Four Amino Acids in the α Subunits Determine the γ-Aminobutyric Acid Sensitivities of GABA<sub>A</sub> Receptor Subtypes

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GABA<sub>A</sub> receptors, mediators of fast inhibitory neurotransmission, are heteropentameric assemblies from a large array of subunits. Differences in the sensitivity of receptor subtypes to endogenous GABA may permit subunit-dependent finely tuned responsiveness to the same GABAAergic inputs. Using both radioligand binding and electrophysiology combined with mutagenesis, we identified a domain of four amino acids within the α subunits that mediates the distinct sensitivities to GABA allowing their selective switch between αβ3γ2 combinations. Replacing this domain in α3 by the corresponding segments of α1–α5 resulted in mutant receptors displaying the GABA EC<sub>50</sub> values of the respective wild-type receptors. Vice versa, the α3 motif forced the low sensitivity to GABA of α3 upon α1β3γ2, α4β3γ2, and α5β3γ2. Binding of the GABA agonist [35S]TBPS was not affected by the exchange of the motif between α1 and α3 subunits. Thus, the equilibrium binding pocket is maintained upon replacement of the four amino acids. Taken together our data suggest that the identified motifs contribute to a structure involved in the transduction of the binding signal rather than to the binding itself.

Fast inhibitory neurotransmission in the mammalian central nervous system is mediated mainly by the GABA<sub>A</sub> receptor, a ligand-gated chloride channel. The receptor complex presumably is composed of five protein subunits, each consisting of an extracellular N-terminal domain with a putative cysteine knot, four largely conserved transmembrane segments (TM), and a variable intracellular region between TM3 and TM4. This topology is characteristic for members of the superfamily of ligand-gated ion channel receptors (1, 2). Several GABA<sub>A</sub> receptor subunits (α1–6, β1–3, γ1–3, δ, ε, θ, π, ρ1–3) have been cloned from mammalian brain (3, 4). Thus, the genetic diversity of multiple GABA<sub>A</sub> receptor subunits permits the assembly of a vast number of receptor heteromeric isoforms. Apparently, the subunit composition determines the pharmacological profile of the resulting receptor subtype (5), but the physiological significance of the GABA<sub>A</sub> receptor heterogeneity in the central nervous system remains largely unknown. Most GABA<sub>A</sub> receptor subunits exhibit distinct regional and cellular distribution throughout the brain, with their expression patterns changing during pre- and postnatal development (6). In addition, in neurons expressing different receptor isoforms, a subunit-selective targeting to cellular domains has been observed (7–9). These findings further strengthen the notion that the receptor subtypes subserve individual functions in GABAAergic neurotransmission. Hence, they may contribute to synaptic variety, as depending on their subunit composition recombinant GABA<sub>A</sub> receptors differ in their sensitivity to the endogenous agonist GABA (10–12). Especially in view of the existence of extrasynaptic GABA<sub>A</sub> receptors (7, 13), a 20-fold difference in GABA sensitivity may play a decisive role in neuronal transmission irrespective of the assumed saturating GABA concentration in the synaptic cleft. Therefore, various GABA<sub>A</sub> receptor subtypes may allow finely tuned responsiveness to the same GABAAergic inputs (14).

In recombinant ternary receptor complexes the sensitivity to GABA is most apparently influenced by the α subunit, with α3-containing isoforms consistently reported to display the lowest sensitivities of all GABA<sub>A</sub> receptor subtypes (10, 14–17). In addition, the GABA-dependent [35S]TBPS binding of α3β3γ2/3 assemblies deviates remarkably from all other subunit combination, that is, high concentrations of GABA are needed to significantly reduce binding of the cage convulsant [35S]TBPS as compared with its respective binding maximum (18). As the deflection point of the concentration-response curves of [35S]TBPS binding for a given subunit combination correlates well with its EC<sub>50</sub> value for GABA (18), these data confirm the low sensitivity of α3-containing receptors and recommend α3 as the starting point to identify molecular determinants of GABA potency in the α subunit.

Here we describe the identification and functional characterization of four-amino acid motifs specific for individual α subunits of the GABA<sub>A</sub> receptor, which control the sensitivity to GABA of the resulting receptor. Exchange between subunits of the whole motif but not of single amino acids resulted in transfer of the respective GABA sensitivities for most receptors of the general composition αβ3γ2, where i ranges from 1 to 6. Thus, our findings may provide insight into the mechanistic features responsible for the molecular diversity of GABA<sub>A</sub>ergic neurotransmission and deliver tools to further dissect the biological significance of GABA<sub>A</sub> receptor heterogeneity.

EXPERIMENTAL PROCEDURES

Construction of Chimeras—The coding strands of the rat GABA<sub>A</sub> receptor α1 and α3 subunits were excised from expression vectors pRK5-rα1 and pRK5-rα3, respectively, and subcloned into pBluescript SK– (Stratagene, Amsterdam, Netherlands). Two conserved NcoI re-
surrounding the NcoI restriction site in pBluescript and the use of the XbaI site in the multiple cloning site of pBluescript.

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4.3. Mutagensis—Eukaryotic expression vectors (pRKS or pRK7) for the rat GABA_A receptor α1–6 subunits (19) were used in this study. The desired point mutations and multiple amino acid changes were introduced into the coding strands using the QuikChange site-directed mutagenesis kit (Stratagene). As applicable we interchanged nucleotides between the latter). They were filled with 10 (or 15) ml of minimum essential medium (MEM, Invitrogen) supplemented with 158 mg/l sodium bicarbonate, 2 ml glucose (Invitrogen), 100 units/ml penicillin-streptomycin (Invitrogen), and 10% fetal calf serum (Invitrogen). Cultures were maintained at 37 °C in a humidified 5% CO_2, 5% O_2 atmosphere for 2–3 (2) days. Transfection with recombinant rat GABA_A receptors were carried out as described in detail elsewhere (20). Briefly, HEK 293 cells were transfected using the phosphate precipitation method with rat GABA_A receptor cDNAs in eukaryotic expression vectors (pRKS or pRK7) for the α, β, and γ subunits (19). For optimal receptor expression (18), final concentrations (μg of vector DNA/1.6 cm2 (or 15-cm) tissue culture plate) were: α1, 2 (or 5); α2 4.8, 6, 1.2 (or 3); α3 16, 5; α4 4, 2; β3 2, 1.5; β2 4, 0.5; and γ2 2, 0.75. Chimera and mutants were used in the concentration of the major parental subunit.

Wild-type α subunits, α subunit chimera, and α subunit mutants were co-transfected with the β3 and γ2S subunits. For the identification of transfected cells for electrophysiological recordings, 1 μg/pL pEGFP-N1 vector (BD Biosciences) was added.

Electrophysiology—Two days after transfection single cervices were taken and the HEK 293 cells were placed in a recording chamber mounted on the movable stage of a fluorescence microscope (Olympus IX70) and perfused at room temperature with a defined saline solution containing (in mM): 130 NaCl, 5.4 KCl, 2 CaCl_2, 2 MgSO_4, 10 glucose, 5 sucrose, and 10 HEPES (free acid), pH adjusted to 7.35, with about 35 mm NaOH. Transfected cells were identified by their fluorescence (enhanced green fluorescent protein), and ligand-mediated membrane currents of these cells were studied in the whole-cell configuration of the patch clamp technique (21). Patch clamp pipettes were pulled from hard borosilicate capillary glass (0.5 mm ID, 1.5 mm OD, Vitrex, Science Products GmbH, Hofheim, Germany) using a horizontal puller (model P-97, Sutter Instruments, CA) in a multi-stage process. The pipettes had an initial resistance of 2–4 MΩ when filled with a solution containing (in mM): 90 KCl, 50 KOH, 2 CaCl_2, 2 MgCl_2, 10 EGTA, 3.1 ATP (di-potassium salt), 0.4 GTP (tri-sodium salt), and 10 HEPES (free acid), pH 7.35.

The junction potential between the pipette and the external solution was less than 2.3 mV and therefore was disregarded. Seal resistances of >1 gM were routinely obtained by applying gentle suction to the pipettes. Membrane rupture was monitored electrically as an increase in capacity. Pipette capacitance, membrane capacitance, and series resistance were compensated for electronically to achieve minimal capacitative transients. A series resistance compensation of >60% was regularly used. Using a fast Y-tube application system, the recombinant receptors were tested at GABA concentrations of 0.01, 0.03, 0.1, 0.3, 1, 3, 5, 10, 30, 60, 100, and 1000 μM. The responses of the cells were recorded by a patch clamp amplifier (EPC-8, HEKA-Electronic, Lambrecht, Germany) in conjunction with a standard personal computer and the pClamp 8.1 software package (Axon Instruments, Foster City, CA). The standard holding potential for the cells was –40 mV. Whole cell currents were low-pass-filtered by an eight-pole Bessel filter at 5 or 10 kHz before being digitized by a Digidata 1322A interface (Axon Instruments) and recorded by the computer at a sampling rate of at least 1 kHz.

Binding Assays—Resuspended cell membranes (50–200 μg of protein/tube) were incubated in a final volume of 0.5 ml of 50 mm Tris citrate buffer, pH 7.3, for [3H]muscimol binding (0.5–30 nm) supplemented with 0.2 μM NaCl for [35S]TBPS (6 nm) binding. GABA was diluted from a 100 nm solution in H2O. Non-specific binding was determined by 100 μM GABA and 20 μM picrotoxin (for [3H]muscimol and [35S]TBPS binding, respectively. After 60 min on ice and 90 min at room temperature ([3H]muscimol and [35S]TBPS, respectively), the assay mixtures were rapidly diluted to 5 ml with ice-cold 10 mm Tris/HCl, pH 7.4, filtered through glass fiber filters, and washed once with 5 ml of 10 mm Tris/HCl, pH 7.4. Filters were immersed in 1 ml of 0.5 ml of 5 M sodium hydroxide. Solv scintillation fluid, and radioactivity was determined in a Beckman liquid scintillation counter using external standardization. Statistical calculations were performed using the GraphPad Prism program (GraphPad Software, San Diego, CA).

RESULTS

GABA Potency of Wild-type αiββy2 Receptors—We transiently expressed ternary rat GABA_A receptors in HEK 293 cells configured from any of the six α subunits with the β3 and γ2S variants. Transfected cells were analyzed by whole-cell patch-clamping. When expressed in HEK 293 cells, the EC50 of GABA-induced chloride current properties varied with the expressed α subunit in a range of <1 to >50 μM in the rank order α6 > α1 > α2 > α4 > α5 >> α3 (Table 1). Thus we have confirmed the exceptionally low sensitivity to GABA conferred by the α3 subunit.
TABLE I

| EC<sub>50</sub> | n    |
|----------------|------|
| a1β3γ2S        | 2.9 ± 0.1 | 10 |
| a2β3γ2S        | 5.2 ± 0.2 | 8  |
| a3β3γ2S        | 48 ± 2    | 10 |
| a4β3γ2S        | 7.6 ± 0.3 | 7  |
| a5β3γ2S        | 11.6 ± 0.5| 7  |
| a6β3γ2S        | 1.0 ± 0.03| 8  |

Properties of a1-a3 Chimeric Subunits in [35S]TBPS Binding—To initialize the search for the molecular determinants of GABA potency in the α subunits, we chose the ubiquitous α1 as a reference to the α3 subunit and constructed chimeras containing variable segments of each subunit (Fig. 1). We analyzed the GABA-dependent [35S]TBPS binding of these chimeric subunits (Fig. 1). We analyzed the GABA-dependent [35S]TBPS binding of these chimeric subunits and compared them with recombinant receptors containing either wild-type α1 or α3 in addition to β3γ2S. [35S]TBPS binding is modulated by most, if not all, compounds interacting with GABA<sub>Δ</sub> receptors and can be inhibited by physiologically relevant concentrations of GABA (for review, see Ref. 3). This proved to be true for all chimeric subunits tested, indicating that they were functionally intact. In keeping with previous data (18) for the wild-type receptors, GABA led to a sharp increase in [35S]TBPS binding at concentrations of 1 μM (α3β3γ2S) and 10 μM (α3β3γ2S), respectively (Fig. 2). [35S]TBPS binding to a1β3γ2S was completely abolished by 100 μM GABA, whereas concentrations up to 1 mM GABA did not effectively block [35S]TBPS binding to a3β3γ2S receptors. The properties of the chimeric subunits α1(TM12a3) and α1(TM14a3) were indistinguishable from those of wild-type α1, whereas the values obtained with the α3(TM2a1) chimera resembled those of wild-type α3-containing receptors. The characteristics of chimera ro31-α3, with only 62 amino acids N-terminal of the putative cysteine loop derived from the α1 subunit, were indistinguishable from those of α1. This finding indicates a minor role of both the transmembrane domains and the intracellular loop in determining the α subunit-specific regulation of [35S]TBPS by GABA and predicts the molecular determinants to be localized in this 62-amino acid-long stretch of the α subunits.

Functional Properties of a1-a3 Chimeras—To test whether the domains identified by the [35S]TBPS binding technique also determine functional sensitivity to GABA, we recorded GABA-evoked currents of receptors containing the chimeric subunits ro31-α3 or ra13-α3. The dose-response curves to GABA were indistinguishable for α3β3γ2S and (ra13-α3)β3γ2S receptors (data not shown), with an EC<sub>50</sub> for GABA of 48 ± 2 and 51 ± 3 μM, respectively. Likewise, the differences in the EC<sub>50</sub> values of α1β3γ2S and (ro31-α3)β3γ2S, 2.9 ± 0.1 and 3.1 ± 0.1 μM, respectively, were insignificant, proving that in α1 and α3 the molecular determinants of GABA sensitivity and those affecting modulation of [35S]TBPS binding by the neurotransmitter are located within the same region of the extracellular N-terminal domain. Moreover, the apparent characteristics of the two subunits are transferable by exchange of the short segment between α1 and α3.

These findings warranted further dissection of this segment to pin down the decisive amino acid residues. The sequence identity of α1 and α3 in this stretch of 62 amino acids is 87%, and thus a maximum of 8 amino acid residues could cause the functional characteristics. Based on the assumption that the particular low sensitivity conferred by the α3 subunit is due to a unique structural feature, we aligned the 62-amino acid stretch of all α subunits (Fig. 3). The α3 subunit differs in only two positions from all other α subunits, i.e., Val<sup>174</sup> and His<sup>188</sup> (relating to the unequivocal numbering of the unprocessed protein), recommending both as prime candidates for subsequent mutagenesis studies.

**Properties of a1-a3 Chimeric Subunits in [35S]TBPS**

**Properties of a1-a3 Chimeric Subunits in [35S]TBPS Bind-**
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Fig. 1. Sketches of the GABA_A receptor subunits. A, the semicircle depicts the putative cysteine loop, and the boxes depict the four transmembrane domains (TM1–4). The positions of the conserved NcoI restriction sites in the α1 and α3 cDNAs and the primer binding site (PCR) for the construction of chimeras are indicated by arrows. The black bar spans the region of the sequence alignment represented in Fig. 3. B, chimeric subunits constructed from α1 and α3. The chimeric nature of the different subunits is indicated by a thin line plus open boxes (α1) and bold lines plus filled boxes (α3), respectively.

Fig. 2. Modulation of [35S]TBPS binding by GABA of receptors containing wild-type and chimeric α1 and α3 variants. [35S]TBPS (6 nM) binding to membranes of wild-type and chimeric α subunits (see Fig. 1) coexpressed with the β3 and γ2S subunits was modulated by increasing concentrations of GABA. Error bars represent the S.E. of three experiments in duplicate. A, receptors with an α1-like GABA sensitivity assemble from the chimeric α1TM12α3, α1TM14α3, and α31–α3 subunits. B, receptors with an α3-like GABA sensitivity assemble from the chimeric α3TM12α1, α3TM14α1, and α13–α3 subunits. The results narrow the stretch of amino acids involved in GABA sensitivity to the segment derived from α1 in chimera α31–α3.
EC$_{50}$ for GABA of αβγ2S (Table I). Likewise, we were able to force the higher sensitivities for GABA conferred by the α4 and α5 subunits upon the α3βγ2S receptor by mutating the original LVDN residues into the 4aa motifs derived from the α4 or α5 sequences (Fig. 5, B and C, and Table I).

When expressing βγ2S together with α3QDD, the dose-response curve of the resulting receptors did not differ from that of α2βγ2S (Fig. 6A, Table I). Introducing the LVDN motif of α3 into the α2 sequence had a remarkable effect on the GABA potency, as the sensitivity for GABA of α2LVDN containing receptors was reduced more than 1000-fold. As the dose-response curve of the mutant receptor complex did not even plateau at the highest GABA concentration tested, the EC$_{50}$ could only be extrapolated to 5 μM (Fig. 6A).

The dose-response curve for GABA of the mutant subunit α6LVDNβ3γ2S shifted in the direction of the dose-response curve of wild-type α3βγ2S (Fig. 6B, Table I), resulting in an EC$_{50}$ value of α6LVDN containing receptors 2.5-fold higher than that of wild-type α6 receptors. The reverse mutant α3LMQNBβ2S was the only receptor of all wild-type and mutant GABA$_\alpha$ subunit assemblies tested that exhibited a biphaseic response to GABA with EC$_{50}$ values of 1.7 ± 0.4 μM for the high affinity and 101 ± 56 μM for the low affinity site (Fig. 6B). Thus, both values were double the magnitude of the α6 and α3 wild type-containing receptors, respectively. The fraction of the high affinity site was determined to be 63 ± 5%. Based on the F-score (F$_{1,12}$ = 10.97), the two-site fit was statistically superior to that of the Hill equation (p = 0.006). In our hands, β3 and β3γ2S alone did not form functional channels (data not shown). Thus, we can exclude any contribution of homomeric β3 receptors or β3γ2S receptors to the biphaseic nature of the α3LMQNB3γ2S dose-response curve.

**Equilibrium Binding of [3H]Muscimol Is Not Affected by the 4aa Motif in α1 and α3**—We measured the binding displacement...
Four-amino acid Motifs Determine GABA Sensitivity

Fig. 5. A 4aa motif determines the GABA sensitivity of α1β3γ2S, α3β3γ2S, α4β3γ2S, and α5β3γ2S receptors. A, dose-response curves to GABA of α1LVDN, α3ITED, wild-type α1, and wild-type α3 subunits coexpressed with β3γ2. Exchange of four amino acids results in the transfer of the respective GABA sensitivities. B, GABA dose-response curves of α4LVDN, α3MRN, wild-type α4, and wild-type α3 subunits coexpressed with β3γ2S. Exchange of four amino acids results in the transfer of the respective GABA sensitivities. C, dose-response curves to GABA of α5LVDN, α3LED, wild-type α5, and wild-type α3 subunits coexpressed with β3γ2S. Exchange of four amino acids results in the transfer of the respective GABA sensitivities. Error bars represent S.E.

Fig. 6. The 4aa motif partially explains the GABA sensitivity in α2β3γ2S and α6β3γ2S receptors. A, GABA dose-response curves of α2LVDN, α3QDD, wild-type α2, and wild-type α3 subunits coexpressed with β3γ2S. Exchange of four amino acids results in the partial transfer of the GABA sensitivity of α6 to α2 and in a biphasic dose-response curve carrying the characteristics of both parental sensitivities with EC50 values of 1.7 ± 0.4 μM for the high affinity site and 101 ± 56 μM for the low affinity site. The fraction of the high affinity binding site was determined to be 63 ± 5%. Based on the F-score (F112 = 16.97), the two-site fit was statistically superior to the Hill equation model (p < 0.006). Error bars represent S.E.

**DISCUSSION**

The structural heterogeneity of GABA_A receptors is widely accepted, but the physiological significance of the receptor variety in *vivo* remains enigmatic, especially as there are no known endogenous analogues for most psychoactive drugs modulating GABAergic inputs. The fact that many receptor subtypes can be distinguished by their respective sensitivity to GABA in *vivo* argues strongly that differential expression of at least the α subunits influences the neuronal responsiveness to this neurotransmitter in *vivo*. Thus, an array of different subunit isoforms would allow an adjustment to varying GABAergic inputs as well as differing responses to uniform signals (14, 22). The diversity of GABA_A receptor subtypes may hence form an important basis for synaptic plasticity in the inhibitory circuitry.

As data concerning specific expression, regulation, composition, and localization of receptor subtypes have to be interpreted in the light of their respective functional properties, it is essential to characterize these properties and to establish the underlying molecular determinants. In the present study we identified a segment of four amino acids in the extracellular N-terminal region of α subunits decisive for the GABA sensitivity of GABA_A receptors in the composition αβ3γ2S. By exchanging the 4aa motif between the α subunits, we were able to transfer the GABA sensitivities of the "donor" wild-type subunits upon most mutants. The exchange was incomplete with substitution of single amino acids, thus pointing to an orchestrated action of more than one amino acid. Although we cannot prove the absolute necessity of all four amino acids in the motif...
for the characteristics of GABA sensitivity, we provide evidence here for the contribution of three of four nonsequential amino acids. Thus, the use of the term 4aa motif is warranted. The subunit-specific 4aa motifs are conserved between the human, rat, mouse, bovine, and chicken α subunits, suggesting their functional importance in the inhibitory circuitry of the brain throughout evolution.

The α1, α3, α4, and α5, and most likely α2 subunits share the exact sequence position of the molecular determinant for GABA potency, whereas the GABA potency of α6 was only incompletely transferred by the 4aa motif. The mutated α3IQDD subunit fully adopted the sensitivity of wild-type α2, thus matching the effects produced with the corresponding α1, α4, and α5 mutants. In contrast, the converse mutant α2LVDN led to more than a 1000-fold rightward shift of the GABA dose-response curve. This points to the 4aa motif as being crucial for GABA sensitivity in α2, but its exchange may disrupt the receptor conformation imperative for a directed shift of the sensitivity to GABA. However, this hypothesis would require the additional assumption that the GABA interaction site of the α2 subunit is more labile than that of all other α subunits.

Among the α subunits, α6 confers the highest sensitivity to GABAA receptors (11, 12, 23, 24). Although the EC50 to GABA was altered in α6LVDN and α6LMQN, the characteristics of the respective wild-type subunits were either incompletely transferred (α6LVDNβ2y2S) or resulted in a unique biphase dose-response curve (α6LMQNβ3y2S) with the higher and lower affinity closely resembling those imposed by the original sequences α6 and α3, respectively (Fig. 6B). This is even more surprising as the exchanged domain comprises just two altered amino acids, with only one of these being unique to all six 4aa motifs (Q). As we can exclude any functional contribution of homomeric β3 receptors or β3y2S receptors to the biphase nature of the α3LMQNβ3y2S dose-response curve (data not shown), our results suggest an additional mechanism in the α6 subunit, besides the 4aa motif, that underlies its extraordinarily high sensitivity. Using chimeric subunits of the α1 and α6 subunit, we now aim to identify its molecular basis.

In heteropentameric GABAA receptor complexes the putative GABA binding site is presumed to be located at the α-β interface. A variety of approaches revealed several residues in the α and β subunits that line the binding pocket. From these experiments it was concluded that in the α subunits most amino acids relevant to the recognition of GABA cluster in two segments of the extracellular N-terminal domain, i.e. Phe205→Ser207 (25-28) and Val205→Asp210 (29). Additionally, a conserved arginine N-terminally adjacent to the 4aa motif (Fig. 3) has been proposed to be a key factor for both binding and action of GABA, as mutant α1 (30) or α6 subunits (31) in which the respective residue was substituted by lysine showed a dramatic increase in the Kd of the [3H]muscimol binding as well as the EC50 to GABA. All amino acid residues up to now associated with the formation of the GABA binding site are highly conserved among the α subunits, most likely because of the search criteria employed. Because of the wide variance in EC50 values of GABAA receptors containing different α subunit isoforms, the sites involved in the action of GABA on the α subunits need to contain highly variable residues in addition to the known set of conserved amino acids. We tentatively suggest that the 4aa motif is part of this variable segment.

Exchange of the 4aa motif was not accompanied by a corresponding shift in equilibrium binding parameters of the GABA agonist [3H]muscimol. As ligand-binding affinity at steady state most likely recognizes receptors in a desensitized and high affinity state, we propose that the 4aa motif does not contribute to the high affinity binding site. However, it may contribute to the low affinity binding site, which has been interpreted as a conformational variant of the high affinity site (32).

A previous study (33) showed that exchange of the isoleucine belonging to the α1TED motif by valine decreases both the sensitivity to GABA and the affinity of agonist GABAA receptor ligands in mutant α1β2y2 receptors. The discrepancies between these data and our results may be caused by the nature of the introduced amino acid. The first position of the 4aa motif in all known α subunits is occupied either by leucine or isoleucine. Whereas replacement of isoleucine by leucine may maintain the structural features of the 4aa motif, introduction of valine could induce conformational changes in both the motif and its flanking segments. Furthermore, we cannot exclude a decisive role of the different β subunits employed in the two studies.

Information about the three-dimensional arrangement of GABAA receptors is based primarily on the structure derived from the soluble molluscan acetylcholine-binding protein (AChBP), a homologue of the N-terminal ligand-binding domain of the nicotinic acetylcholine receptor α subunits (34). By definition, acetylcholine receptors share structural and functional characteristics with other members of the superfamily of ligand-gated ion channels. Therefore, it is permissible to model the extracellular region of the GABAA receptor according to the crystal structure of AChBP (35, 36). All available models include the 4aa motif in a secondary structure named "loop E," claimed to be part of the GABA binding pocket at an α-β plus-minus interface (35). Depending on the alignment chosen, these four residues are predicted either to link the β5 and β6 strands (34) or to participate in β5′ (35, 36). As all predictions rely on accurate sequence alignments anchored around con-
served residues, they become more speculative in regions of low
or absent homology. The 4aa motif is present only in the
GABA_A receptors and possibly the glycine receptors (see Fig. 3)
but is missing in other members of the ligand-gated ion channel
superfamily. Thus, the alignment of the 4aa motif remains
uncertain, and extrapolations from the structure of the AChBP
to the function of GABA_A receptors should be handled with
cautions (34–36).

The activation of GABA_A receptors involves the processes of
binding to the sensitized receptor, signal transduction, and
finally channel opening. Our study was not geared toward
identifying the precise molecular role of the 4aa motif. Rather,
we expect that the data presented here will help to further
elucidate the binding process and signal transmission
mechanism of GABA_A receptors. Moreover, the results of our study
may encourage a knock-in approach in which exchange of the
4aa motif between α subunit genes should result in the expres-
sion of mutant receptors with altered sensitivity to GABA.
Resulting phenotypic abnormalities and possible compensations
could be attributed directly to unbalanced neuronal re-
sponses to GABAergic inputs and hence might settle the sig-
nificance of the α subunit diversity in the physiology and
pathophysiology of the brain.

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