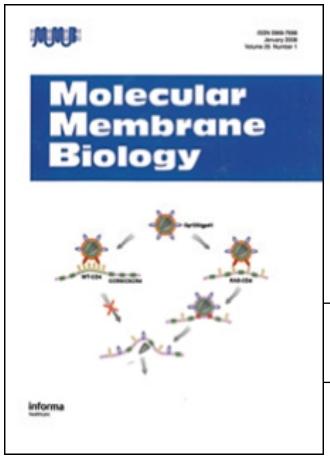


Molecular Membrane Biology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713693962

# Assembly of $GABA_A$ receptors (Review)

Isabella Sarto-Jackson [a]; Werner Sieghart [a]
[a] Division of Biochemistry and Molecular Biology, Center for Brain Research, Medical University Vienna, Austria

First Published on: 13 March 2008

To cite this Article: Sarto-Jackson, Isabella and Sieghart, Werner (2008) 'Assembly of $GABA_A$ receptors (Review)', Molecular Membrane Biology, 25:4, 302 — 310

To link to this article: DOI: 10.1080/09687680801914516
URL: http://dx.doi.org/10.1080/09687680801914516

*Molecular Membrane Biology*, May 2008; 25(4): 302–310





# Assembly of $GABA_A$ receptors (Review)

ISABELLA SARTO-JACKSON & WERNER SIEGHART

*Division of Biochemistry and Molecular Biology, Center for Brain Research, Medical University Vienna, Austria*

*(Received 29 November 2007 and in revised form 9 January 2008)*

**Abstract**

$GABA_A$ receptors are the major inhibitory transmitter receptors in the central nervous system. They are chloride ion channels that can be opened by γ-aminobutyric acid (GABA) and are the targets of action of a variety of pharmacologically and clinically important drugs. $GABA_A$ receptors are composed of five subunits that can belong to different subunit classes. The existence of 19 different subunits gives rise to the formation of a large variety of distinct $GABA_A$ receptor subtypes in the brain. The majority of $GABA_A$ receptors seems to be composed of two α, two β and one γ subunit and the occurrence of a defined subunit stoichiometry and arrangement in αβγ receptors strongly indicates that assembly of $GABA_A$ receptors proceeds via defined pathways. Based on the differential ability of subunits to interact with each other, a variety of studies have been performed to identify amino acid sequences or residues important for assembly. Such residues might be involved in direct protein-protein interactions, or in stabilizing direct contact sites in other regions of the subunit. Several homo-oligomeric or hetero-oligomeric assembly intermediates could be the starting point of $GABA_A$ receptor assembly but so far no unequivocal assembly mechanism has been identified. Possible mechanisms of assembly of $GABA_A$ receptors are discussed in the light of recent publications.

**Keywords:** *$GABA_A$ receptor, heterogeneity, assembly, intermediates, mechanism*

## $GABA_A$ receptor heterogeneity

$GABA_A$ receptors are the major inhibitory transmitter receptors in the central nervous system. They are chloride ion channels that can be opened by γ-aminobutyric acid (GABA) and are the targets of action of a variety of pharmacologically and clinically important drugs, such as benzodiazepines, barbiturates, neuroactive steroids, anesthetics and convulsants [1]. From the effects of these drugs it can be concluded that these receptors regulate the excitability of the brain, anxiety, vigilance, and learning and memory.

$GABA_A$ receptors are composed of five subunits that can belong to different subunit classes. Six α-, three β-, three γ-, one δ-, one ε-, one π-, one θ- and three ρ-subunits as well as splice variants of some of these subunits have been cloned from the mammalian nervous system [2], and depending on their subunit composition, receptors exhibit distinct pharmacological and electrophysiological properties [1,3]. Individual subunits exhibit a distinct but overlapping and often widespread distribution throughout the nervous system [4] resulting in a large variety of $GABA_A$ receptor subtypes in the brain. Immunoprecipitation studies indicated that α and β subunits can assemble with all the other $GABA_A$ receptor subunits investigated, but that γ and δ subunits seem not to co-exist in the same receptor subtype [5]. The possible existence of receptors containing two different α and/or two different β subunits as well as a γ, δ, ε, or π subunit or receptors containing θ or ε subunits in unusual combinations with other subunits contributes to the extreme heterogeneity of $GABA_A$ receptors [5–9].

This heterogeneity was supported by recombinant receptor studies. After transfection of MDCK or HEK cells with single subunits, only β3 and less efficiently β1 subunits could be detected on the cell surface to a significant extent [10,11]. In addition, a robust expression of GABA-activated homo-oligomeric chloride channels was observed with ρ1 and possibly ρ2 and ρ3 subunits [12,13] but different ρ subunits might also co-assemble to form hetero-oligomeric receptors. Channels composed of α and β subunits formed more efficiently than homo-oligomeric channels [5] and co-expression of α, β, and γ subunits resulted in a robust formation of

Correspondence: Werner Sieghart, PhD, Division of Biochemistry and Molecular Biology, Center for Brain Research, Medical University Vienna, Spitalgasse 4, A-1090 Vienna, Austria. Tel: +43 1 4277 62950. Fax: +43 1 4277 62959. E-mail:werner.sieghart@meduniwien.ac.at

ISSN 0968-7688 print/ISSN 1464-5203 online 
DOI: 10.1080/09687680801914516

GABA-gated chloride channels and properties resembling those of the majority of native receptors [1]. As suspected from the co-precipitation data of subunits from the brain, these receptors seem to be composed of two α, two β and one γ subunit [5,14] where alternating α and β subunits are connected by the γ subunit. Concatenated $GABA_A$ receptor subunits [15] as well as homology modeling studies [16] allowed to distinguish between two possible mirror image arrangements of these subunits and to identify the absolute subunit arrangement of αβγ receptors (Figure 1). The occurrence of a defined subunit stoichiometry and arrangement in αβγ receptors strongly indicates that assembly of $GABA_A$ receptors proceeds via defined pathways.

Recombinant receptor studies also indicated that receptors containing two different α or β subunits are able to assemble and to exhibit properties different from receptors that contain only a single α or β subunit type. However, on heterologous co-expression of more than three subunits probably a variety of different receptors with one, two, three, four, and if present, even five different subunits are assembled and complicate interpretation of data [5]. Only a few studies are available on recombinant receptors containing ε, π, or θ subunits and assembly of these receptors so far was not investigated.

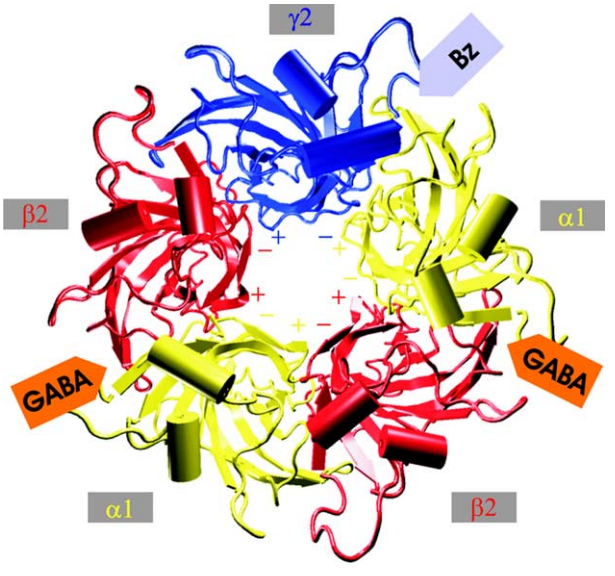


Figure 1. Model structure of $GABA_A$ receptor extracellular domains. The absolute arrangement for α1, β2 and γ2 containing $GABA_A$ receptors is shown, the view is from extracellular. The (+) (plus) and (−) (minus) sides of the subunits are identified on the inner circumference of the channel. The interfaces at which benzodiazepines (BZ) and GABA bind are labeled by the respective labels (from [16] with permission).

## $GABA_A$ receptor assembly

*Receptor assembly occurs in the endoplasmic reticulum and is supported by chaperones*

The assembly of hetero-oligomeric receptors of the ligand-gated ion channel superfamily (Cys-loop receptors) comprising the nicotinic acetylcholine receptors, $GABA_A$ receptors, glycine receptors, and 5-hydroxytryptamine, type 3 receptors, is a complex, multi-step process [17]. The assembly of subunits seems to occur in the endoplasmic reticulum (ER) and to involve interaction with chaperone molecules [10,18]. For the assembly of $GABA_A$ receptors, the chaperones calnexin, immunoglobulin heavy-chain-binding protein (BiP), and protein disulphide isomerase seem to be required. Calnexin recognizes immature glycans (mono-glucosylated proteins), BiP recognizes exposed hydrophobic regions and protein disulphide isomerase catalyses the formation of disulphide bridges between appropriate cysteine residues. Together, these chaperone proteins monitor the fidelity of protein folding and assembly. Unassembled $GABA_A$ receptor subunits are bound efficiently to BiP and calnexin and are retained in the ER (by virtue of luminal KDEL ER retention signals within the chaperone proteins) until degraded [19]. On the other side of the membrane, the masking of ER retention signals during assembly ensures the cytoplasmic fidelity and transport competence of receptors. Correctly assembled receptors are released and traffic to the cell surface [20].

*Amino acid residues important for assembly*

Based on the differential ability of subunits to interact with each other, a variety of studies have been performed to identify amino acid sequences or residues important for assembly. Two different approaches were applied. A 'positive approach' aimed to enhance interaction of subunits or subunit fragments by replacing sequences of assembly-incompetent with homologous sequences of assembly-competent subunits ('knock-in'). If the chimera could then be co-precipitated with the other subunit, it could be assumed that the incorporated sequence was important for assembly. In a 'negative approach', a 'knock-out' strategy aimed to reduce interaction of subunits by replacing amino acid sequences or residues of assembly-competent with homologous sequences of assembly-incompetent subunits. With this strategy, loss of binding of a truncated, chimeric or a mutated subunit to another subunit suggested that the sequence or residue was important for assembly.

Subunits or subunit fragments that can initially interact with another subunit but are unable to

participate in the formation of completely assembled receptors can also be identified by their interfering with receptor assembly [21]. Their interaction with assembly competent subunits generates non functional assembly intermediates that usually are degraded. This results in a deprivation of subunits needed for assembly and thus, in a reduced receptor expression at the cell surface. The absence of an effect, however, does not necessarily imply that a construct does not contain assembly signals. The mutant subunit may not be folded, processed or trafficked in a way that permits interaction with the target protein.

Experimental evidence as well as information from structural models of $GABA_A$ receptors [16] generated using the acetylcholine binding protein (AChBP) [22] as a template indicate, however, that $GABA_A$ receptor subunits contain more than one N-terminal sequence making direct contacts with the same neighbouring subunit [23]. This adds an additional complication for experimental studies. Whereas the very N-terminally located sequences important for assembly can be easily studied by investigating assembly between N-terminal fragments and full length subunits, using a positive as well as a negative approach, this no longer is possible for the identification of further putative binding sequences located more C-terminally. If the shorter N-terminal fragment already contains a sequence important for assembly, it will assemble with the other subunit whether or not an additional sequence important for assembly is present or incorporated in the longer N-terminal fragments. In such a case only knockout approaches are possible that usually result in weak effects due to the presence of additional binding sites.

It has to be kept in mind, however, that on identifying residues as being important for assembly, no information is gained on their function. Such residues might be involved in direct protein-protein interactions, but they might also have stabilized regions elsewhere in the protein that then could have interacted with the adjacent subunit. And replacement of these residues by homologous residues of assembly incompetent subunits might have caused a disturbance in protein packing or subunit folding by incorporating the residues into a new environment, leading to artefacts and wrong conclusions. Similarly, when interactions between truncated and full length subunits are investigated, contact regions within the truncated fragments could be exposed that normally would be buried and thus not be available for interaction in full-length subunits. Overall, however, the methods used are complementary to each other and applied together provide good information on residues important for assembly. Following this discussion, the term 'assembly signals' seems inappropriate: the presence of these residues just only directly or indirectly allows a contact or confers the necessary flexibility to the protein for direct contact between subunits.

To identify single amino acid residues that are directly involved in protein-protein interactions, residues forming direct contacts with each other in homology models of $GABA_A$ receptors were replaced by cysteines in the hope that they might form spontaneous disulfide bridges between subunits that then could be identified by Western blot analysis under suitable conditions. Absence of crosslink between subunits, however, does not exclude a direct interaction between residues because formation of a disulfide bond between cysteines requires an appropriate distance and angle between interacting $-SH$ groups that not necessarily can be achieved even if the two residues are sitting close together. In addition, it has to be kept in mind that contact sites between subunits in the fully assembled receptors not necessarily are identical with the sites making primary contacts between subunits, because such primary contact sites might become abandoned after subsequent conformational changes of the proteins needed for further assembly of subunits.

*Residues important for homo-oligomeric assembly of $GABA_A$ receptors.* As mentioned above, only ρ1 or β3 subunits seem to be able to robustly form homo-oligomeric receptors [11,12]. When co-expressed in Xenopus oocytes, the N-terminus of ρ1 interfered with ρ1 but also with with ρ2 receptor formation indicating that the N-terminal regions of ρ subunits contain the initial signals for both homo-oligomeric and hetero-oligomeric assembly [24]. Using a series of $GABA_A$ receptor β1 subunit chimeras containing different regions of the ρ1 N-terminus for interference with ρ1 and ρ2 subunit assembly into functional $GABA_A$ receptors, it was demonstrated that transfer of 70 residues of ρ1 (ρ1(137–206)) to the β1 subunit created a chimera that disrupted ρ1 but not ρ2 assembly into functional receptors. These observations suggest that different sequences are important for the formation of ρ1 homo- and ρ1/ρ2 hetero-oligomeric assembly [13].

Interestingly, the chimera β1ρ1(137–206) did not confer the same level of dominant-negative effect on ρ1 subunit assembly as the chimera β1ρ1(137–264). In addition, chimera β1ρ1(137–182) containing the first half, or chimera β1ρ1(183–206) containing the second half of the 70 amino acid region were less effective at reducing ρ1 currents than the chimera β1ρ1(137–206) with the entire 70 amino acid region. This could be interpreted by the assumption

that several N-terminal ρ1 sequences are important for assembly.

The presence of different domains ('assembly boxes') important for homo- and hetero-oligomeric assembly has also been demonstrated for glycine receptors [25]. However, only a few sequences important for glycine receptor assembly are located within the 70 amino acid region identified as important for the homo-oligomeric assembly of ρ1. In addition, two amino acid residues of the nACh receptor ε subunit, ε106 and ε115 important for assembly with the nACh receptor α subunit are located within the ρ1 sequence of the chimera β1ρ1(137–182) [26].

Some of the β3 subunit residues important for homo-oligomeric assembly have been identified by exploiting the differing capabilities of the β2 and β3 subunits to form functional homomeric receptors at the cell surface. Substitution of four homologous residues within the β2 subunit by β3 residues (β2D171G, β2N173K, β2T179E, β2K180R; $\beta2^{GKER}$) in a region homologous to contact forming residues located at the (−)side of the AChBP, was sufficient to enable homo-oligomerization of β2 subunits in HEK or COS cells. The reciprocal mutation (β2 residues to replace those of β3; $\beta3^{DNTK}$) abolished the ability of β3 to assemble with itself. These residues, however, were not critical for the formation of hetero-oligomeric receptors composed of α1β2 or α1β3 subunits [27].

In another study it was demonstrated that by replacing amino acid residues 76–89 in β3 subunits by the homologous α1 residues the formation of α1β3 receptors was drastically reduced [28]. This β3 sequence, however, was not important for the formation of homo-oligomeric β3 receptors (Sarto-Jackson and Sieghart, unpublished work). So homo-oligomeric assembly possibly might be guided by sequences promoting homo-oligomeric and others preventing hetero-oligomeric assembly.

*Residues important for hetero-oligomeric assembly of GABA$_A$ receptors.* Sequences important for a selective assembly of different subunits are responsible for the formation of a distinct subunit stoichiometry and arrangement within the receptors. They might form direct contact sites or ensure an adequate subunit structure. Although homologous sequences might be important for selective assembly in different subunit types the individual contact forming residues are

Table I. Sequences important for $GABA_A$ receptor assembly.

| Residues important for assembly | Assembly with | (+)/(−) side experimental evidence | Reference | Homologous residues important for assembly | (+)/(−) side on AChBP |
|---|---|---|---|---|---|
| α1(54–68) | β3 | (−) | [32] | β3(52–66)<br>γ2(67–81)<br>γ3(70–84)<br>AChBP T45/W53 | (+)/(−) |
| α1(58–67) | β3 | nd | [30] | AChBP W53 | (−) |
| α1R66 | β2 | (−) | [33] | | (−) |
| α1W69 | β2 | (+) | [31] | | (−) |
| α1(80–100) | γ2(91–104) | (+) | [34] | | (+)/(−) |
| α1W94 | β2 | (+) | [31] | | |
| β3(52–66) | α1 | (−) | [32] | α1(54–68)<br>γ2(67–81)<br>γ3(70–84)<br>AChBP T45/W53 | (+)/(−) |
| β3(76–89) | α1 | (−) | [28] | γ2(91–104)<br>AChBP S75/P77 | (−) |
| β3(G171, K173, E179, R180) | β3 | nd | [27] | AChBP E163/Y164 | (−) |
| γ2R43 | β2 | (+) | [37] | AChBP I19 | (+) |
| γ2(67–81) | α1 | (−) | [32] | α1(54–68)<br>β3(52–66)<br>γ3(70–84)<br>AChBP T45/W53 | (+)/(−) |
| γ2(83–90) | α1+β3 | (+)/(−) | [21] | | |
| γ2(91–104) | α1(80–100) | (−) | [21] | β3(76–89)<br>AChBP S75/P77 | (−) |
| γ2T125, γ2P127 | β3 | (+) | [35] | AChBP L98/P100 | (−) |
| γ2S171 | nd | nd | [41] | | (+) |
| γ3(70–84) | α1+β3 | (+)/(−) | [23] | α1(54–68)<br>β3(52–66)<br>γ2(67–81)<br>AChBP T45/W53 | (+)/(−) |

most likely different (i.e., not conserved – [22]) and are possibly also not located at homologous positions in different subunits (Table I).

*Residues important for the selective assembly of the α–β interface.* The discovery of an α6 splice variant (α6S) lacking 10 amino acids within the N-terminal extracellular domain, which was unable to form ligand-binding sites upon co-expression with β2 and γ2 subunits [29], prompted investigation of its assembly properties. It was demonstrated that only the α6L splice variant containing the 10 amino acids 58–67 was able to form α6β2 or α6β3 surface receptors, whereas the α6S subunit was retained within the ER. Interestingly, this sequence located at the α(−) side, is highly conserved in all $GABA_A$ receptor subunits and might thus be essential also for the assembly of other subunits. Consistent with this possibility, immunoprecipitation experiments using either the short version of α1 (with deleted residues 58–67), or a version in which these α1 residues were replaced by the corresponding residues of ρ1, revealed that these residues are also important for oligomerization of α1 with β3 subunits probably forming the α1(−)β3(+) interface [30]. Not surprisingly, however, they were without effect on the production of αγ complexes that probably form a contact between the α1(+) and γ2(−) side as in intact $GABA_A$ receptors.

The importance of amino acid residues within the region α1(54–69) for assembly with β subunits was independently confirmed by several other studies [30–33]. Within this sequence, α1Q67 [30] and α1W69 [31] were conserved widely in the superfamily. These residues, thus, might rather have a structural role in stabilizing an adequate conformation of the subunit than representing a direct contact site.

The sequence β3(52–66), that is homologous to the above mentioned sequence α1(54–68), was demonstrated to be important for assembly with α1 subunits [32]. In addition, the sequence β3(76–89), and here especially β3(85–89) seemed also to be important for assembly with α1 subunits [28]. Interestingly, residues β3N85 and β3V87 are homologous to residues S75/P77 in the AChBP that are involved in direct inter-subunit contacts. This region might thus represent a direct assembly site in $GABA_A$ receptor subunits.

*Residues important for the selective assembly of the α–γ interface.* In other studies it was demonstrated that the construct γ2(1–234) consisting of the complete extracellular N-terminal domain was able to reduce the expression of α1β3γ2 receptors on the cell surface by 50% [21]. A subsequent reduction in the size of the truncated subunit indicated that fragments γ2(1–130), γ2(1–117), and γ2(1–113) also were able to interfere with assembly of these receptors, but to an increasingly smaller extent. The observation that the longest N-terminal fragment exhibited the strongest interference with $GABA_A$ receptor assembly, can be explained by the possibility that longer fragments are more able to stabilize their binding sites for α1 or β3 subunits. Alternatively, additional contact sites for these subunits might be located between γ2(113–234).

γ2(1–113) was the smallest N-terminal fragment that could reduce the expression of α1β3γ2 receptors on the cell surface [21]. This N-terminal γ2 subunit fragment was able to assemble with α1 subunits by forming a high affinity [$^3$H]flunitrazepam binding site indicating that the respective assembly site must be located at the γ2(−) side. By incorporating amino acid sequences from this γ2 fragment into a homologous α1(1–100) fragment that could not bind to α1 subunits, the sequence γ2(91–104) was identified to be important for the assembly of $GABA_A$ receptor γ2 with α1 subunits [21].

Using a similar approach, the sequence γ2(67–81) [32] and the homologous sequence γ3(70–84) [23], here especially the sequence γ3(70–78) were also found to induce assembly with α1 subunits. The importance of these sequences was verified by demonstrating that chimeric subunits in which the respective γ2 or γ3 sequences were substituted by the homologous ρ1 sequence drastically reduced the formation of receptors containing γ2 or γ3 subunits at the cell surface [23,32].

Finally, the putative contact site of the sequence γ2(91–104) was identified on the neighbouring α1 subunit (α1(80–100)) by incorporating various α1 sequences into the N-terminal fragment β3(1–115) that was unable to assemble with γ2 subunits. So far, these are the only sequences identified that might form direct contacts with each other [34]. Single residues that actually make the contacts have not been identified yet, but the residues γ2S100 and γ2M102 (as well as their counterparts α1N87 and α1M89), are homologous to residues S75/P77 in the AChBP that actually contribute to direct interaction of a given monomer. In addition, residue α1D97 is homologous to residue D85 in the AChBP that forms direct contacts with the neighbouring subunit and, as expected, is located at the (+) side of the respective monomer.

*Residues important for the selective assembly of the β–γ interface.* Using comparative models of the extracellular domains of $GABA_A$ receptor subunits [16] several residues were predicted to be exposed for inter-subunit contacts. These residues were individually substituted by cysteines and a possible

disulphide bond formation between subunits was investigated on co-transfection into HEK cells. A spontaneous cross-link between subunits could not be detected. However, investigation of the mutated subunits together with wild-type subunits indicated that two constructs containing the mutation γ2T125C or γ2P127C led to a reduced formation of receptors at the cell surface by interfering with the formation of γ2β3, but not of γ2α1 intermediates. Thus, amino acid residues γ2T125 and γ2P127 seem to be important for assembly with β3 subunits. If these residues form direct and final contacts between subunits, they should thus be located at the γ2(+) side [35]. The homologous residues of the AChBP, however, are located at the (−) side. Since in this particular region, the (+) and the (−) side of the protein are only separated by 3 amino acid residues, it is possible that a slightly different protein fold shifts the position of these residues. Alternatively, the residues investigated here are structurally important or represent only initial contact sites that are no longer present in the completely assembled pentameric receptor.

Finally, the point mutation γ2R43Q recently demonstrated to be associated with epilepsy [36] is located at the γ2(+) side and has been reported to prevent binding to β2 subunits, suggesting a failure of β2–γ2 interface formation during receptor assembly [37]. Similar results were observed when the homologous mutation was present in either the α1 or β2 subunits [37]. Given the complete conservation of the residue R43 in all members of the cys-loop superfamily, this implies a more structural role for this amino acid residue in receptor assembly.

*Residues important for non-selective assembly.* The sequences γ2(83–90) [21] or γ3(70–84) [23] identified in a 'knock-in' approach in assembly incompetent truncated subunits induced association with full-length α1 as well as β3 subunits. These sequences thus might either have stabilized or exposed the binding sites to α1 and β3 subunits or might be important for association of subunit intermediates at an early stage of assembly, where discrimination between neighbouring subunits might not be necessary.

### *Mechanism of GABA$_A$ receptor assembly*

*Homo- and hetero-oligomeric assembly intermediates could represent different starting points of assembly.* The occurrence of assembly intermediates was investigated in the brain of adult and young rats by sucrose density gradient centrifugation [38]. Whereas in the brain of adult rats $GABA_A$ receptor α1, β3, and γ2 subunits sedimented as a single peak corresponding to completely assembled pentameric receptors, in the brain of young rats the sedimentation pattern was much more heterogeneous. Thus, α1, β3, and γ2 subunits could be identified in the subunit dimer, trimer, tetramer and pentamer peaks. These results indicate that different hetero- and/or homo-dimers are formed in the brain.

When HEK cells transfected with single $GABA_A$ receptor subunits were investigated by sucrose gradient centrifugation, it was demonstrated that only a small amount of subunit monomers, and unexpectedly, higher oligomeric complexes were present within the cell. Thus α1 subunits formed homo-di-, tri- and tetramers, β3 subunits formed all different homo-oligomers up to pentamers, while γ2 subunits formed homo-di- and trimers (Ehya and Sieghart, unpublished work). This indicates that in each of these subunits contact sites for homo-oligomerization are present. The failure to form homo-pentameric α1 or γ2 receptors then might indicate that on homo-oligomerization possible conformational changes of subunits necessary for accommodating five identical subunits into completely assembled receptors are not induced. In addition, the fact that in fully assembled hetero-pentameric αβγ receptors no direct contacts between two α, two β, or two γ subunits do occur, suggests that homo-oligomeric interaction might either be used as a default mechanism in the absence of other subunits, or the sites for homo-oligomeric interaction only serve as primary contact sites for initial stabilization of subunits until another more competent assembly partner arrives.

When α1 and γ2 subunits, or β3 and γ2 subunits were transfected together, in both cases the majority of assembly intermediates are found in the dimer peak [14], thus reducing the heterogeneity of assembly intermediates. These data also indicated that once formed, the hetero-dimers cannot form higher oligomers in the absence of the missing third subunit. When α1 and β3 subunits were transfected together, predominantly subunit tetramers and pentamers were formed, whereas co-transfection of α1, β3 and γ2 subunits resulted in a single peak of pentameric receptors. These results supported previous conclusions [10] that only α1β3γ2 and α1β3, but not α1γ2 or β3γ2 subunits, give rise to completely assembled pentameric $GABA_A$ receptors that then are transported to the cell surface. The formation of α1β3, α1γ2, and β3γ2 dimers on heterologous co-expression might indicate that each one of these dimers could become the starting point of receptor assembly. The formation of tetrameric and pentameric receptors in cells transfected with α1β3 subunits, only, and the exclusive formation of

pentameric receptors in cells transfected with α1, β3, and γ2 subunits, seem to indicate that the speed of incorporation of a γ2 subunit into tetrameric α1β3 assembly intermediates is faster than that of a β3 or α1 subunit. These data do not necessarily indicate that the γ2 subunit is the last subunit incorporated into $GABA_A$ receptor intermediates during assembly, but they suggest a hierarchical assembly mechanism, in which some preferred subunit combinations dominate receptor assembly.

*Spontaneous cross-link of mutated α1 subunits provides hints on a possible mechanism of $GABA_A$ receptor assembly.* Using comparative models of $GABA_A$ receptors for predicting amino acid residues in α1 and γ2 subunits that might form direct contacts with each other [16] it was demonstrated that on co-transfection of α1H109C with wild-type β3 and γ2 subunits, mutated α1H109C subunits spontaneously cross-linked with each other forming subunit dimers that were then transported to the cell surface [39]. In addition, completely assembled and functional $GABA_A$ receptors composed of α1H109C and wild-type β3 and γ2 subunits were observed at the cell surface, exhibiting wild-type stoichiometry and subunit arrangement and containing two opposing α1H109C subunits cross-linked by a disulfide bond bridging the channel mouth. The segment around residue α1H109 protrudes furthest toward the channel lumen and in contrast to the sequence of the AChBP, $GABA_A$ receptor subunits contain an insertion of three amino acid residues in this region that are most likely also located luminally. The position of α1H109 might be relatively flexible resulting in a fairly mild distortion of $GABA_A$ receptors containing cross-linked α1 subunits.

The formation of completely assembled receptors containing cross-linked α1H109C subunits most directly can be explained by an interaction of two α1 subunits being at least one starting point of wild-type receptor formation. This possibility is supported by the fact that the α1 subunits are synthesized side by side by different ribosomes on the same mRNA and thus, probably have a higher chance to interact with each other than with β3 or γ2 subunits that are synthesized via a different mRNA-ribosome complex. In addition, α1H109C homo-dimer formation and cross-link seemed to occur more rapidly than the assembly between α1H109C and β3 or γ2 subunits as indicated by the large surplus of cross-linked α1H109C dimers over completely assembled receptors at the cell surface. Dimerization of α1 subunits via their respective (+) and (−) sides would have caused a dead end of assembly. But interaction of α1 subunits via the side forming the channel mouth might have been weakly stabilized,

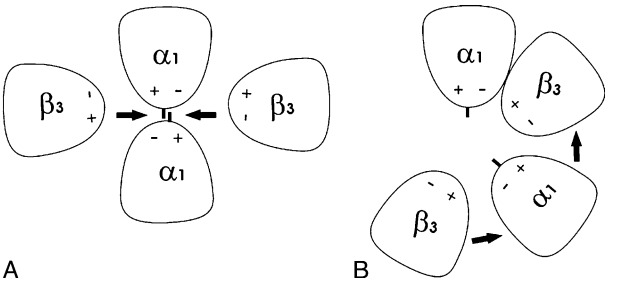


Figure 2. Schematic drawing of possible mechanisms of $GABA_A$ receptor assembly. The mutated and sterically exposed residues α1H109C are indicated by a black line at the luminal side of the subunit. + and − indicate the principal and complementary side of a subunit, respectively. (A) In one mechanism the two α1H109C subunits interact via their luminal side and offer the possibility that β3 subunits encountering the dimer from the one or the other side could assemble by forming two correct interfaces with two different α1 subunits. (B) In the second mechanism $GABA_A$ receptor assembly starts with the formation of a heterodimer of α1 and β3 (or γ2) subunits via their respective (+) and (−) sides. As soon as the correct (+)/(−) interface has been established, each additional subunit then has to assemble in the correct order. Taken from [39] with permission.

by the interaction of the two sterically exposed α1H109 residues, thus enhancing the probability of interaction with a β3 subunit that can approach from the one or the other side (Figure 2A).

Formation of two correct α/β interfaces via the (+) or (−) sides of the two α1 subunits then probably causes opening of the weak luminal interactions between the two α1 subunits, thus allowing the fourth and fifth subunits to enter and complete $GABA_A$ receptor assembly. To finally result in receptors with the correct subunit stoichiometry and arrangement, it is mandatory, however, that subunits assembling with the trimer preferentially bind either to the accessible α1(+) side (γ2 subunits) or the α1(−) side (β3 subunits). Overall, such a mechanism is consistent with the finding that even in the presence of a covalent cross-link of two α1H109C subunits completely assembled receptors with a wild-type stoichiometry and arrangement are formed presumably before the cross-linked dimers could be transported to the cell surface. Covalent cross-linking with subsequent transport to the cell surface could thus have trapped a labile assembly intermediate that could not have been detected without the use of mutated subunits [39].

Alternatively, the assembly of $GABA_A$ receptors could have started with the formation of a heterodimer, either by a direct interaction of α1 and β3 (or γ2) subunits via their respective (+) and (−) sides, or again via an initial contact with a subsequent rearrangement forming the final (+)/(−) interaction. As soon as the correct (+)/(−) interface has been established in the dimer, each additional subunit then has to assemble in the correct order

(Figure 2B) to give rise to a receptor with correct subunit stoichiometry and arrangement. In this case, cross-linking of the non-neighboring α1H109C subunits could have occurred at the hetero-trimeric assembly stage via luminal contacts of the two α1H109C subunits before correct formation of all interfaces of the α1-β3-α1 trimer. If assembly of $GABA_A$ receptors started with the formation of hetero-dimers, then the simultaneous formation of a large surplus of cross-linked α1H109C homo-dimers represented an independent and competitive event that reduced the amounts of α1 subunits available for receptor assembly. Cross-linked homo-dimers could then have been a dead end of assembly or could have led to completely assembled receptors containing cross-linked α1H109C subunits before the homo-dimers were transported to the cell surface. The described sequential assembly mechanism is similar to that proposed for the nACh receptor [18]. For the nACh receptor an additional assembly mechanism has been discussed in which two subunit dimers could combine with a fifth subunit to form the completely assembled pentameric receptor [40]. Further experiments will have to decide between all these possibilities.

## Acknowledgements

Work from the laboratory of the authors has been supported by the Austrian Science Fund.

## References

[1] Sieghart W. 1995. Structure and pharmacology of γ-aminobutyric acidA receptor subtypes. Pharmacol Rev 47:181–234.

[2] Simon J, Wakimoto H, Fujita N, Lalande M, Barnard EA. 2004. Analysis of the set of GABA(A) receptor genes in the human genome. J Biol Chem 279:41422–41435.

[3] Hevers W, Luddens H. 1998. The diversity of $GABA_A$ receptors. Pharmacological and electrophysiological properties of $GABA_A$ channel subtypes. Mol Neurobiol 18:35–86.

[4] Pirker S, Schwarzer C, Wieselthaler A, Sieghart W, Sperk G. 2000. GABA(A) receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. Neuroscience 101:815–850.

[5] Sieghart W, Sperk G. 2002. Subunit composition, distribution and function of GABA(A) receptor subtypes. Curr Top Med Chem 2:795–816.

[6] Jechlinger M, Pelz R, Tretter V, Klausberger T, Sieghart W. 1998. Subunit composition and quantitative importance of hetero-oligomeric receptors: $GABA_A$ receptors containing alpha6 subunits. J Neurosci 18:2449–2457.

[7] Benke D, Fakitsas P, Roggenmoser C, Michel C, Rudolph U, Mohler H. 2004. Analysis of the presence and abundance of $GABA_A$ receptors containing two different types of alpha subunits in murine brain using point-mutated alpha subunits. J Biol Chem 279:43654–43660.

[8] Jones BL, Henderson LP. 2007. Trafficking and potential assembly patterns of epsilon-containing $GABA_A$ receptors. J Neurochem 103:1258–1271.

[9] Bonnert TP, McKernan RM, Farrar S, le Bourdelles B, Heavens RP, Smith DW, Hewson L, Rigby MR, Sirinathsinghji DJ, Brown N, Wafford KA, Whiting PJ. 1999. theta, a novel gamma-aminobutyric acid type A receptor subunit. Proc Natl Acad Sci USA 96:9891–9896.

[10] Connolly CN, Krishek BJ, McDonald BJ, Smart TG, Moss SJ. 1996. Assembly and cell surface expression of heteromeric and homomeric gamma-aminobutyric acid type A receptors. J Biol Chem 271:89–96.

[11] Connolly CN, Wooltorton JR, Smart TG, Moss SJ. 1996. Subcellular localization of gamma-aminobutyric acid type A receptors is determined by receptor beta subunits. Proc Natl Acad Sci USA 93:9899–9904.

[12] Cutting GR, Lu L, O'Hara BF, Kasch LM, Montrose-Rafizadeh C, Donovan DM, Shimada S, Antonarakis SE, Guggino WB, Uhl GR, Kazazian HHJ. 1991. Cloning of the gamma-aminobutyric acid (GABA) rho 1 cDNA: a GABA receptor subunit highly expressed in the retina. Proc Natl Acad Sci USA 88:2673–2677.

[13] Enz R, Cutting GR. 1999. GABAC receptor rho subunits are heterogeneously expressed in the human CNS and form homo- and heterooligomers with distinct physical properties. Eur J Neurosci 11:41–50.

[14] Tretter V, Ehya N, Fuchs K, Sieghart W. 1997. Stoichiometry and assembly of a recombinant $GABA_A$ receptor subtype. J Neurosci 17:2728–2737.

[15] Baumann SW, Baur R, Sigel E. 2002. Forced subunit assembly in alpha1beta2gamma2 $GABA_A$ receptors. Insight into the absolute arrangement. J Biol Chem 277:46020–46025.

[16] Ernst M, Brauchart D, Boresch S, Sieghart W. 2003. Comparative modeling of $GABA_A$ receptors: limits, insights, future developments. Neuroscience 119:933–943.

[17] Green WN, Millar NS. 1995. Ion-channel assembly. Trends Neurosci 18:280–287.

[18] Green WN, Claudio T. 1993. Acetylcholine receptor assembly: subunit folding and oligomerization occur sequentially. Cell 74:57–69.

[19] Bollan K, Robertson LA, Tang H, Connolly CN. 2003. Multiple assembly signals in gamma-aminobutyric acid (type A) receptor subunits combine to drive receptor construction and composition. Biochem Soc Trans 31:875–879.

[20] Mizielinska S, Greenwood S, Connolly CN. 2006. The role of $GABA_A$ receptor biogenesis, structure and function in epilepsy. Biochem Soc Trans 34:863–867.

[21] Klausberger T, Fuchs K, Mayer B, Ehya N, Sieghart W. 2000. GABA(A) receptor assembly. Identification and structure of gamma(2) sequences forming the intersubunit contacts with alpha(1) and beta(3) subunits. J Biol Chem 275:8921–8928.

[22] Brejc K, van Dijk WJ, Klaassen RV, Schuurmans M, van der Oost J, Smit AB, Sixma TK. 2001. Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors. Nature 411:269–276.

[23] Sarto I, Klausberger T, Ehya N, Mayer B, Fuchs K, Sieghart W. 2002. A novel site on gamma 3 subunits important for assembly of GABA(A) receptors. J Biol Chem 277:30656–30664.

[24] Hackam AS, Wang TL, Guggino WB, Cutting GR. 1997. The N-terminal domain of human GABA receptor rho1 subunits contains signals for homooligomeric and hetero-oligomeric interaction. J Biol Chem 272:13750–13757.

[25] Griffon N, Buttner C, Nicke A, Kuhse J, Schmalzing G, Betz H. 1999. Molecular determinants of glycine receptor subunit assembly. Embo J 18:4711–4721.

[26] Gu Y, Camacho P, Gardner P, Hall ZW. 1991. Identification of two amino acid residues in the epsilon subunit that

promote mammalian muscle acetylcholine receptor assembly in COS cells. Neuron 6:879–887.

[27] Taylor PM, Thomas P, Gorrie GH, Connolly CN, Smart TG, Moss SJ. 1999. Identification of amino acid residues within GABA(A) receptor beta subunits that mediate both homomeric and heteromeric receptor expression. J Neurosci 19:6360–6371.

[28] Ehya N, Sarto I, Wabnegger L, Sieghart W. 2003. Identification of an amino acid sequence within GABA(A) receptor beta3 subunits that is important for receptor assembly. J Neurochem 84:127–135.

[29] Korpi ER, Kuner T, Kristo P, Kohler M, Herb A, Luddens H, Seeburg PH. 1994. Small N-terminal deletion by splicing in cerebellar alpha 6 subunit abolishes $GABA_A$ receptor function. J Neurochem 63:1167–1170.

[30] Taylor PM, Connolly CN, Kittler JT, Gorrie GH, Hosie A, Smart TG, Moss SJ. 2000. Identification of residues within GABA(A) receptor alpha subunits that mediate specific assembly with receptor beta subunits. J Neurosci 20:1297–1306.

[31] Srinivasan S, Nichols CJ, Lawless GM, Olsen RW, Tobin AJ. 1999. Two invariant tryptophans on the alpha1 subunit define domains necessary for GABA(A) receptor assembly. J Biol Chem 274:26633–26638.

[32] Sarto I, Wabnegger L, Dogl E, Sieghart W. 2002. Homologous sites of GABA(A) receptor alpha(1), beta(3) and gamma(2) subunits are important for assembly. Neuropharmacology 43:482–491.

[33] Bollan K, King D, Robertson LA, Brown K, Taylor PM, Moss SJ, Connolly CN. 2003. GABA(A) receptor composition is determined by distinct assembly signals within alpha and beta subunits. J Biol Chem 278:4747–4755.

[34] Klausberger T, Sarto I, Ehya N, Fuchs K, Furtmuller R, Mayer B, Huck S, Sieghart W. 2001. Alternate use of distinct intersubunit contacts controls $GABA_A$ receptor assembly and stoichiometry. J Neurosci 21:9124–9133.

[35] Sarto-Jackson I, Ramerstorfer J, Ernst M, Sieghart W. 2006. Identification of amino acid residues important for assembly of GABA receptor alpha1 and gamma2 subunits. J Neurochem 96:983–995.

[36] Macdonald RL, Kang JQ, Gallagher MJ, Feng HJ. 2006. GABA(A) receptor mutations associated with generalized epilepsies. Adv Pharmacol 54:147–169.

[37] Hales TG, Tang H, Bollan KA, Johnson SJ, King DP, McDonald NA, Cheng A, Connolly CN. 2005. The epilepsy mutation, gamma2(R43Q) disrupts a highly conserved inter-subunit contact site, perturbing the biogenesis of $GABA_A$ receptors. Mol Cell Neurosci 29:120–127.

[38] Klausberger T, Ehya N, Fuchs K, Fuchs T, Ebert V, Sarto I, Sieghart W. 2001. Detection and binding properties of GABA(A) receptor assembly intermediates. J Biol Chem 276:16024–16032.

[39] Sarto-Jackson I, Furtmueller R, Ernst M, Huck S, Sieghart W. 2007. Spontaneous cross-link of mutated alpha1 subunits during GABA(A) receptor assembly. J Biol Chem 282:4354–4363.

[40] Verrall S, Hall ZW. 1992. The N-terminal domains of acetylcholine receptor subunits contain recognition signals for the initial steps of receptor assembly. Cell 68:23–31.

[41] Jin P, Walther D, Zhang J, Rowe-Teeter C, Fu GK. 2004. Serine 171, a conserved residue in the gamma-aminobutyric acid type A ($GABA_A$) receptor gamma2 subunit, mediates subunit interaction and cell surface localization. J Biol Chem 279:14179–14183.

This paper was first published online on iFirst on 13 March 2008.