necessary cDNAs encoding N-terminally-tagged EGFP $GABA_A$ receptor subunits in the appropriate eukaryotic expression vectors (see the Methods section for details on how the constructs were made and Figure 3. 1). In addition to the vector backbone (described below), non-coding sequences at the 5' end, including the signal peptide, were amplified from 57 nucleotides upstream of the translational start codon, and these features were kept the same in all constructs to minimize the potential differences in expression levels. Save for 74 nucleotides downstream of the translational stop codon, all the 3' untranslated mRNA sequences were deleted. Using the published ^{GFP}□2 subunit as a model (Kittler et al 2000, GFP tagged at the N terminus between amino acids 4 and 5 of the mature peptide), I inserted an EGFP reading frame into the equivalent position of the mouse □ subunit cDNA, between amino acids 4 and 5 of the mature peptide. Using the ^{GFP}□2 and ^{GFP}□ cDNAs as templates, I used *in vitro* mutagenesis, followed by "cutting and pasting" of restriction fragments to construct two GFP-(N-terminally) tagged $GABA_A$ receptor subunit cDNA chimeras (^{GFP}□2TM3-TM4□ and ^{GFP}□□M3-TM4□2). I subcloned all cDNAs into mammalian expression vectors (the CMV promoter-based pRK5 and the Thy1 promoter-based vector (see Methods). I thus had a series of eight subunit cDNAs: (Figure 1) pRK5^{GFP}□2, Thy1^{GFP}□2, pRK5^{GFP}□2TM3-TM4□, Thy1^{GFP}□2TM3-TM4□, pRK5^{GFP}□□M3-TM4□2, Thy1^{GFP}□□M3-TM4□2, pRK5^{GFP}□ and Thy1^{GFP}□. All constructs were completely sequenced to verify the reading frames. The next step was to test in transfected HEK cells that the cDNAs expressed the expected full-length proteins and that they assembled on the cell membrane (these experiments are described in the Appendix). This was indeed the case (see Figures 5.1, 5. 2 and 5.3 in the Appendix).

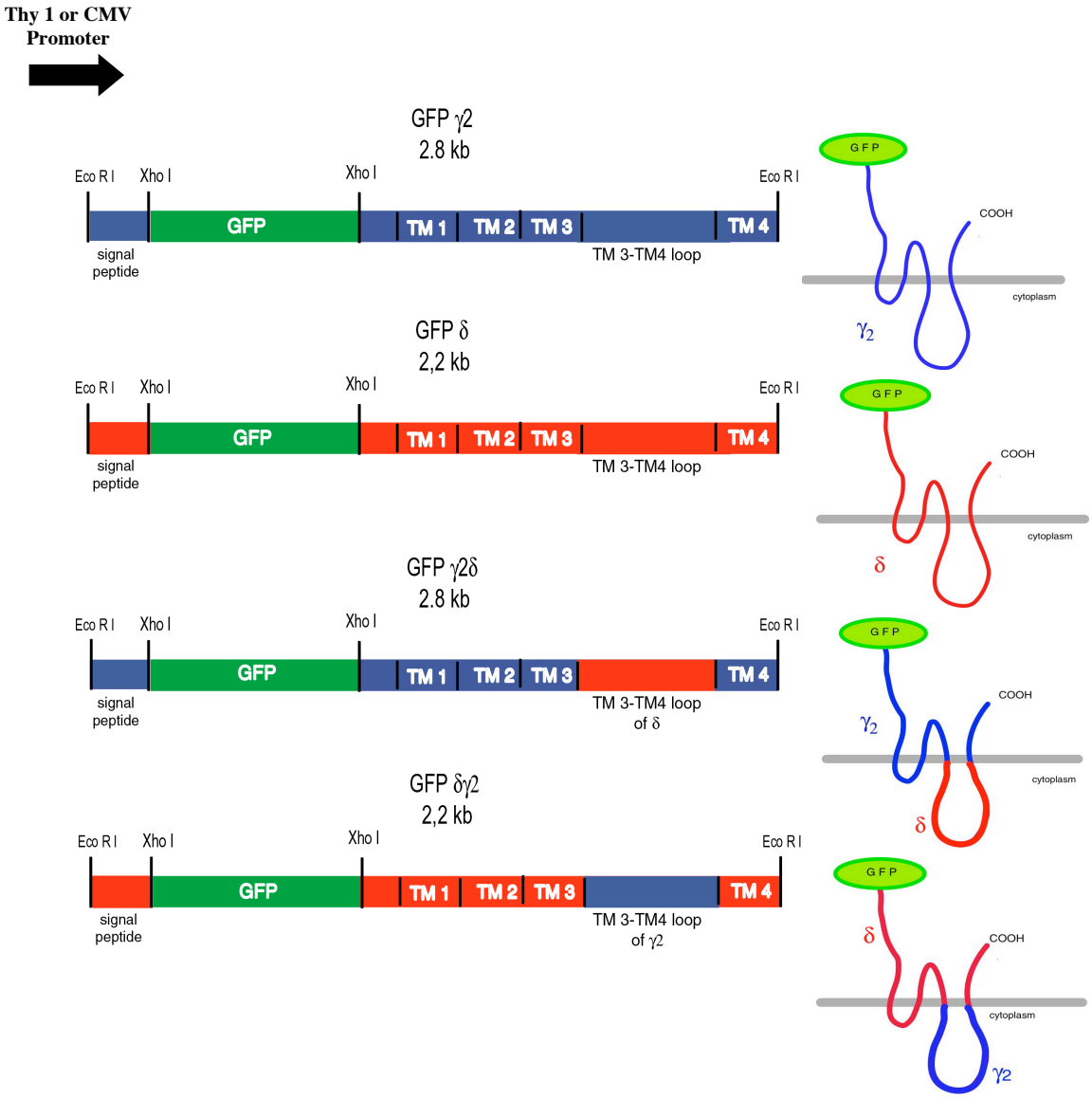


Figure 3.1: Diagram of cDNAs encoding for chimeric murine $GABA_A$ receptors subunits. The cDNAs of ^{GFP}□2, ^{GFP}□, ^{GFP}□2□ (^{GFP}□2TM3-TM4□), ^{GFP}□□2 (^{GFP}□TM3-TM4□2) are shown on the right from top to bottom respectively. The corresponding protein conformations of them on the plasma membrane is shown on the left.

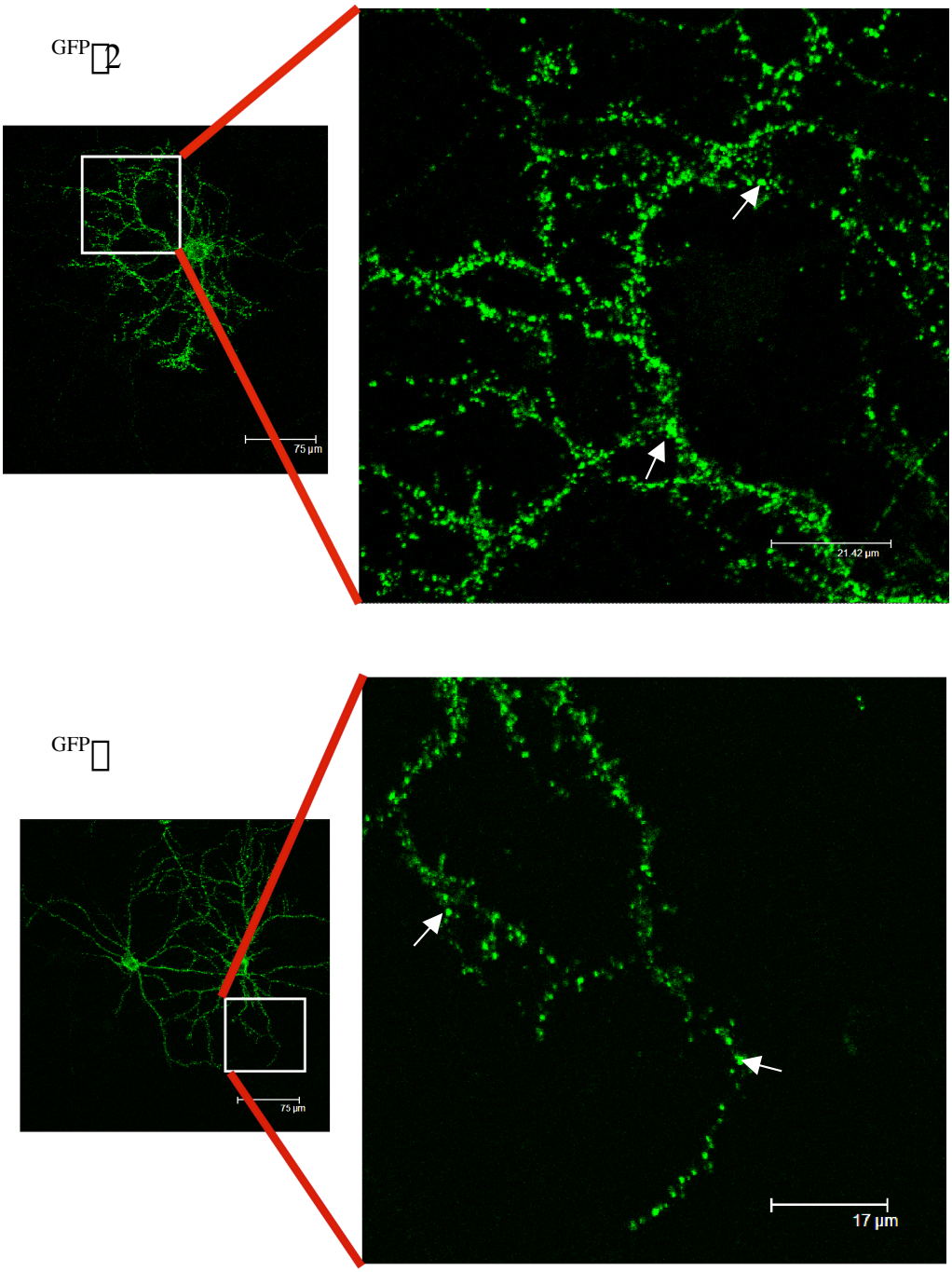


Figure 3.2: Expression of ^{GFP} and ^{GFP} in the cultured hippocampal neurons DIV19. Scanning confocal images of immunostained hippocampal neurons DIV 19 transfected with ^{GFP} (on the top right) and ^{GFP} (on the bottom right) subunits show that the subunits are probably co-assemble with the endogeneos subunits, targeted to the cell membrane and form clusters, as shown by the white arrows in the high resolution images of dentritic spines (left) of the boxed regions (right).

3.2 GABA $_A$ receptors formed with $^{GFP}\beta 2$, $^{GFP}\gamma 2$, $^{GFP}\gamma 2$TM3-TM4$\gamma 2$, and $^{GFP}\gamma 2$TM3-TM4$\gamma 2$ are targeted to the outer membrane of cultured hippocampal neurons

As previously described by others (Kittler et al., 2000), the $^{GFP}\beta 2$ subunit when expressed in transfected hippocampal neurons assembles into receptor clusters at inhibitory synapses, as well as at extrasynaptic sites. This is the same result as found for native $\beta 2$ subunits (Somogyi et al 1996); thus the large N- terminal GFP tag does not interfere with the targeting of the $\beta 2$ subunit (Kittler et al, 2000).

To examine the targeting of the γ subunit, I transfected the $^{GFP}\gamma$ subunit cDNA expression vector into primary cultured hippocampal neurons (see Methods and Appendix for details). I transfected the hippocampal neurons at DIV7 and used these neurons for immunocytochemistry at DIV 19, as the membrane targeting of $GABA_A$ receptors are facilitated at older ages (data not shown). As a positive control and reference I ran parallel transfections with the $^{GFP}\beta 2$ subunit cDNA expression vector (Kittler et al 2000). It was suggested that for neurons exogenous expression of $GABA_A$ receptors are facilitated with the co-transfection of $\gamma 1$ and $\gamma 2$ subunits (Kittler et al 2000). As for HEK cells (Figure 5.3 in the Appendix), neurons were triple transfected with $\gamma 1$, $\gamma 2$ and the test subunit expression vectors. I optimized the procedure first with the $^{GFP}\beta 2$ subunit (Kittler et al, 2001). Initial experiments were tried with the CMV promoter-based expression vector, but I switched to an expression plasmid, which uses the neural-specific Thy1 promoter (Caroni, 1997), and without co-transfecting the $\gamma 1$ and $\gamma 2$ subunit expression vectors, as this gave better subunit expression (data not shown). In agreement with my findings, Christie et al (2005) also found that cotransfection of exogenous γ and δ subunits is not required; they found that expressed tagged $\gamma 2$ subunits in rat hippocampal neurons can coassemble with endogenous $GABA_A$ receptor subunits and form clusters of similar density and size to the endogenous $GABA_A$ receptor clusters, probably because the

endogenous subunits are rate limiting for receptor assembly (Christie et al 2005). This effect is shown in the following figures: ^{GFP}□2 (Figure 3.2), ^{GFP}□2TM3-TM4□ (Figure 3.3), ^{GFP}□ (Figure 3.2), and ^{GFP}□□M3-TM4□2 (Figure 3. 3) receptor subunits probably co-assemble with endogenous subunits and target to the cell membrane in cultured hippocampal neurons (most likely pyramidal neurons). For all subunit constructs, GFP immunoreactivity of unpermeabilized cells corresponds to the membrane targeting of subunits (see the arrows in the figures); receptors with these tagged subunits form clusters with different sizes and probably with different densities for each of the constructs.

3.3 $GABA_A$ receptors formed with the ^{GFP}□2, ^{GFP}□, ^{GFP}□2TM3-TM4□ and ^{GFP}□□ 3□□ 4□2 subunits have similar cluster sizes in cultured hippocampal neurons

$GABA_A$ receptors assembled from recombinant ^{GFP}□2 and ^{GFP}□ receptor subunits expressed in hippocampal neurons form clusters that in each case have considerable size variation, from relatively large to smaller clusters (see Table 3.1 and Figure 3.4). The comparison of relative values of cluster sizes (radius) show that the 'small-size' clusters were more frequent in each case as assessed using the 'integrated morphometry analysis tool' of the Metamorph 6.0 software (see Methods for details and Figure 3.4); more than 60 % of all clusters formed by each subunit construct at the cell surface of the neurons have a size less than 2.5 units (small clusters), at least 25% of the clusters have a size between 2.5 and 7.5 units (medium size of clusters) and rest of them have a size more than 7.5 units (large clusters, rarest form of cluster size for each case). As shown in the Table 3.1, ^{GFP}□ expressing neurons tend to form the biggest size of clusters: the average of the maximum mean radius

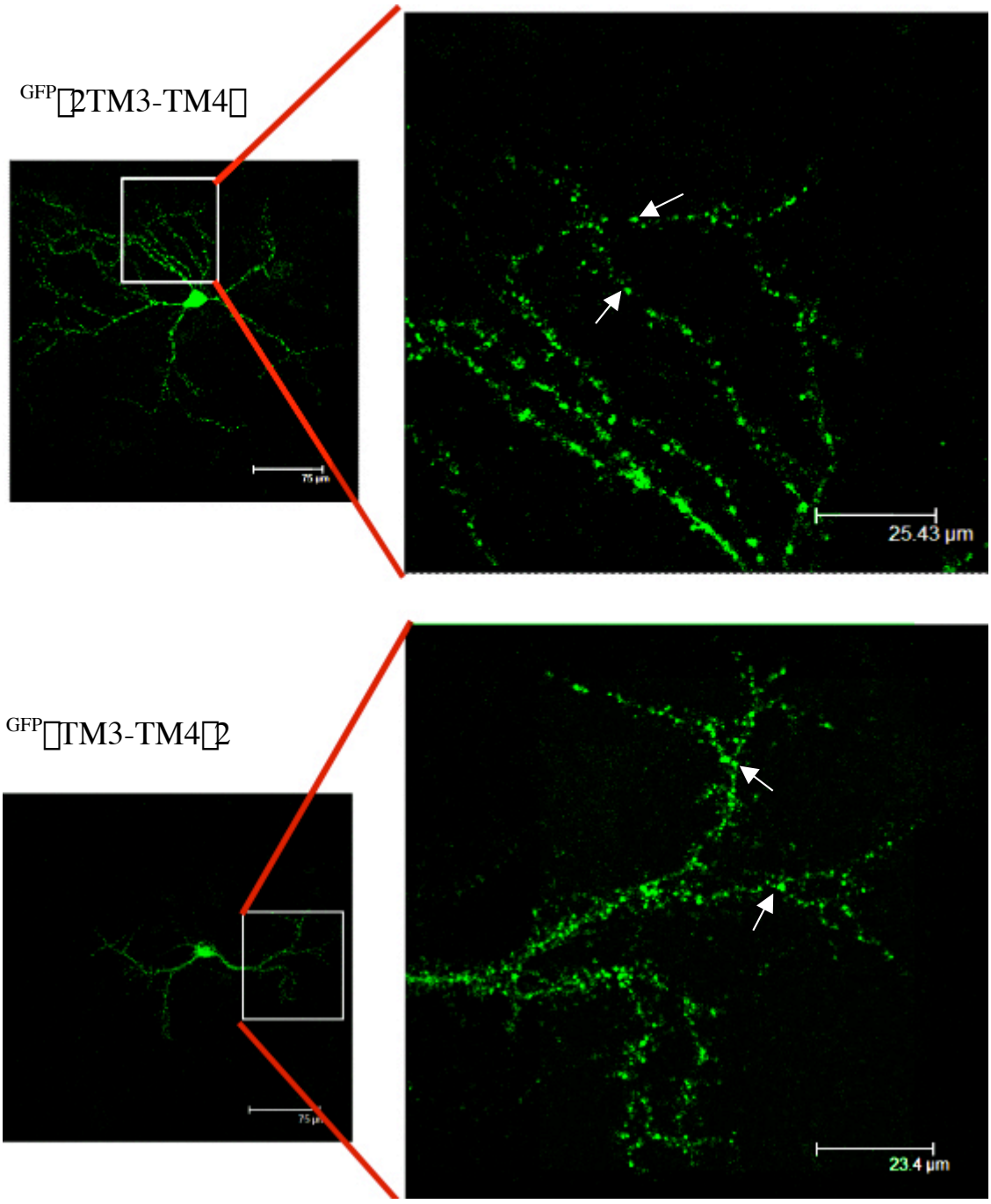


Figure 3.3: Expression of GFP□2□□ 3□□□ 4□ and GFP□□□ 3□□□ 4□2 expressed in the cultured hippocampal neurons DIV19. Scanning confocal images of immunostained hippocampal neurons DIV 19 transfected with GFP□2TM3-TM4□ (top) or GFP□TM3-TM4□2 (bottom) subunit show that these chimeras are most probably co-assemble with the endogeneous subunits, targeted to the cell membrane and form clusters, as shown by the white arrows in the high resolution images (right) of the boxed regions (left).

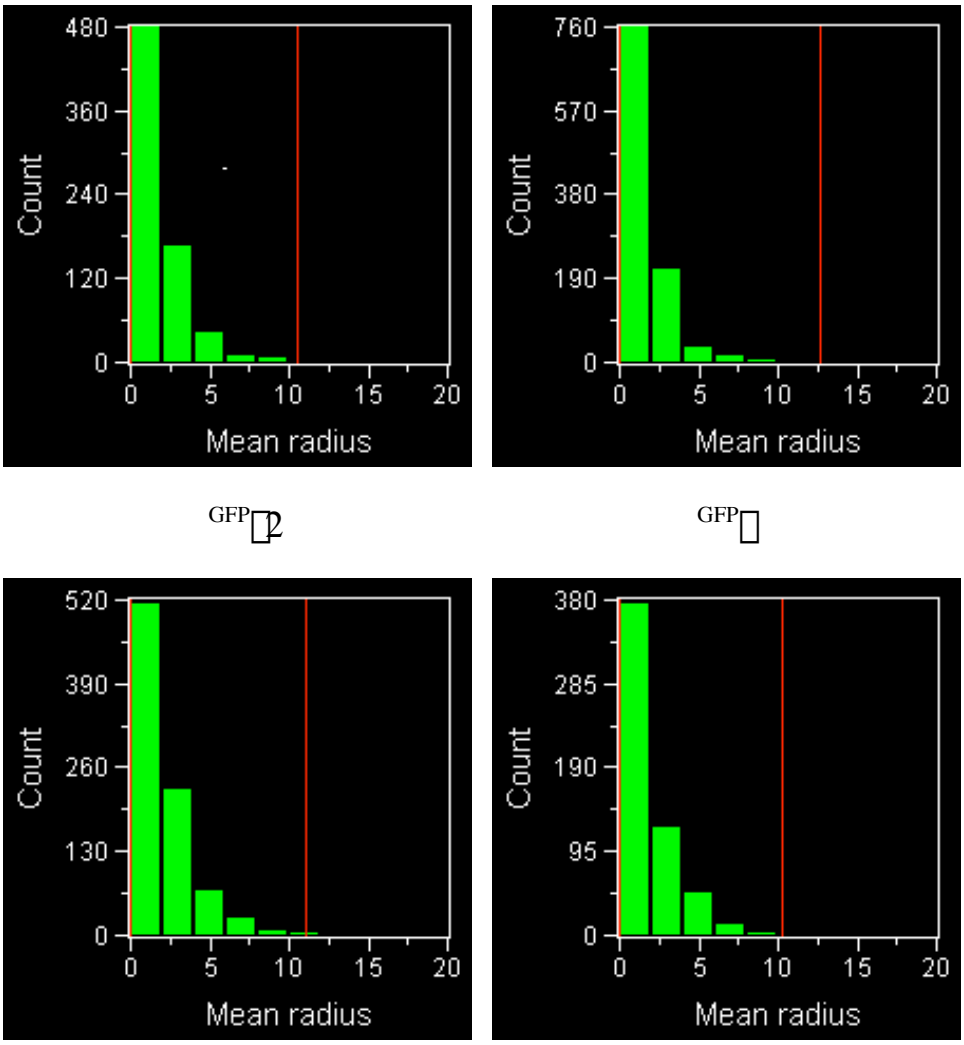
Chimeric $GABA_A$Rs	Cluster size (relative values)	
	min	max
^{GFP}□2	<0.5	8.91
^{GFP}□2□□ 3□□□ 4□	<0.5	10.06
^{GFP}□	<0.5	11.24
^{GFP}□□□ 3□□□ 4□2	<0.5	7.89

Table 3.1: Cluster sizes (in relative values) for each chimeric constructs expressed in cultured hippocampal neurons.

of the biggest clusters is 11.24 units, measured from n= 1823 clusters expressed on 2 or more dendrites of at least 3 neurons. On the other hand the maximum mean radius of ^{GFP}□2 clusters was relatively lower, 8.91 units (calculated from n=1569 clusters of 2 or more dendrites of at least 3 neurons). However, as it is seen in the Figure 3.4., the trend for the variation in cluster sizes were similar for each of the constructs and any differences in relative cluster size for different subunits were not statistically significant (Student's t-test, for each case P > 0.05).

3.4 The number of clusters obtained by forming $GABA_A$ receptors with ^{GFP}□2 in primary hippocampal neurons is more than those with ^{GFP}□

By eye it was already clear that immunoreactivity was brightest for neurons transfected with the ^{GFP}□2 construct, moderate in the case of ^{GFP}□2□M3-TM4□ and less for ^{GFP}□ or ^{GFP}□□M3-TM4□2. Indeed, as confirmed by quantification of cluster densities using the 'count objects manually' tool of the Metamorph software, transfected neurons showed a higher degree of expression of ^{GFP}□2-based subunits than ^{GFP}□□based subunits. By the criterion of number of puncta



GFP□2 GFP□

GFP□2□□ 3□□□ 4□ GFP□□□ 3□□□ 4□2

Figure 3.4: Integrated morphometry analysis of the relative size of $GABA_A$ receptor clusters found in hippocampal neurons transfected with either GFP□2 (n=730), GFP□2□□ 3□□□ 4□ (n=1005), GFP□ (n=825) or GFP□□□ 3□□□ 4□2 (n=570). At least 60 %, of clusters at the cell surface have a size less than 2.5 units (small clusters), at least 25% of them have a size between 2,5 and 7.5 units (medium size clusters) and 5 % or less have a mean radius more than 7.5 units (large clusters). The n number corresponds to the total number of clusters analyzed for each subunit construct. Constructs were expressed with the Thy1 promoter.

Chimeric $GABA_A$ Rs	Cluster number/100 μm dendrite
$^{GFP}\alpha 2$	101±8
$^{GFP}\alpha 2\beta 3\gamma 4$	97±5
$^{GFP}\beta$	55±8
$^{GFP}\alpha\beta 3\gamma 4\alpha 2$	50±6

Table 3.2: Cluster numbers of chimeric $GABA_A$ Rs expressed on the cell surface of transfected hippocampal neurons, DIV 19 shows that the $^{GFP}\alpha 2$ and $^{GFP}\alpha 2$TM3-TM4β subunits have higher cluster densities in contrast to $^{GFP}\beta$ and $^{GFP}\beta$TM3-TM4$\alpha 2$. At least two dendrites with 50 to 150 μm in length were analyzed from 5 to 8 neurons expressing each of the chimeric constructs,When compared with $^{GFP}\alpha 2$, P value is more than 0.05 for $^{GFP}\alpha 2\beta 3\gamma 4$ and less than 0.05 for $^{GFP}\beta$ and $^{GFP}\alpha\beta 3\gamma 4\alpha 2$.

per 100 μm dendrite, a significant difference ($P < 0.05$, Student's t-test) in the number of clusters of $^{GFP}\alpha 2$ and $^{GFP}\beta$ was evident (see Table 3.2). The number of clusters of $^{GFP}\alpha 2$ was 101±8 per 100 μm of the dendrite; on the other hand the cluster number of $^{GFP}\beta$ was 55±8 per 100 μm dendrite. The cluster number of $^{GFP}\alpha 2$TM3-TM4β was similar to $^{GFP}\alpha 2$, i.e., 97±5 per 100 μm dendrite, suggesting that the large intracellular TM3-TM4 loops of either the $\alpha 2$ or β subunit do not influence the differential levels of cluster density of the $\alpha 2$ and β subunit-containing $GABA_A$ receptors on the cell surface. The number of receptor clusters containing $^{GFP}\alpha\beta 3\gamma 4\alpha 2$ subunits was 50±6 clusters per 100 μm dendritic segment, similar to the cluster number obtained by expressing $^{GFP}\beta$.

3.5 $GABA_A$ receptors with the $^{GFP}\gamma 2$ subunit are targeted to inhibitory synapses of primary cultured hippocampal neurons, but receptors with the $^{GFP}\gamma$ subunit less so.

The sorting, assembly, targeting, and clustering of $GABA_A$ receptors are probably mediated by different domains of the subunits interacting with distinct proteins; at the time when I began this work, it seemed the most reasonable to hypothesize that the large cytoplasmic TM3-TM4 domains of these subunits helps specify the subcellular targeting of the receptor. Given that γsubunit-containing $GABA_A$ receptors are located largely extrasynaptically (Nusser et. al., 1998, Wei, et al, 2003), perhaps the TM3-TM4 intracellular domain of this subunit determines this extracellular location. To examine this possibility, I analyzed quantitatively the colocalization of receptors containing recombinant $\gamma 2$ and γ subunits with swapped TM3-TM4 domains (Figure 3.1) with the presynaptic markers: glutamic acid decarboxylase 67 (GAD 67) which selectively marks GABAergic terminals and synaptophysin which marks both glutamatergic and GABAergic terminals. Only the images with GAD 67 are shown (Figures 3.5, 3.6, 3.7 and 3.8), but the quantification of the co-localization of subunit type with presynaptic marker is given for both GAD 67 and synaptophysin terminals in Figure 3.9. Under our culture conditions, most terminals in the primary hippocampal cultures are GABAergic.

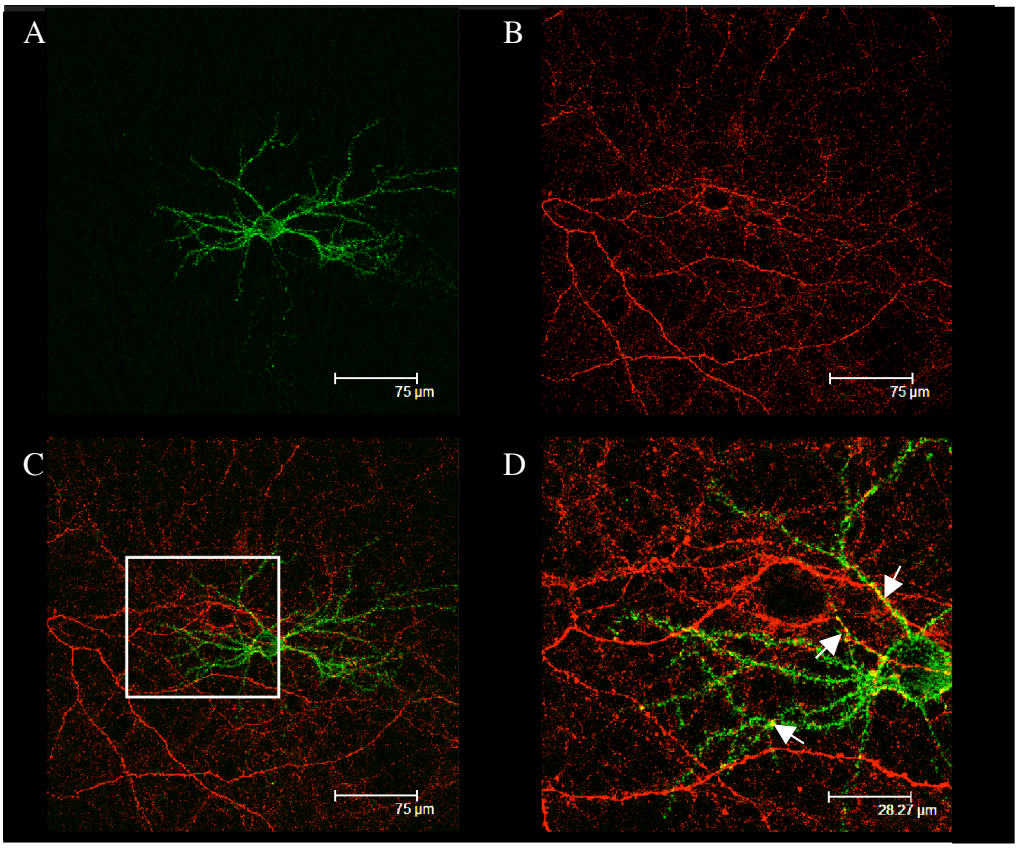


Figure 3.5: Cultured hippocampal neurons transfected with $^{GFP}\square_2$ and innervated with GABAergic terminals. Scanning confocal images of hippocampal neurons transfected with $^{GFP}\square_2$ followed by immunostaining with GFP antibody and GAD 67 antibody to localize synaptic and nonsynapting targeting of chimeric receptors on the membrane. Green color represents the GFP immunoreactivity of $^{GFP}\square_2$ (A) and red color corresponds to GAD terminals innervetad with corresponding neurons expressing the chimera (B). The overlay (yellow) of green and red is shown in panel C. High resolution image of dendritic spine in C is shown in D, where there is a high colocalization of GAD and $^{GFP}\square_2$ is observed (see yellow dots shown by white arrows).

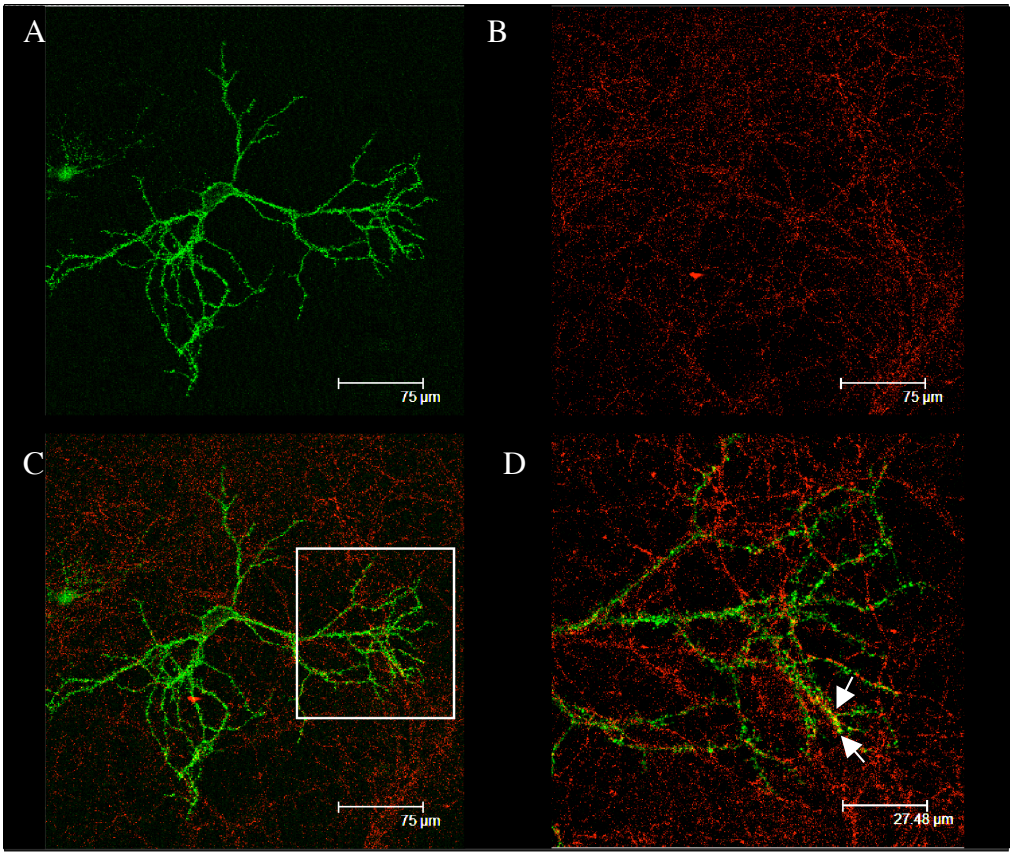


Figure 3.6: Cultured hippocampal neurons transfected with $^{GFP}\gamma_2$ and innervated with GABAergic terminals. Hippocampal neurons DIV19 expressing the $GABA_A$ receptor subunit, $^{GFP}\gamma_2$ as visualized by immunostaining with GFP and GAD 67 antibodies to localize synaptic and nonsynaptic targeting of $GABA_A$ receptors on the membrane. A, Green, $^{GFP}\gamma_2$; B, red, GAD 67-postive terminals; C, overlay (yellow). High resolution of the boxed region in C is shown in D, where a limited colocolazitation of GAD-67 and $GABA_A$ receptors with $^{GFP}\gamma_2$ occurs (yellow dots marked by white arrows).

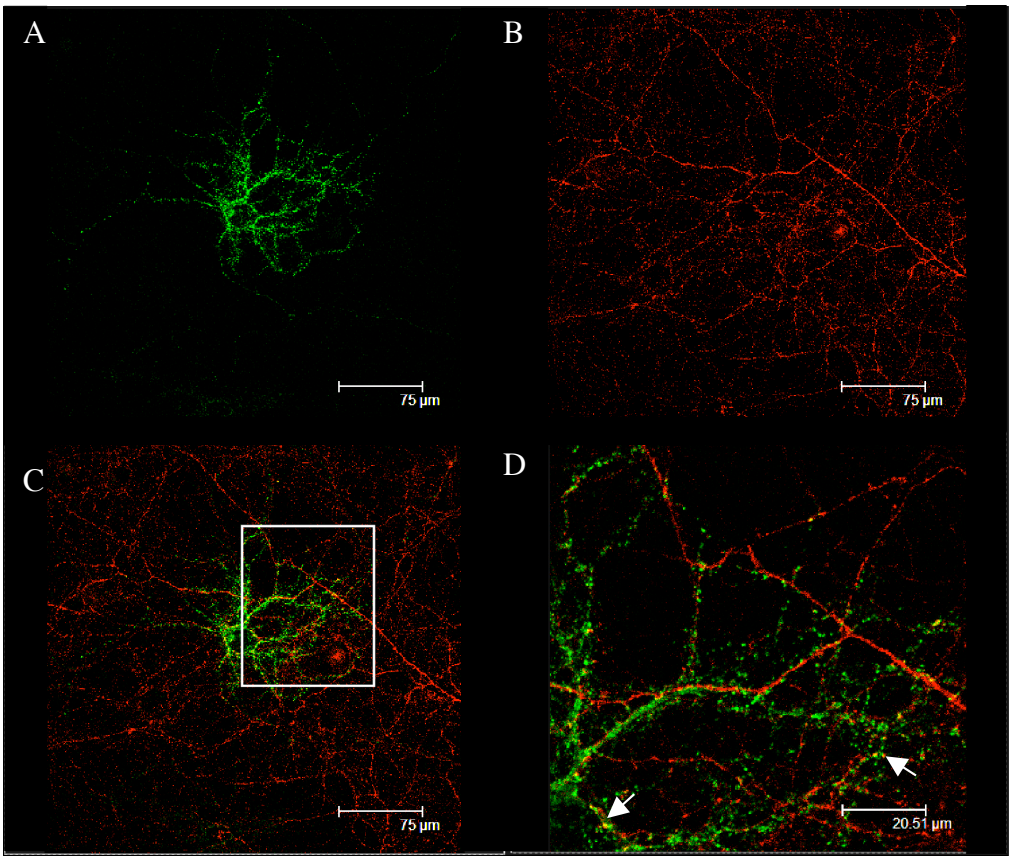


Figure 3.7: Cultured hippocampal neurons transfected with $^{GFP}\beta$2TM3-TM4γ and innervated with GABAergic terminals. Hippocampal neurons (DIV19) expressing the $GABA_A$ receptor subunit $^{GFP}\beta$2TM3-TM4γ as visualized by immunostaining with GFP and GAD 67 antibodies to localize synaptic and nonsynaptic targeting of $GABA_A$ receptors on the membrane. A, green, GFP; B, red, GAD 67-positive terminals; C, overlay (yellow); D, high resolution of the boxed region in C, where there is a 'speculative' colocalization of GAD 67 and receptors with the $^{GFP}\beta$2TM3-TM4γ subunit see yellow dots shown by white arrows).

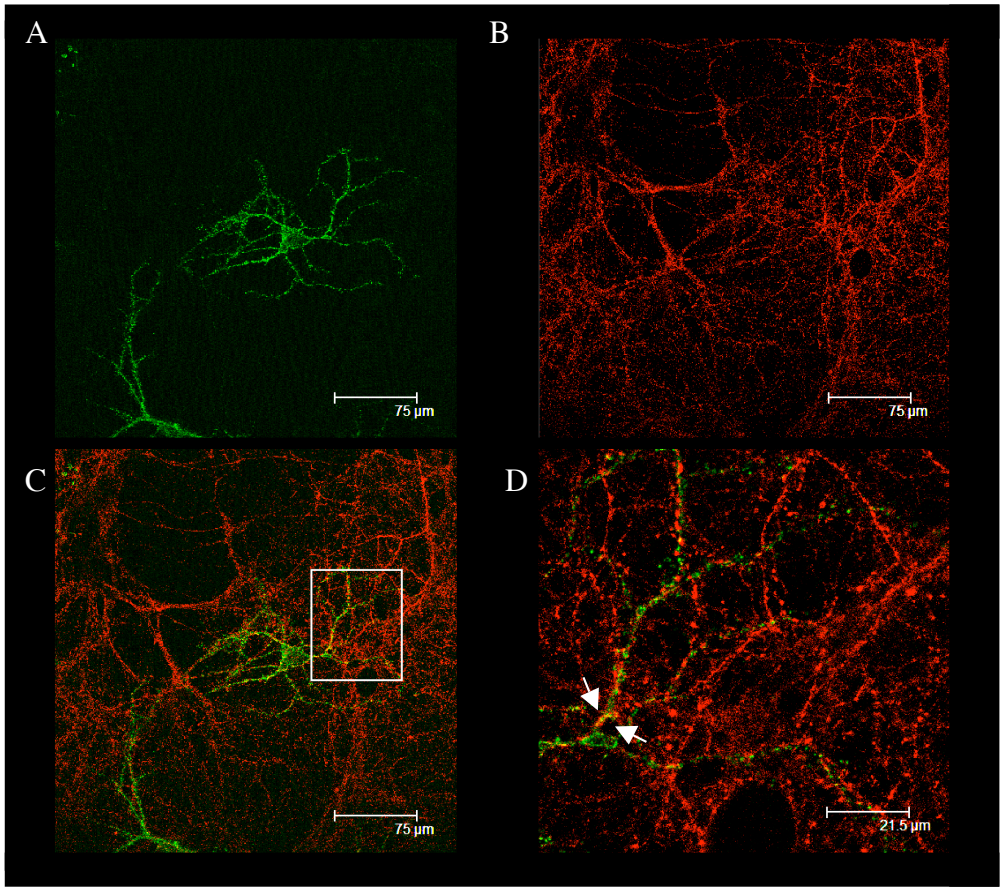


Figure 3.8: Cultured hippocampal neurons transfected with ^{GFP}TM3-TM4 and innervated with GABAergic terminals. Scannining confocal images of hippocampal neurons (DIV19) immunostained with GFP and GAD 67 antibodies to localize and compare the synaptic and nonsynapting targeting of $GABA_A$ receptor chimeras s on the membrane. A, green, GFP; B, red, GAD 67-positive terminals; C, overlay (yellow); D, high resolution of the boxed region in panel C, where there is a limited colocalization of GAD 67 and ^{GFP}TM3-TM4 (see yellow dots shown by white arrows).

As shown in Figure 3.5, there are many ^{GFP} clusters that are not synaptic besides to synaptic ones (shown by whote arrows). Nevertheless, the average percentage of co-localized weighted voxels of ^{GFP} with GAD 67 immuno-positive terminals is the highest (more than 55 %, Figure 3. 9A). Similarly, the average percentage of co-localized weighted voxels of ^{GFP} with synaptophysin immuno-positive terminals is almost 70 % (Figure 3.9B). By contrast, I found that the ^{GFP} subunit is often located extrasynaptically (Figure 3.6). Receptor

clusters with the γ subunit, as represented by GFP immunoreactivity (green) (Figure 3.6A), often do not colocalize with GAD 67 immunoreactivity (red) (Figure 3.6B), when an overlay image is made (Figures 3.6C, D). Indeed, by contrast with $^{GFP}\gamma2$, the average percentage of co-localized weighted voxels of $^{GFP}\gamma$ with GAD 67 and synaptophsyin immuno-positive terminals was significantly less (approximately 40% for GAD-67 and 45% for synaptophsyin, Figures 3.9A and B), according to unpaired Student's t test with $p<0.05$. Nevertheless, there are still quite a few γ subunit-containing clusters opposite presynaptic markers.

3.6 $GABA_A$ receptors with the $^{GFP}\gamma2$TM3-TM4γ and $^{GFP}\gamma$TM3-TM4γ2 subunits form mostly nonsynaptic clusters

For hippocampal neurons expressing the $^{GFP}\gamma2$ (Figure 3.7A) or $^{GFP}\gamma$TM3-TM4γ2subunits (Figure 3.8A), there was often no apparent co-localization of receptor clusters with GAD 67 immunoreactivity (Figures 3.7B, 3.8B), as assessed by the overlay images (Figures. 3.7C, D, 3.8C, D).

However this phenomenon can not be judged as totally non-synaptic because, as shown by the white arrows in Figures 3.7D & 3.8D, there are also some receptor puncta colocalized with GAD 67 immunoreactivity (yellow color).

The percentage of co-localized weighted voxels for both the $^{GFP}\gamma2$ and $^{GFP}\gamma$TM3-TM4γ2subunits co-localized with GAD-67 and synaptophysin is similar to that for $^{GFP}\gamma$, approximately 40% and 45% respectively (Figures 3.9A and B; $p < 0.05$ when compared with $^{GFP}\gamma2$ according to unpaired Student's t test).

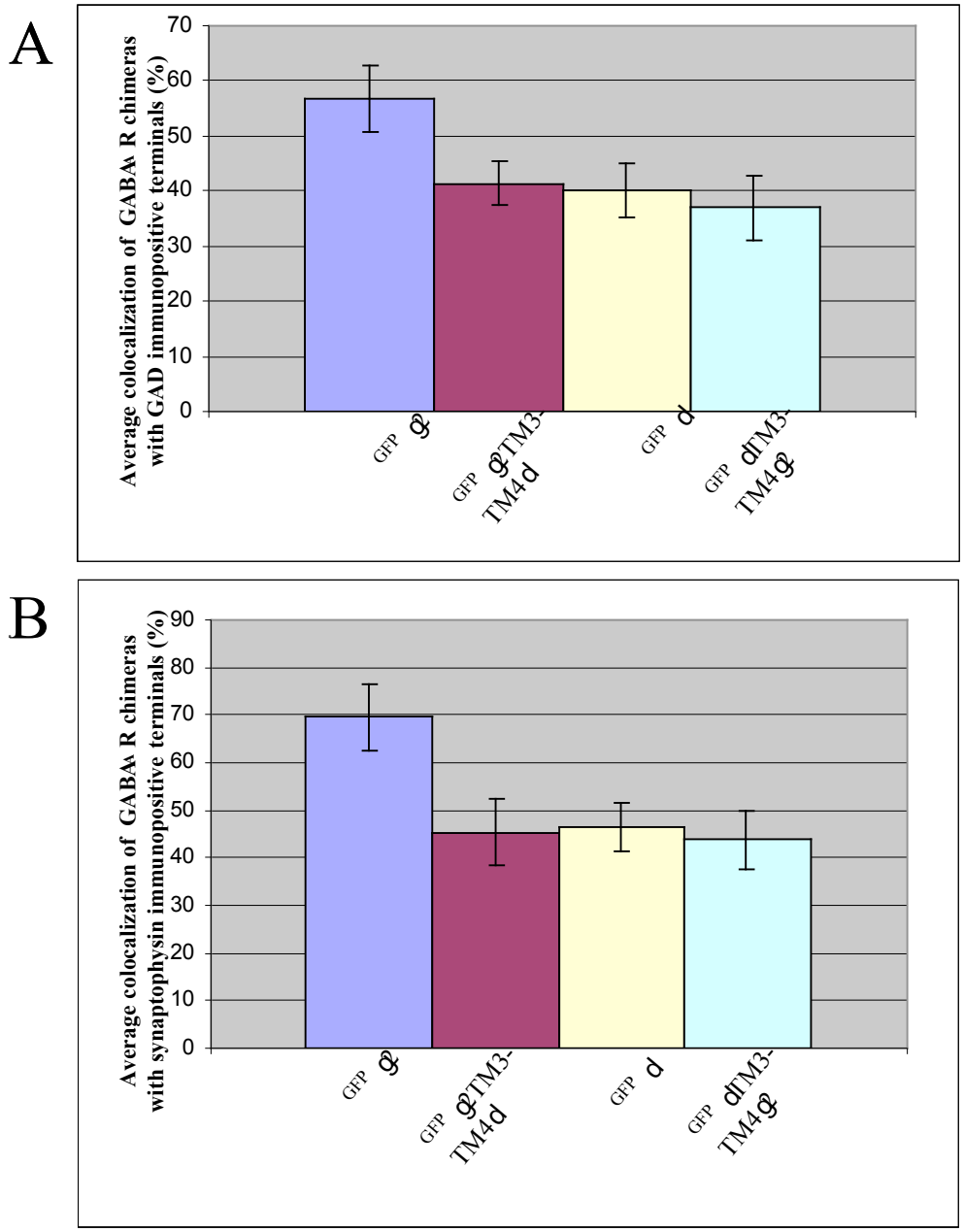


Figure 3.9: Average % colocalization frequency of GAD 67 (graph A) and synaptophysin (graph B) with recombinant □2 and □ $GABA_A$ receptors expressed in cultured hippocampal neurons DIV 19. Data in these charts represent the percentage colocalized weighted voxels (volume of co localized voxels over the entire volume of the image above threshold) and are shown as mean ± SEM of 1-2 dendiritic segments of at least 5 cells (n=5) innervated with presynaptic markers from triplicate experiments using three independent cultures. Statistical significance was calculated according to the unpaired Student's t-test with $P < 0.05$

4 DISCUSSION

How $GABA_A$ receptor subtypes segregate so precisely to different subcellular locations is not understood. In this study, I have expressed the recombinant γ2 and δ $GABA_A$ receptor subunits in cultured hippocampal neurons to analyze the differential cell surface expression and sub-membrane segregation of synaptic and extrasynaptic $GABA_A$ receptors. My data demonstrate that the synaptic targeting of γ2-containing $GABA_A$ receptors does not depend on the cytoplasmic TM3-TM4 domain of the γ2 subunit. This result was actually surprising, until Alldred et al published their work last year (2005) showing that the synaptic localization of receptors with the γ2 subunit requires the TM4 domain rather than the large TM3-TM4 cytoplasmic loop. The premise of our work, when we started, was that the loop region would be responsible. On the other hand, I showed here that the TM3-TM4 cytoplasmic domain of the δ subunit is either a factor for the extra-synaptic clustering of the δ containing $GABA_A$ receptors (this would be an active mechanism) or alternatively that this loop region may not contain any information at all on receptor targeting, and subunits with this domain may simply lack sufficient "information" to be placed in synapses (passive exclusion). I cannot currently distinguish between these two possibilities. Nevertheless, by comparing the δ subunit TM3-TM4 loop amino acid sequences across the whole span of vertebrate evolution, I discovered that the loop was remarkably conserved. This would suggest that the loop's tertiary structure and specific proteins that bind to it are important for some function(s) of δ-containing receptors. A passive exclusion mechanism might have lead to a degeneration of the loop sequence, as found for example when the $GABA_A$ α6 subunit TM3-TM4 loop is compared across the same evolutionary distance (Bahn et al 1996).

4.1 Synaptic and non-synaptic receptors in general

For efficient and fast (millisecond) synaptic transmission neurotransmitter receptors are concentrated in synapses. This is universally true, whether when

considering the Cys loop receptor family (nAChRs clustered at the muscle end plate opposite motor neuron terminals, $GABA_A$Rs and GlyRs opposite their respective terminals in the central nervous system), or the ionotropic glutamate receptor family (AMPA and NMDA receptors) at the PSD (see Introduction). Only modulatory type receptors mediating much slower responses (often 7TM G-protein coupled receptors e.g. metabotropic glutamate or $GABA_B$ receptors) or Cys-loop receptors in the CNS associated with modulatory transmitters that cause fast responses (nAChRs and maybe 5HT via $5HT_3$ receptors) are nonsynaptically located. Nevertheless, it has become appreciated in the last few years that two types of $GABA_A$ receptor transmission co-exist on some types of CNS neurons, mediating the tonic and phasic components (see Introduction, Sections 1.1.5 and 1.1.6). $GABA_A$ receptors with □ subunits, most likey □4□□ in the forebrain and □6□□ on cerebellar granule cells are exclusively extrasynaptic, whereas a wide variety of □□□2 type receptors occur in both synaptic and extrasynaptic locations. There is no □2-containing receptor that is exclusively synaptic, although one might get this impression from some published papers; but the □2 subunit does confer the ability of the receptor to be placed stably in the synapse or at least, if considering that lateral diffusion of receptors is happening continuously, that the □2 subunit increases the receptor dwell time in the synaptic zone. Mice with no □2 subunits have no synaptic clustering of $GABA_A$ receptors (Essrich et al, 1998). Synaptic clustering of $GABA_A$ receptors is such an important feature that without this, life cannot be sustained past the early postnatal period (Gunther et al 1995). The □1 and □3 subunits are likely to perform a similar role (Seeburg et al, 1990), but as these subunit genes are only expressed in very specialized cell populations (Wisden et al 1992), they contribute to only a small fraction of total brain $GABA_A$ receptors. An additional feature is that the □2 and □ subunits confer a full single channel conductance on the □□□ or □□□2 receptor complex.

The significance of the □2 subunit for $GABA_A$ receptor pharmacology was understood from the beginning (Prtitchett et al 1989): but although the □ subunit sequence was published in the same year as □2 (Shivers et al 1989), □ remained a "dark horse" for a long time. However, Shivers et al 1989 did presciently note

that the distribution of δ subunit mRNA resembled ^{3}H muscimol autoradiographic distributions (see Introduction, section 1.1.4.1.1) and that δ receptors may be associated with brain structures that contained glomeruli e.g. oflfactory bulb, thalamus and cerebellum (which was an accurate and insightful prediction). Nevertheless, the first reports on δ subunit expression in *Xenopus* oocytes and HEK cells were unremarkable, and many research groups could not get stable expression of the subunit in HEK cells. The δ knockout mouse study was also not initially with much impact, being considered at first as something having a rather subtle phenotype (occasional seizures and changed steroid sensitivity; Spigelman et al 2003, Mihalek et al 1999, Vicini et al 2002). However, it is now fair to say that with the discovery and elaboration of studies of $GABA_A$ receptor mediated tonic (extrasynaptic) inhibition (see Introduction, sections 1.1.6 and 1.1.7), and the likely influence of tonic inhibition in various pathological states (reviewed by Mody, 2005), the δ subunit is currently centre stage of many $GABA_A$ receptor studies. Thus it becomes important to dissect the mechanism for the targeting of these δ subunit-containing receptors, hence the topic of my thesis.

4.2 Targeting of $GABA_A$ receptors to the synapse: what is known so far about the γ2 subunit's role

Originally, it was anticipated that targeting of $GABA_A$ receptors could be explained by a simple "anchoring protein" concept. In the same Cys loop receptor family, muscle nicotinic acetylcholine receptors (nAchRs) use rapsyn, glycine receptors (GlyRs) (very close relatives of $GABA_A$ receptors) use gephyrin. Ionotropic receptors are anchored with complexes of PDZ domain proteins. In all cases, particularly for the nAchRs and GlyRs receptors, a protein directly binds the large TM3-TM4 loop of one of the subunits and anchors it there (Huebsch and Maimone, 2003; Kirsch et al 1993; Kirsh and Betz, 1995). Indeed this approach, including my own, has dominated the field to search for TM3-TM4 loop binding proteins (Loebrich et al 2006; Wang et al 1999; Keller et al 2004). No direct loop-binding proteins have so far turned out to be suitable

candidates, except possibly radixin (see Loebrich et al 2005). With the discovery that gephyrin knockout mice had far fewer synaptic $GABA_A$ receptors (see below; Kneussel et al 1999), it seemed natural to suppose that the problem had been solved. The first optimism was soon dampened when it was found that gephyrin did not directly bind $GABA_A$ receptors, and that only a few subtypes of receptor were affected, and that the first study had been over interpreted (Kneussel et al 1999). It further came as a total surprise that the TM4 transmembrane domain of □2 was actually the relevant region of the protein for synaptic targeting (Alldred et al 2005). Thus the field is full of interesting challenges. So far, nobody can explain how $GABA_A$ receptors are targeted. Most likely the "anchoring protein" concept still holds, just that the real candidates are not yet identified.

As a further complexity, the □2 subunit-containing $GABA_A$ receptors are targeted and clustered to different compartments of the neuronal cell membrane depending on the particular □ subunit in the □□□2 combination. For example, $GABA_A$ receptors with □2 subunits are enriched on the axonal hillock of hippocampal pyramidal cells, although they are also present in other doamins (Nusser et al, 1996); □5□□2 receptors are enriched as extrasynaptic clusters (Brunig et al 2002), but about 25% of them are postsynaptic. Ectopically expressing □6□□2 receptors in hippocampal and neocortical pyramidal cells produces mostly extrasynaptic receptors (Wisden et al., 2002). Thus there is an extraordinary complex processing of these receptors. It is not just a simple interaction with the □2 subunit. The □ subunits must also play a role, and these signals must sometimes override the □2 signals depending on which □ subunit is involved. The actin-binding protein radixin anchors extrasynaptic □5 subunits, and by implication □5-containing receptors to the actin cytosketon via a short segment in the □5 TM3-TM4 loop, provided that the radixin protein is activated by phosphorylation (Loebrich et al 2005); radixin is enriched extrasynaptically.

The phenomenon of $GABA_A$ receptor targeting is actually quite hard to study because the distributions, with the exception of the □ subunit, do not seem to be

absolute: γ2 receptors are enriched in the synapse, but there are plenty of them extrasynaptically (Somogyi et al 1996). There is also plenty of gephyrin clusters extrasynaptically (Danglot et al, 2003). The γ2 subunit is enriched in the axon hillock, but it is also in other areas (Nusser, et al, 1996). The assays of mutations of protein domains that affect targeting are therefore based on statistical probabilities: even the receptors with no γ2 are found in the synapse. In contrast, electron microscopy data clearly show that δ subunit containing $GABA_A$ receptors are *never* located on the synapses (Nusser, et. al.,1998; Wei et al, 2003). Therefore I would argue that the synaptic targeting of δ subunit and associated $GABA_A$ receptor trafficking might fundamentally differ form γ2 containing receptors. In this way the δ subunit would be directly targeted always specially to the extra-synaptic sites from the beginning and clustered selectively.

The intracellular domain of γ2 subunit interacts with several proteins that might facilitate receptor trafficking. For example it interacts with the $GABA_A$ receptor trafficking factor GABARAP (Wang and Olsen, 2000), but since GABARAP is enriched in Golgi, it appears not to be a major factor for the synaptic clustering. In fact, mice with no GABARAP protein have normally trafficked and clustered $GABA_A$ receptors, and have no adverse phenotype (O'Sullivan et al 2005). Other GABARAP-like proteins could compensate, although these are not upregulated in the knockout; alternatively GABARAP is simply not important for $GABA_A$ receptor function, in spite of the many studies where forced expression of GABARAP and $GABA_A$ receptors influences $GABA_A$ receptor function (reviewed by O'Sullivan et al 2005).

Depolymerizing the neuronal microtubule cytoskeleton causes $GABA_A$ receptors to de-cluster (reviewed by Petrini et al 2004). The microtubule binding protein gephyrin is a major postsynaptic protein which is highly colocalized with γ2-containing postsynaptic receptors as well as with glycine receptors (Sassoe-Pognetto, 2000; Schmitt et al, 1987; Prior et al 1992). Although the intracellular domain of the β subunit of glycine receptors interacts directly with gephyrin to assemble the aggregation of postsynaptic glycine receptors (Meyer et. al., 1995;

Kneussel et. al., 1999; Kirsch et. al., 1996), this seems to be not the case for postsynaptic clustering of □2 containing $GABA_A$ receptors (Gunther et. al., 1995; Essrich et. al., 1998; Schweizer et. al., 2003; reviewed in Loebrich et al 2006). Indeed, current data (Kirsch, 1999; Kneussel et. al., 1999; Levi, et. al., 2004; Jacob et. al. 2005) suggest that gephyrin could be used for the maintenance or refinement rather than construction of inhibitory synapses (reviewed and elaborated on by Alldred et al 2005).

The palmitoyltransferase GODZ (Keller et. al., 2004) interacts with a cysteine motif located in the cytoplasmic TM3-TM4 domain of the □2 subunit (Rathenberg et. al., 2004). Inhibiting this interaction decreases the steady state cell surface receptor number of $GABA_A$ receptors and synaptic clustering which suggests this domain as a prominent factor for receptor density via its palmitoylation. Perhaps the TM3-TM4 cytoplasmic domain of □2 subunit is involved in the modulation of the ratio of density of synaptic versus non-synaptic □2 containing receptors, rather than the total receptor number for a given state. This implies that it must be a modulatory element for synaptic plasticity rather than an essential factor for membrane targeting and synaptic clustering of the receptors, (although most of the proteins i.e., $GABA_A$ receptor acting proteins, $GABA_A$ receptor-associated adaptors, kinases, phosphateses, and extracellular signals (reviewed by Luscher and Keller, 2004) that have been identified as the modulators of the trafficking and cell surface expression of $GABA_A$ receptors interact mainly with □ subunits).

4.3 The cytoplasmic domain (TM3-TM4 loop) of the γ2 subunit, when placed into the context of a β subunit backbone, is not directly involved in synaptic clustering or targeting of $GABA_A$Rs and does not influence cluster number

I have shown that the synaptic clustering of $GABA_A$ receptors does not depend on the cytoplasmic domain of the γ2 subunit. On the other hand, another group using a similar experimental paradigm to ours (except they used C-termally EGFP-tagged subunits rather than N-terminal tags) has also recently addressed the importance of the TM3-TM4 domain as a major factor for synaptic $GABA_A$ receptor targeting; they found a significant reduction in the synaptic targeting of receptors containing ^{GFP}γ2TM3-TM4β chimeric subunits compared with receptors containing ^{GFP}γ2 (Christie, et. al., 2005). Our data agree with the reduced clustering at synaptic sites for the ^{GFP}γ2TM3-TM4β subunit, but since we have used both ^{GFP}βTM3-TM4β and ^{GFP}βTM3-TM4γ2 chimeric subunits as test constructs in our experiments (Christe et al could not get β-based chimeras to express), we demonstrate that the 'deviation' of the ^{GFP}γ2TM3-TM4β construct from the synaptic targeting profile of the ^{GFP}γ2 control construct was not because of lack of the TM3-TM4 domain of the γ2 subunit but due to the existence of the corresponding domain of β subunit. I showed using a ^{GFP}βTM3-TM4γ2 subunit that the TM3-TM4 domain of γ2 was not sufficient to provide a preferential synaptic clustering on the rest of the β subunit backbone (β subunit N-terminal area, four transmembrane domains, i.e., TM1, TM2, TM3, TM4 and C terminus, but not the cytoplasmic loop). Therefore the cytoplasmic domain of γ2 subunit, at least by itself, is not a major factor for synaptic clustering of $GABA_β$ receptors. In agreement with our data, Alldred et al., (2005) have recently reported that the fourth transmembrane domain (TM4) of this subunit is actually the critical domain for synaptic targeting of γ2 containing $GABA_A$ receptors. Similarly, the TM4 domain of α and βsubunits of nAChRs have been proposed to interact directly with the phospholipid bilayer of the cell membrane, facilitating the postsynaptic clustering of these receptors, presumably in collaboration with

rapsyn binding the large TM3-TM4 loop (Bruses et. al, 2001; Hering et. al., 2003; Pediconi et. al., 2004, Alldred et. al., 2005).

As a further finding, I discovered that the TM3-TM4 β2 cytoplasmic domain is not critical for receptor cluster density: if it were, the chimeric β2 subunit possessing the large TM3-TM4 cytoplasmic domain of the γ subunit (^{GFP}β2TM3-TM4γ) would have given $GABA_A$ receptors with a corresponding decrease in the receptor density; however my data show that this was not the case, rather the receptor density was almost unchanged. Similarly, γ subunits with the cytoplasmic domain of β2 subunit did not influence receptor density as this had almost the same value for the receptor density as just the γ.

4.4 The large TM3-TM4 cytoplasmic domain of the γ subunit: a putative domain for the selective extrasynaptic targeting of γ subunit-containing $GABA_A$ receptors

I found a statistically significant reduction in the synaptic clustering of $GABA_A$ receptors with the ^{GFP}β2TM3-TM4γ subunit compared to receptors with ^{GFP}β2. In other words, the ^{GFP}β2TM3-TM4γ subunit chimera behaves like ^{GFP}γ as regards preferential nonsynaptic localization on the cell surface of hippocampal neurons. How does the cytoplasmic domain (TM3-TM4 loop) of γ subunit specify the nonsynaptic targeting of associated receptors? Does it lack the necessary motifs for synaptic clustering, and is therefore restricted from the synapses and located extrasynaptically by default (passive mechanism)? Alternatively, does the γ subunit have some specific motifs that anchor receptors containing it selectively to the nonsynaptic zones (active mechanism)? Indeed, one of the test constructs (^{GFP}β2TM3-TM4γ) that we have used in our culture system to study the membrane targeting pattern may hint at an active mechanism for the nonsynaptic clustering of γ-containing $GABA_A$ receptors. This chimera consists of the large TM3-TM4 intracellular loop from the γ subunit but the rest of the subunit is composed of the original β2 peptides, including the β2 TM4 domain critical for the synaptic

clustering. Therefore this ^{GFP}(β2TM3-TM4α) chimera represents the model for the competition of two different domains (β2TM4 and αTM3-TM4 loop) with opposing effects on the synaptic versus nonsynaptic targeting. When expressed exogeneously in the cultured hippocampal neurons, this chimeric subunit most probably co-assembles with endogeneous α and β subunits to give a mixture of receptors located synaptically (β2 TM4 influence) and extrasynaptically (α loop influence), with the majority of receptors extrasynaptic. This possibly competitive action between domains might explain why many receptors (40%) are still in the synapse. On the other hand, as the ^{GFP}(αTM3-TM4β2) behaves similarly to ^{GFP}(β2TM3-TM4α), giving the same extrasynaptic versus synaptic distribution, and would rather undermine the previous argument. To more rigorously test this, I would have to make subunit chimeras with say the α subunit up until TM3 followed by the entire β2 subunit including TM4 sequences, and vice versa.

4.5 ^{GFP}α subunit-containing $GABA_A$ receptors form clusters on the cell surface of hippocampal neurons

I have shown that similar to β2 containing $GABA_A$ receptors, α subunit-containing $GABA_A$ receptor chimeras are not- diffusely distributed, as perhaps might have been expected, but instead, when expressed ectopically in hippocampal pyramidal cells, form clusters. This result with α subunits contrasts with the results obtained by Christie et al 2005, who found that the recombinant α subunit, tagged with GFP at its C-terminus, when expressed ectopically in hippocampal pyramidal neurons was diffusely distributed extrasynsptically. They used C-terminally tagged α with EGFP; this might give a different result.

Why do extrasynaptic αβcontaining $GABA_A$ receptors form clusters? For extrasynaptic α5β2 $GABA_A$Rs, which are also highly clustered, the clustering does not seem to be important for the associated tonic inhibition (discussed in Loebrich et. al. 2006). The α receptors in clusters may exist as “GABA-sensitive”

rafts in the extrasynaptic membrane. Interestingly, in the case of δ-containing $GABA_A$ receptors in hippocampal cultures, de-clustering by inhibiting the polymerization of microtubules leads to a decrease in the associated tonic inhibition, possibly indicating that the degree of clustering regulates tonic inhibition (Petrini et. al., 2004). However, the findings in this paper are based on an apparent strong reactivity with a δ subunit antibody that stains putative pyramidal cells; as δ is not normally expressed in hippocampal pyramidal cells in vivo, or at least only at a low level, this paper should be interpreted with caution.

Do clusters of δ-containing $GABA_A$ receptors occur on native neurons? Yes. Sun et al 2004 found δ subunit clusters on dentate granule cell bodies, in rat hippocampal slices, and also some diffuse membrane staining; the δ subunit clusters rarely occurred opposite GAD-positive terminals, frequency of about 1% (Sun et al 2004). However, using the same antibody, the same group also found non-clustered δ immunoreactivity on cultured hippocampal neurons (Mangan et al 2005), but one wonders what kind of staining they were really looking at, and if this was really specific, a common problem with antibodies.

4.6 Caveats to the hippocampal neuron transfection experiments

4.6.1 Limitations on forced ectopic expression of subunits

The δ subunit is not normally expressed in either developing or adult hippocampal pyramidal cells in vivo, or if it is, the levels are very low (Wisden et al 1992; Sperk et al 1997). In my study, forced expression of the δ subunit in these cultured pyramidal cells is thus ectopic. Further, the CMV and Thy1-2 promoters are quite powerful; overexpressing a subunit could flood the natural subunit assembly mechanisms of the cells and produce artifactual results. Ideally, one would need to repeat these experiments using an in vivo setting, expressing the subunit chimeras at physiological amounts in cell types which normally express the δ subunit (e.g. dentate granule cells, cerebellar granule cells, certain thalamic relay cells). The danger of interpreting in vitro experiments by forced

subunit expression is highlighted by the δ6 knockout mouse (Jones et al 1997). With no δ6 protein, the δ protein disappears from granule cells (Jones et al 1997). There is plenty of δ1 subunit left in the granule cells, but it does not save the δ subunit from degredation, even though in HEK cells δ1 assembles well with the δ. Cell-type mechanisms for subunit assembly must be taken into account.

4.6.2 Limitations of light microscopy

From the images I obtained with the confocal microscope, I concluded that many δ-related subunits were in the synapse, as judged by apparent co-localization with presynaptic markers. However, the subunits could be perisynaptic in an annulus i.e. they could be surrounding the synapse rather than actually in the synapse. The light miscroscope does not give accurate resolution. This would change the interpretation of my experiments. This annular arrangement exists for native δ subunits on dentate granule cells as seen by electron microscopy (Wei et al, 2003).

4.7 Evolutionary conserved regions in the TM3-TM4 loop of the δ and δ2 subunits of $GABA_A$Rs

I was interested to use a bioinformatics approach to examine if there may be some functional motifs in the cytoplasmic domain (TM3-TM4 loop) of the δ subunit that might confer some protein-protein interactions. Amino acid sequence comparisons of gene orthologues across large stretches of evolutionary time (e.g. 550 million years separating fish and mammals) might indicate particularly important sequences, as they would have survived purifying natural selection, assuming the mechanism of receptor targeting also to be conserved. By aligning multiple sequences of the predicted cytoplasmic TM3-TM4 domain of the δ subunit across vertebrate species, I identified fully or partially evolutionary conserved aminoacid residues (Figure 4.1). Moreover, some of these residues (green and yellow boxes in the Figure 10) seem to be frequently occurring motifs

in the proteins according to ENSEMBL database (see Methods section 2.5). I identified a putative site (S378, X379, R380) for protein kinase C (PKC), which is evolutionary conserved in the subunit of vertebrates including fish species and chicken (Figure 4.1). Therefore this motif might have an important influence on receptor function, but it is difficult to propose any functional role for this motif without experimental evidence.

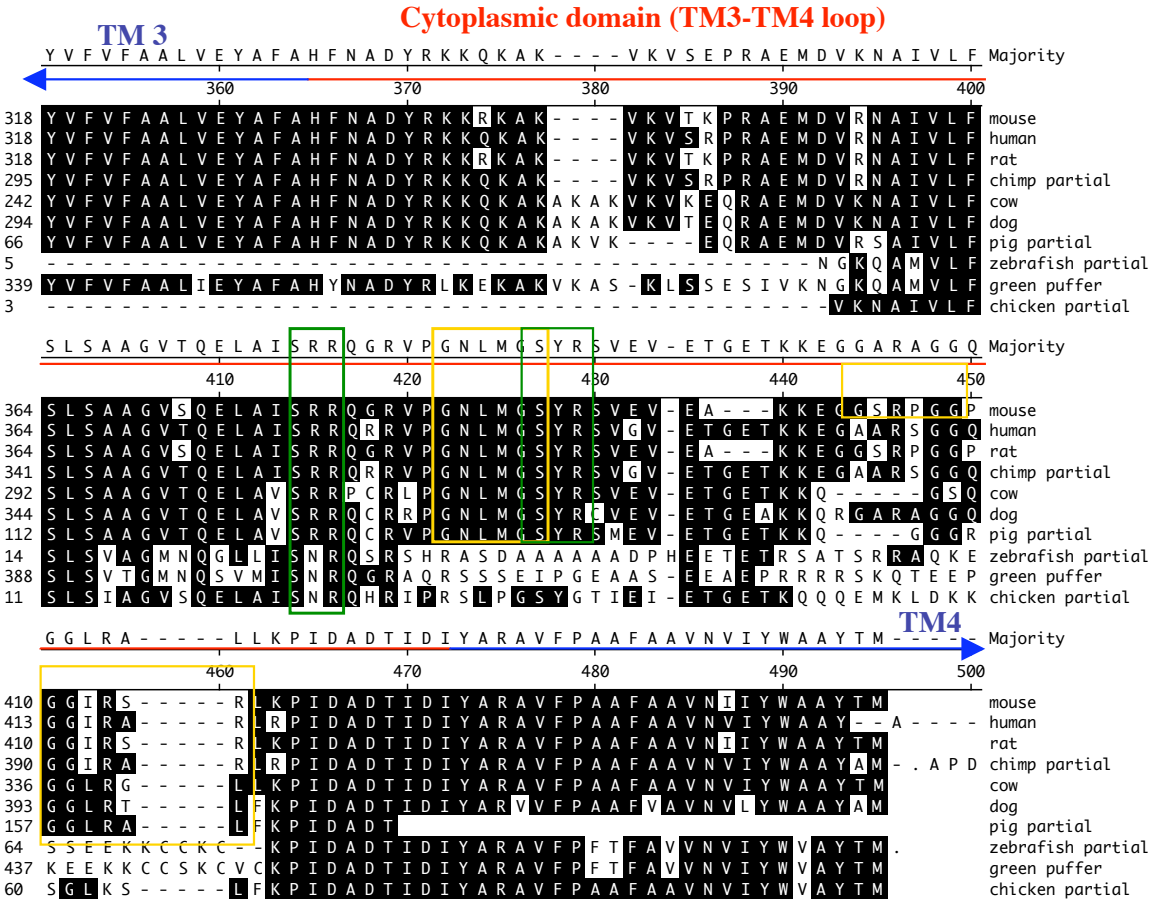


Figure 4.1: Aminoacid alignment of cytoplasmic domain (TM3-TM4 loop) of subunit across vertebrates. 2 putative motifs (SXR) for phosphokinase C (PKC) site is shown by green boxes. One of these, the residues S 378, X379, R380 (according to mouse) is evolutionary conserved. The VLFSLS at the proximity of S378 is also 100% conserved. There are also 3 myristoylation sites as shown by big or small yellow boxes. Two of these sites are partially conserved (big

However, it is generally known that $GABA_A$ receptor phosphorylation is a major mechanism for the modulation of receptor activity i.e., the channel kinetics or receptor internalization, via the residues within the intracellular domains of mainly β and γ2 receptor subunits by a range of serine theronine and tyrosine kinases in a subunit and neuron-specific manner (reviewed by Kittler and Moss, 2003).

Interestingly, multiple aminoacid alignment of cytoplasmic domain (TM3-TM4 loop) of γ2 subunit (Figure 4.2) is much more conserved when compared with that of β subunit (Figure 4.1). The homology between cytoplasmic domain of γ2 subunit from human and chicken lines which are thought to have diverged about 300 million years ago could be aligned with 100% similarity. In the fugu fish, which diverged from the human line more than 400 million years ago partially sequenced regions could be aligned with almost 90 % similarity (Figure 4.2). Although the cytoplasmic domain of γ2 subunit does not appear to be determinant for synaptic clustering of $GABA_A$ receptors (see section 4.3), the appareantly conserved aminoacids shown in the Figure 4.2 addresses that the this domain (TM3-TM4 loop) have some very important roles in the receptor function. In deed current data in the literature suggest that extracellular signals and activity-dependent modulation of GABAergic synapses is mediated by phosphorylation and Ca^{2+} dependent signaling cascades, especially on the β subunit variants as well as γ2 subunit in the TM3-TM4 loop region (rewieved in Luscher and Keller, 2004). Therefore the strong conservation in the TM3-TM4 loop region of γ2 subunit among the whole span of vertebrate evolution is inline with the view supporting its potential for synaptic plasticity. However, further investigation needs to be done to refine this point of view, because the effect of any interacting partners on the trafficking, stability and function of $GABA_A$ receptors is not known.

With this study I have identified the TM3-TM4 loop of the β subunit as a key element for the extrasynaptic targeting of corresponding $GABA_A$ receptors; these

receptors appear to have a fundamentally different targeting mechanism than synaptic γ2-containing $GABA_A$ receptors.

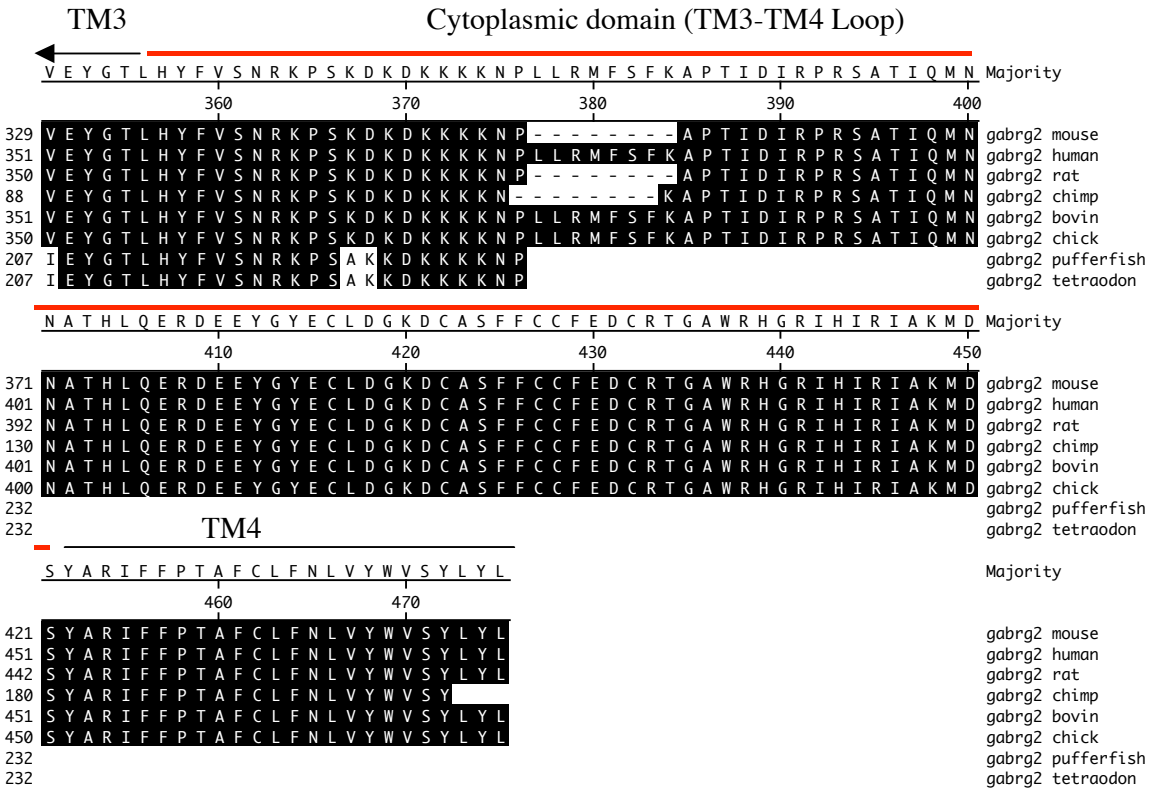


Figure 4.2: Aminoacid alignment of cytoplasmic domain (TM3-TM4 loop) of γ2 subunit across vertebrates. The cytoplasmic domain of γ2 subunit is highly conserved among the vertebrate evolution. Although pufferfish and tetraodon species genes have not been sequenced totally to evaluate this homology more precisely, the partial sequences are significantly similar with the other species.

Determination of functional motifs and interacting partners with mutagenesis experiments and GST pull down assays will be useful for furthering our, so far very limited, knowledge of mechanisms involved in regulating protein sorting in neurons, and eventually feed into the larger picture of how neuronal diversity is generated.

5 APPENDIX

The Appendix contains supplementary material on methods relating to subcloning of DNA fragments, *in vitro* mutagenesis of plasmids, culture of HEK cells and neurons, and immunodetection of $GABA_A$ receptor subunits expressed in HEK cells.

5.1 Plasmid preparation

Plasmids were isolated from 1.5 ml cultures (minipreps) by alkaline lysis with SDS according to Protocol 2 given in volume I of Sambrook and Russell, 2001. For all plasmid preps I used DH5 (Invitrogen) for bacterial transfomation. For plasmids which were to be transfected into neurons I used maxipreps (QIAfilter Plasmid Maxi Kit, Qiagen).

5.2 DNA gel electrophoresis

DNA fragments from digested plasmids were separated by electrophoresis on 0.8 to 1.0 % agarose gels (containing 0,5 g/ml ethidium bromide) in 1 x TAE buffer (800mM Tris-HCl, 400mM NaOAc, 40mM EDTA, pH 8.3 adjusted with acetic acid).

5.3 Destroying enzyme sites in plasmids by blunting and religation

1 μg of plasmid (e.g. pRK5GFP 2) (1 g is typically 1 l of a standard plasmid miniprep) was linearized with SalI in 20 μl reaction mixture for 1 h at 37°C and then blunted using Klenow enzyme (Roche) (10 μl pRK5GFP 2 digestion, 1 mM each dNTP, 2 μl 10X filling buffer, 1 U Klenow enzyme and H_2O up to 20 μl) for 15 min at room temperature. Klenow was heat inactivated at 70°C for 20 min. The vector was then recircularized: to 8 μl of heat inactivated Klenow mixture, I added ligation buffer, ATP and T4 ligase; after 4 h at room temperature the ligation was transformed into DH5 *E. coli* cells. Randomly picked colonies

were minicultured and the isolated plasmid DNA was restricted with SalI, electrophoriesed in agar gels; supercoiled (i.e. non-cutting) plasmids were chosen.

5.4 Removing inserts from plasmids by digestion and religation.

1 μg of plasmid (e.g. pRK5^{GFP}□2(Sal)^2) (1 □g is typically 1 □l of a standard plasmid miniprep) was digested with SalI in 20 μl reaction mixture for 1 h at 37°C, and then heat inactivated at 70°C for 20 min. The vector was then recircularized: to 8 μl of heat inactivated digestion mixture, I added ligation buffer, ATP and T4 ligase; after 4 h at room temperature the ligation was transformed into DH5□ *E. coli* cells. Randomly picked colonies were minicultured and the isolated plasmid DNA was restricted with SalI, electrophoriesed in an agar gel, and appropriate plasmids selected by size of fragment.

5.5 Construction of Thy1 GFP-GABA$_A$ receptor subunit expression vectors

The Thy-1.2 expression cassette plasmid (Caroni, 1997) was linearized with XhoI, which is a unique insertion site downstream of the Thy1 promoter, blunted with Klenow polymerase, and SmaI-EcoRI adaptors ligated onto this plasmid linearized backbone, and then EcoRI fragments of GABA$_A$ receptor cDNAs ligated into this to recircularize the plasmid (Wisden et al 2002). In detail, this was done as follows: 2 □l (2 □g) of Thy1.2 plasmid was linearized with XhoI in a 20 □l digestion volume; 10 □l of this digest was then blunted with Klenow (see above sections for details on blunting), the Klenow then heat killed at 70ºC; 10 □l of this linearized blunted Thy1 vector was mixed with 6 □l of SmaI-EcoRI adaptors at 0.4 □g/□l (Stratagene), 2 □l of 10X ligase buffer (plus rATP) and 2 □l of T4 ligase (NEB) and left at 16ºC overnight. The reaction volume was then increased to 100 □l with TE buffer (0.01 M Tris/0.1 mM EDTA, pH 7.6, sterile) and then spun through a Chromaspin 1000 TE column (Clontech) to remove the excess adaptors. To ligate in the EcoRI gel purified GABA$_A$ cDNAs, I mixed

10 µl of the column eluate, 20 µl of EcoRI gel pure cDNA insert (see Methods), 70 µl water, 1 µl glycogen stock (as carrier), 10 µl 3 M sodium acetate, and 250 ml 100 % ethanol, followed by spinning, washing the pellet in 70 % ethanol, and resuspending in 8 µl water, 1 µl 10X ligation buffer (plus rATP) and 1 µl T4 ligase; the reaction was left at 16 °C overnight. The ligation mix was transformed into DH5α cells, and colonies with inserts selected by colony screening using the cDNA as a hybridization probe. The correct orientation of inserts was verified by restriction mapping and confirmed by sequencing with Thy1 primers upstream of the XhoI site.

5.6 Colony screening protocol for ligations

The TM3-TM4 loop inserts in the pRK5GFP-2TM3-TM4α and pRK5GFP-TM3-TM4-2 subclones were identified by colony screening of ligations. Agar plates containing transformed colonies were kept 1 h at 4 °C before colony lifting. Afterwards, the colonies were lifted on Hybond N+ (Amersham) membranes (after the membranes and plates had been co-marked with pinholes) and the membranes were transferred (colony side up) for 5 min on Whatmann paper soaked in 2 x SSC/5% SDS solution for cell lysis (Buluwela et al., 1989). The filters were then microwaved at high power for 2.5 minutes, so crosslinking DNA with the membrane (Buluwela et al., 1989). Colony lifted agar plates were kept 24 hours at room temperature allowing the regrowth of the colonies for later picking. The membranes were hybridized overnight at 42°C with ^{32}P-labeled (random-primed) TM3-TM4 α2 or α loop fragment probes (5 µl of [α-^{32}P]dCTP, Specific activity: 110 TBq/mmol, Radioactive concentration: 370 MBq/ml from Amersham is used for the radiolabeling of DNA probe with Rediprime II DNA Labelling Kit (Amersham)): briefly, the DNA probe (approximately 1 µg is diluted 44 µl TE buffer) is denaturated at 95 °C for 5 min and then kept in ice for 5 minutes and added to the Rediprime labelling kit. After the addition of radioactive dCTP, the final mixture was mixed with the micropipette and incubated at 37 °C for one hour. The un-incorparated oligonucleotides were removed by Chromaspin 100 (1000) TE columns from

Clontech. After measurement of the degree of radioactive labelling, the probe is denaturated before the hybridisation (hybridization buffer contained 50% formamide/5 x SSC). After hybridization, filters were washed once with 2 x SSC solution at room temperature for 5 min and twice with 0.1 x SSC at 65 °C (for restriction fragment probes) and 1 x SSC at 60 °C for oligonucleotides (30 to 45 mers); filters hybridized with shorter oligonucleotides were washed with 1 x SSC at 42 °C. Hybridized filters were exposed 2 h to autoradiographic film (X-OMAT, Kodak) at -70 °C in cassettes with scintillation screens. Positive colonies were picked and minicultured; plasmids were isolated and analysed by restriction enzyme digestion and gel electrophoresis. (1 X SSC: 150 mM NaCl/15 mM NaCitrate).

5.7 In vitro mutagenesis

Site-directed mutagenesis was carried out using oligonucleotides (Invitrogen) and the Stratagene Quick Change mutagenesis kit, in which oligonucleotides with the desired mutation are annealed to plasmid DNA to prime *in vitro* DNA synthesis with Pfu DNA polymerase, and selection of mutants is done by DpnI digestion. Briefly, with the use of a thermal re-cycler, complementary primers (each 125 ng) carrying the desired mutation in the middle of (at least) 24-mer oligonucleotides (with at least 40 % GC content) were allowed to anneal 1 minute at 55-60 ºC to denaturated parental DNA plasmid (5-50 ng of dsDNA template; usually I used 1-5 μl of a 1:100 diluted miniprep) in a reaction volume of 50 μl in the presence of dNTPs, Pfu Turbo and reaction buffer as described in the protocol of the supplier. Then the DNA polymerase (Pfu Turbo, with proofreading ability) extends and incorporates the mutagenic primers resulting in nicked circular strands (for 30 seconds at 68 ºC). After several cycles (18 times) of the same procedure that have been performed in the thermal re-cycler, the reaction mixture is allowed to cool in ice for a couple of minutes and then at 37□C, it is treated with Dpn1, an enzyme that selectively digests the methylated or parental DNA and thus allowing to isolate the mutated DNA. Enzymatic digestion were carried out for 1 hour and then the Dpn1 was inactivated at 65 ºC

for 15 minutes, before subsequent transfomation into competent bacteria (provided by the mutagenesis kit). The DNA extracted from the colonies was sequenced to verify mutagenesis.

5.8 In vitro expression of $GABA_A$ Receptors in HEK 293 cells

5.8.1 Preparation of plating medium for HEK 293 cells:

The coverslips were washed with 70 % ethanol followed by dry-heat sterilization in a box between layers of kimwipes. The surface of the coverslips was then covered with about 300 μl of Fibronectin (5-10 μg/ml) in 24-well plates, before washing with 1x PBS and letting them dry for half an hour and subsequent plating the HEK cells.

5.8.2 Plating of HEK 293 cells

Frozen aliquots of Human Embryonic Kidney Cells (i.e., HEK cells, A293; ATCC CRL 1573) were stored in liquid nitrogen. When needed, they were thawed quickly in a water bath at 37 °C, transferred into a tube containing warm medium and spun down at 800 rpm/RT for 5 min. The pellet was re-suspended in 10 ml warm medium and plated onto a 94 mm ∅ dish and cultivated in an incubator at 37 °C and 5 % CO_2. When they reached a suitable density they were split; after a washing step with 1x PBS to remove dead cells, the remaining cells were transferred with warm medium into a centrifugation tube to spin them down. Afterwards they were resuspended in warm medium and plated on 12-or 24-well plates in the desired density. HEK cells were maintained in DMEM (Gibco) supplemented with 10 % fetal bovine serum. The plates were incubated at 37 °C and 5 % CO_2 until the day of transfection (normally after one day from the seeding).

5.8.3 Transfection of HEK 293 cells by $Ca_3(PO_4)_2$ precipitation

Ingredients	per1 ml culture
Plasmids (200 ng /□l)	6 □l
H_2O	37.75 □l
$CaCl_2$ (2M)	6.25 □l
2 x BBS	50 □l
vortex and wait 80-90 seconds…	
Medium DMEM (Gibco)	0.9 ml

Table 5.1: Transfection protocol for HEK cells

Exponentially growing HEK cells, seeded at 4 x 10^4 cells/well/24 well-plate, were transfected by $Ca_3(PO_4)_2$ precipitation using equimolar ratios of expression constructs (Conolly et al., 1996). The transfection procedure (1ml for 2 coverslips on 24 well plate and 1 coverslip on 12 well plate) is described on the Table 5.1. After transfection, cells were kept at 37 °C, 3 % CO_2 incubator overnight, then the medium was renewed and the cells were transfered to 5 % CO_2 incubator. Cells were analyzed 48 hrs after transfection.

(2x BBS: 50 mM BES (NW 213) Sigma cat. #B-9879, 280 mM NaCl, 1.5 mM Na_2HP0_4, pH: adjusted to 6.89 with NaOH).

5.9 Immunostaining of HEK 293 cells

A293 cells on Fibronectin coated coverslips were washed with warm PBS two times and then cells are fixed with cold 4 % PFA for 10 minutes at 4 °C (coldroom). After two or three washings with cold PBS (described in Methods, section 2.3) the cells are permeabilized (if desired) in 0.2 % Triton in 1xPBS. Nonspecific binding of the first antibody was eliminated by blocking in the cells with 4% NGS in the presence of 0.2 % Triton in PBS for 30 min. The primary antibody was applied in recommended dilution (anti-green fluorescent protein rabbit serum, 1:10000 dilution, Molecular Probes, USA), in (0.1% Triton, 4 % NGS) PBS and for at least two hours at RT, or over night. Afterwards cells were washed twice in PBS. The secondary antibody (CY-3 anti-rabbit, Jackson Laboratories) was applied in 1.5 % NGS for 1-2 hours at room temperature before washing again two times with PBS and a final washing step in water, the coverslips were mounted on slices for microscopy with Mowiol.

5.10 Immunoprecipitation and Western Blot of Protein Extracts from HEK cells

5.10.1 Preparation of cell lysates

Transfected HEK cells in 90 cm dishes were maintained for 2 days in the appropriate incubator, then the cells were washed with 5 ml PBS (warm) once, before the harvesting of cells with 1 ml of chilled Lysis Buffer (25 mM HEPES, 150 mM NaCl, pH 7.4, 1 % Triton (w/v)) including Protease inhibitor cocktail tablet (Roche). The cells were collected in prechilled 1 ml reaction tubes and incubated at 4 °C on an overhead shaker for at least half an hour before centrifugation at full speed on a table-top centrifuge for 10 minutes at 4 °C. The supernatant was collected in to clean cold eppendorf tubes and the pellet was discarded. (The supernatant could be stored at –80 ºC at this step for future use.)

5.10.2 Immunoprecipitation

5.10.2.1 Preclearing of cell lysate

20 µl of washed protein A slurry (approximately 50 µl of protein A slurry washed 2 times with 800 µl of cold 0.1 % Triton, 25 mM Hepes, 150 mM NaCl pH 7.4, including protease inhibitor cocktail, each time spinned at 2500 rpm for 1 min at 4 ºC on a table top centrifuge, the beads were collected by sterile micropipette tips cut from the tip-end) was added to 950-1000 µl of cell lysate and incubated for 30 minutes at overhead-shaker at cold room before spinning them at 14000 rpm for 10 minutes at 4 ºC.

5.10.2.2 Protein-Antibody Coupling

1.5 µg of anti-GFP antibody (Anti-AFP mAb 3E6, AFP 5002, Qbiogene Inc.) was added to each supernatant obtained from the previous step containing cold precleared lysates (around 800 µl) and incubated for 2-3 hours (or overnight) at 4 °C at overhead-shaker.

5.10.2.3 Protein A-Antibody Coupling

After the protein antibody coupling in the previous step, 20 µl of pre-washed protein A slurry (Protein A Agarose, Fast Flow, 50 % v/v, P3476-5ML, Sigma) was added to each sample and incubated 1 hour at 4 ºC, overhead shaker. The samples were spun at 2500 rpm for 1 min at 4 ºC; and washed three times with chilled HEPES buffer containing 0.1 % Triton (w/v), 25 mM HEPES; 150 mM NaCl, pH 7.4 with protease inhibitor cocktail (Roche). Elution was achieved by adding appropriate amount of loading buffer i.e., 20 µl of 2x loading buffer (for western blot) and boiling the samples at 95 ºC for 5 min. The samples were spun for 1 minute on a table top centrifuge before loading on the SDS-PAGE gel.

5.10.3 Western blot

The gels were applied 120 Volts for stacking gel and at 140 Volts for resolving gel at RT, for about 1– hours with 1x Running Buffer (10X Running Buffer Stock: 30.3g. Tris; 144g Glycine; 10g SDS dissolved in 1 liter distilled H_2O, pH 8.3).

	4 % Stacking Gel	10 % Resolving Gel
DDI H_2O (ml)	6.1	4.1
30 % Degassed Acrylamide/Bis(ml)	1.3	3.3
Gel buffer* (ml)	2.5	2.5
10 % w/v SDS (ml)	0.1	0.1
TEMED (µl)	12	9
10 % (w/v) APS (µl)	100	80

Table 5.2: Ingredients of the acrylamide gels for PAGE. *Stacking Gel Buffer: 0.5 M Tris-HCl, pH 6.8. *Resolving Gel Buffer: 1.5 M Tris-HCl, pH 8.8

Blotting:

The gel slabs were removed from the glass blocks carefully and assembled in to transfer apparatus for protein transfer to membranes (Hybond-P, Amersham, UK); which were pre-soaked in methanol and then in running buffer (as described in the user manual) before setting up the blot. Transfer was achieved at 90 mA over night at room temperature with Transfer Buffer: 100 ml methanol, 800 ml H_2O, 100 ml of 10x Transfer buffer (10 X stock: 30.3 g Tris, 144 g Glycine dissolved in 1 liter distilled water).

Blocking:

20 ml of blocking solution was used per membrane at least 30 minutes at room temperature, shaking.

Blocking solution: 10 % Milk (w/v) in 0.05 % PBST

20 gr milk powder

1 ml of 10 % PBST (10 % Tween (Sigma) v/v in PBS -137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.4)

final volume 200 ml PBS

Primary Antibody:

GFP Ab (Anti-green fluorescent protein rabbit serum; Molecular Probes, USA), 1:10000 dilution, in 8 ml of 0.05 % PBST, incubated at room temperature with shaking at 35 rpm for 2 hours. Then the membranes were washed 3 times for 5 minutes with at least 10 ml of 0.05 % PBST, at room temperature 40 rpm.

Secondary Antibody:

1:20000 diluted peroxidase labelled anti-rabbit IgG (Vector Laboratories, CA) in 8 ml 0.05 % PBST, 1 hour at room temperature, shaking at 35 rpm, then washed three times as before.

The membranes were treated with 1 ml ECL plus Western Blot Detection System (Amersham, UK) reagent for maximum 5 minutes and then exposed appropriate time duration for development of the films.

5.11 Expression of □ and □2 $GABA_A$ Receptor Subunit Chimeras in HEK cells

5.11.1 Chimeric □ and □2 cDNAs are translated into protein without truncation

HEK cells were transfected with CMV-promoter-based expression plasmids of the $GABA_A$ receptor subunits; to ensure stable receptor expression, I co-transfected cells with □1 and □2 subunit cDNA expression plamids in addition to the test subunit (i.e. triple transfections). GFP expression was detectable for all subunits during live cell fluorescence imaging (data not shown). Transfected HEK cells also stained strongly with a GFP-specific-antibody (see section below). However, western blot analysis of HEK cell extracts transfected with chimeric $GABA_A$ receptor cDNAs did not reveal any bands of the expected size; note that a high amount of immuno-positive aggregated protein was visible on top of the gel in each case. But after immunoprecipitation of HEK cell extracts with GFP antibody, the staining of immunoblot against GFP gave signals in the size range of ^{GFP}□2 or ^{GFP}□ and chimeric receptors (Figures 5.1a, and b, Figure 5.2). The native murine □2 subunit has a size of 55 kDa and native □ subunit has a size about 50 kDa and GFP is 25 kDa. Therefore chimeric ^{GFP}□2 is 80 kDa and ^{GFP}□2TM3-TM4□ is 83 kDa, ^{GFP}□ is 75 kDa and ^{GFP}□□□ 3□□□ 4□2 is 77 kDa.

a) GFP Immunoreactivity of protein extracts from HEK cells transfected with ^{GFP}□2 or ^{GFP}□2□ cDNAs

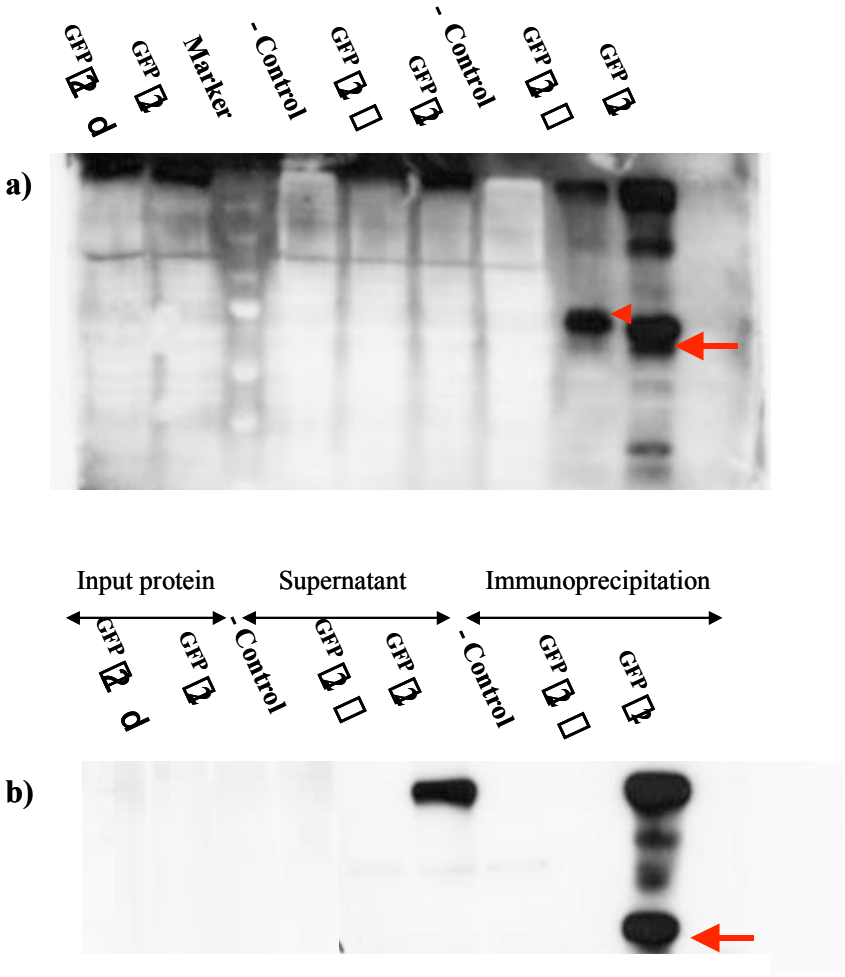


Figure 5.1: Immunoblotting of HEK cell protein extracts obtained after transfection of HEK cells with cDNAs of ^{GFP}□2 and ^{GFP}□2TM3-TM4□. GFP immunoreactivity (shown by red arrowhead for the GFP□2□ and red arrow for GFP□2 signals) is shown in a and □2 (specific for TM3-TM4 loop) immunoreactivity (shown by red arrow) is shown in b. Protein extracts obtained from transfected or untransfected HEK cell lysates (negative control) were loaded to SDS-PAGE gels for immunoblotting directly after protein extraction (input protein) or after immunoprecipitation with GFP antibody (immununoprecipitation), the supernatant from immuno-precipitated samples were also loaded (supernatant).

b) GFP immunoreactivity of protein extracts from HEK cells transfected with^{GFP} and ^{GFP} 3 4

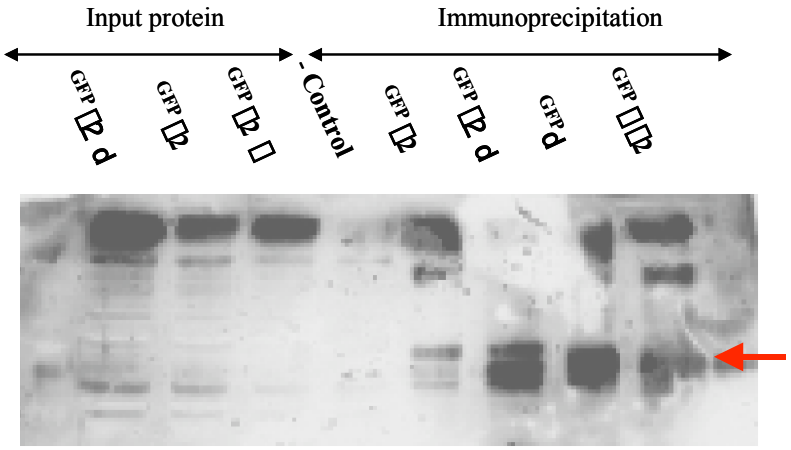


Figure 5.2. Immunoblotting of HEK cell protein extracts obtained after transfection of HEK cells with ^{GFP} and ^{GFP}TM3-TM4 chimeric cDNAs. Cells transfected with cDNAs of ^{GFP}, and ^{GFP} 3 4 were used as positive controls. Protein extracts obtained from transfected or untransfected HEK cell lysates (negative control) were loaded to SDS-PAGE gels for immunoblotting directly after protein extraction (input protein) or after immunoprecipitation with GFP antibody (immununoprecipitation).

5.11.2 Chimeric $GABA_A$ receptors are targeted to the cell surface of HEK cells

To check the expression and membrane targeting of chimeric and GFP-tagged $GABA_A$ subunits, transfected HEK cells were stained with a GFP-selective antibody and examined under the laser scanning confocal microscope. In Figures 5.3A and B, staining with the GFP antibody in unpermealized conditions for cultured HEK cells transfected with and (in the presence of 1 and 2) is shown. The surface membrane-targeted receptors are visible in red, the internal GFP expression is detected as green, which may correspond to synthesized subunits not targeted to the membrane, or that are in the process of being transported (or removed) to/from the outer membrane.

Similarly, *in vitro* expression of the ^{GFP} or ^{GFP}TM3-TM4 2 subunits in the presence of 1 and 2 subunit gave GFP immunoreactivity as shown by red color in the outer membrane (cell surface) as well as internal receptors shown by green color (Figures 5.3C and D).

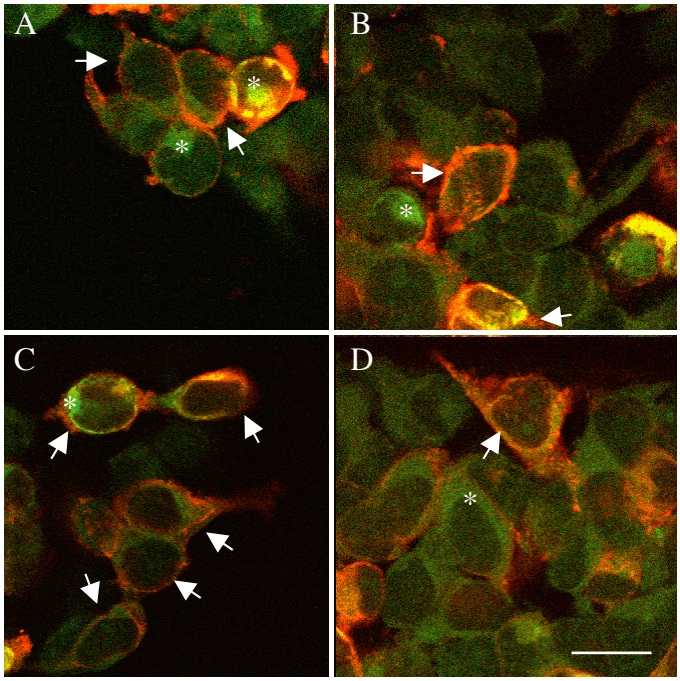


Figure 5.3: Expression of chimeric constructs in HEK cells. Scanning confocal images of fixed and immunostained cells transfected with ^{GFP} 2 (A), ^{GFP}2 3 4(B), ^{GFP} (C), ^{GFP} 3 4 2 (D), in the presence of 1 and 2 subunits of $GABA_A$ receptors show that chimeric receptors (red), are targeted to plasma membrane as shown by white arrows while some of the receptors also restricted in the endoplasmic reticulum as shown by "*", (green). (Scale bar: 15 m)

5.12 Primary cell culture of neurons

Primary cultures of neurons are prepared according to Choi et al. (1987) with some modifications. Briefly, hippocampal cells from each individual mouse embryos (E-16) were dissociated and plated with a density of 40.000 on poly-L-Lysine coated coverslips (300 l of 5 mg/ml per coverslip) in 24 well plates. Cultures were kept at 37 °C in a 5 % CO_2 incubator. The cultures were fed 24 hours after plating and the medium was changed every four days. During feeding,

the culture medium was never changed completely, except for first exchange of the medium with FDX to prevent glial cells overgrowing. Later, for each feeding only half of the medium was replaced. Cells were transfected at DIV7 and used for experiments at DIV19.

1x PBS without Calcium amd magnesium, sterile filtered	PAA Laboratories GmBH, H15-002
PBS/HEPES/Glucose, pH 7.38	
100 ml 10x PBS	Gibco, 14200-067
6 g D-(+)-Glucose	Sigma, G-7021
7.38 g HEPES	Roth, 9105-2
	adjust the pH to 7.38 with 2N NaOH
Culture Medium:	
500 ml Neurobasal Medium without L-glutamine	Gibco, 21103-049
10 ml B-27 Supplement (50x)	Gibco, 17504-044
1.25 ml L-glutamine	Gibco, 25030-024
5 ml Penicillin-Streptomycin	Gibco, 15140-114
(0.2M)Penicillin-Streptomycin	
0.25 % Trypsin in PBS w/o Calcium & Magnesium sterile filtered	PAA, L11-002
Poly-D-Lysine Hydrobromide 1mg/ml	Sigma, p-1149

Table 5.3 Specific Reagents and Solutions used for the cell culture

5.13 Accession numbers of peptides for $GABA_A$ receptor subunits

Species	Database	Accession #
Mus musclulus (mouse)	NCBI protein database	NP_803127
Homo sapiens (human)	NCBI protein database	NP_944494
Rattus novergicus (rat)	NCBI protein database	P18508
Pan troglodytes (chimpanzee)	ENSEMBL genomic database	ENSPTRP00000045735*
Bos taurus (cow)	NCBI protein database	P22300
Gallus gallus (chick)	ENSEMBL genomic database	P21548
Fugu rubripes (pufferfish)	ENSEMBL genomic database	NEWSINFRUP00000159131*
Tetraodon nigroviridis	ENSEMBL genomic database	GSTENT00033745001*

Table 5.4 Peptides used in the □2 subunit cytoplasmic domain (TM3-TM4 loop) alignment ***:** Non-annotated, partial genomic sequence

Species	Database	Accession #
Mus musclulus (mouse)	NCBI protein database	AAL37410
Homo sapiens (human)	NCBI protein database	AAB70007
Rattus novergicus (rat)	NCBI protein database	AAH87714
Pan troglodytes (chimpanzee)	NCBI genomic database	NW_101530.1*
Bos taurus (cow)	NCBI protein database	XP-585530
Canis familiaris (dog)	NCBI protein database	XP-857845
Sus sucrofa (pig)	NCBI protein database	CAI61946
Danio rerio (zebrafish)	ENSEMBL genomic database	XP_70009.1*
Gallus gallus (chicken)	ENSEMBL genomic database	NP_032098.01* Contig52.113

Table.5.5 Peptides used in the □ subunit cytoplasmic domain (TM3-TM4 loop) alignment**. ***: Non-annotated, partial genomic sequence

6 ABBREVIATIONS

Å	Ångstrom (10^{-10} m)
□^{32}P-dCTP	deoxycytosine triphosphate labelled with ^{32}P in alpha position
□2L	long isoform (splice variant) of gamma 2 subunit of $GABA_A$ receptors
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APS	ammonium persulphate
BDNF	brain derived neurotrophic factor
BLAST	basic local alignment search tool
bp	base pair
BSA	bovine serum albumin
BZ	benzodiazepine
cDNA	complementary DNA
Cl^-	chloride
CNS	central nervous system
Cys	cysteine
D	aspartic acid
DDI	double distilled
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EC_{50}	effective concentration of an agent required for 50% desired effect on the ion channel site
em	electron microscopy
EDTA	ethylenediamine tetraacetic acid
EGFP	enhanced green fluorescence protein

F	phenylalanine
GABA	□-aminobutyric acid
GAD 67	glutamic acid decarboxylase enzyme isoform
$GABA_ARs$	□□aminobutyric acid type A receptors
GFP	in this study GFP refers to EGFP
GlyRs	glycine receptors
GODZ	golgi-specific DHHC zinc finger protein
H	histidine
5HT	5-hydroxytryptamine
HEK	human embryonic kidney
HEPES	N-(2-Hydroxyethyl)piperazine-N'-ethanesulfonic acid
IC_{50}	Inhibition concentration of an agent required for 50 % inhibition of ion channel site
IgG	immunoglobulin G
IPSC	inhibitory postsynaptic current
K	lysine
KO	knock out
L	leucine
Lys	lysine
mM	milli molar
M	Molar
M	methionine
mRNA	messenger RNA
nACHRs	nicotinic acetylcholine receptors
NGS	normal goat serum

NMDA	N-methyl-D-aspartate
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBST	tween/phosphate buffered saline
PCR	poymerase chain reaction
PDZ domain	postsynaptic density/disc large/zona occludens-1 homology domain
PFA	paraformaldehyde
PKA	cyclic AMP-dependent protein kinase A
PKC	protein kinase C
PSD	postsynaptic density
R	arginine
RNA	ribonucleic acid
RT	room temperature
RT-PCR	reverse transcription coupled to polymerase chain reaction
S	serine
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SSC	standard saline citrate
SSRI	selective serotonon re-uptake inhibitor
SDS-PAGE	denaturating polyacrylamide gel electrophoresis
T	threonine
TBLASTN	translating BLAST

TE	tris/EDTA buffer
TM	transmembrane
TEMED	N, N, N‘, N‘-tetramethyl-ethylendiamine
Tris	tris-(hydroxymethyl)-aminomethane
TrKB	tyrosine kinase receptor
U	unit
X	degenerate aminoacid residue
V	valine
Y	tyrosine
Zn^{2+}	zinc

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