Single Expressed Glycine Receptor Domains Reconstitute Functional Ion Channels without Subunit-specific Desensitization Behavior*  

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Background: Functional GlyRα1 receptors can be reconstituted from nonfunctional subunit domains.  

Results: GlyRα3 and GABA A/β1 rescues are less efficient with the alternative splicing cassette in the TM3–4 loop of GlyRα3 responsible for desensitization and rescue efficiency.  

Conclusion: Desensitization of GlyRα3 requires nondisrupted intracellular domains.  

Significance: Independent domain reconstitution does not always recapitulate the full functional properties of receptors.  

Cys loop receptors are pentameric arrangements of independent subunits that assemble into functional ion channels. Each subunit shows a domain architecture. Functional ion channels can be reconstituted even from independent, nonfunctional subunit domains, as shown previously for GlyRα1 receptors. Here, we demonstrate that this reconstitution is not restricted to α1 but can be transferred to other members of the Cys loop receptor family. A nonfunctional GlyR subunit, truncated at the intracellular TM3–4 loop by a premature stop codon, can be complemented by co-expression of the missing tail portion of the receptor. Compared with α1 subunits, rescue by domain complementation was less efficient when GlyRα3 or the GABA A/β1 subunit β1 was used. If truncation disrupted an alternative splicing cassette within the intracellular TM3–4 loop of α3 subunits, which also regulates receptor desensitization, functional rescue was not possible. When α3 receptors were restored by complementation using domains with and without the spliced insert, no difference in desensitization was found. In contrast, desensitization properties could even be transferred between α1/α3 receptor chimeras harboring or lacking the α3 splice cassette proving that functional rescue depends on the integrity of the alternative splicing cassette in α3. Thus, an intact α3 splicing cassette in the TM3–4 loop environment is indispensable for functional rescue, and the quality of receptor restoration can be assessed from desensitization properties.  

The glycine receptor (GlyR) is a ligand-gated chloride channel that mediates fast neuronal inhibition predominantly in adult mammalian brain stem and spinal cord. It is a member of the Cys loop receptor (CLR) superfamily also including the nicotinic acetylcholine receptor, the γ-aminobutyric acid receptors type A and C, as well as the 5-hydroxytryptamine type 3 receptor. These receptors share a conserved three-dimensional structure with a large extracellular ligand-binding domain followed by four transmembrane domains (TM1–4), connected via intra- and extracellular loops, and a short extracellular C terminus. A functional CLR is composed of five homologous subunits arranged around a central ion-conducting pore (1). The adult GlyR consists of two α and three β subunits (2). The extracellular ligand-binding domain of the glycine receptor harbors two disulfide bonds, one of which is eponymous for the receptor family and a second disulfide bond located in loop C is described for the glycine receptor and the glutamate-gated chloride channel (GluCl) from Caenorhabditis elegans (3, 4). The large intracellular loop between TM3 and TM4 (TM3–4 loop, also referred to as ICD) is of highest diversity among CLRs.  

Alternative splice sites located within the ICD of GlyRα1 and GlyRα3 contribute to GlyR variability. In contrast to the GlyRα1, splice variants GlyRα3K (spliced form) and GlyRα3L (unspliced form) differ in desensitization properties. Homodimeric GlyRα3L ion channels are essentially nondesensitizing, whereas GlyRα3K desensitizes fast (5). The alternative splicing cassette in α3 is composed of 15 residues and carries possible phosphorylation sites. A series of mutations within this insert has shown that amino acids harboring hydroxyl groups (Thr-358/Tyr-367/Ser-370) are important mediators in the desensitization process. In addition, the insert present in α3L seems to stabilize the overall spatial structure of the domain thereby regulating receptor gating (6, 7). Further studies on the α1 TM3–4

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The abbreviations used are: GlyR, glycine receptor; ICD, intracellular domain; CLR, Cys loop receptor; TM, transmembrane; ER, endoplasmic reticulum.
loop demonstrated the importance of this domain for forward trafficking, nuclear translocation, and post-translational modifications such as interaction with Gβγ proteins (8–10). Common to these various pathways are motifs carrying basic residues at the N- and C-terminal end of the intracellular TM3–4 loop. The importance of the basic stretch 318RRKRR at the N-terminal end of the TM3–4 loop for trafficking and function of the GlyRα1 was demonstrated in a study of the mouse mutant oscillator; where a microdeletion generates a premature STOP codon within the alternative splicing cassette of the GlyRα1 subunit (11, 12). This mutation leads to a neuromotor phenotype and death 3 weeks after birth due to the absence of functional adult α1 subunits. A recent in vitro study on modular GlyR domain architecture revealed that the function of the truncated oscillator GlyRα1 protein as well as an equivalent wild type truncated α1 can be rescued by co-expression of an independent C-terminal tail construct representing the lacking GlyRα1 domain. Therefore, functional GlyRα1 receptors can be rebuilt from independently co-expressed domains (13). Protein truncations have also been observed for GlyRs and GABA_A/C receptors being associated with either the human neuromotor disorder hyperkplexia or a special form of epilepsy GEFS+ (14–16).

Here, we wanted to investigate whether assembly of a functional ion channel from independent domains illustrates a general principle for CLR members not restricted to GlyRα1. GlyRα3 and GABA_A/C ρ1, a closely related inhibitory anion channel, were used for intrafamilial domain complementation among various GlyR subunits (α1 and α3) and interfamilial rescue between GlyR α1 and GABA_A/C ρ1. Furthermore, we show that desensitization is affected by domain complementation and is indeed a measure for the quality of receptor reconstitution.

Our data show that co-expression of receptor domains derived from GlyRα1 and α3 displayed almost mutual compatibility. In contrast, GABA_A/C ρ1 domains were incompatible with GlyR domains. Functional domain complementation revealed that the TM3–4 loops are major determinants of rescue efficiency. Differences in the desensitization between GlyRα3 K- and L- splice variants determined by the 15-residue spliced insert within the TM3–4 loop are not maintained by domain co-expressions. Thus, the spliced insert may not be disrupted for restoration of wild type-like receptor properties by subunit complementation.

**EXPERIMENTAL PROCEDURES**

**Sequence Alignment, Homology Modeling, and Building of Hybrid Complexes—**Homology models of the transmembrane domain (TM) without loops of α1 GlyR, α3 GlyR, and ρ1 GABA_A/C were generated by using the crystal structure of the glutamate-gated chloride channel (Gluclo) from C. elegans at 3.35 Å resolution (Protein Data Bank entry 3RIF (3)) as a template. The sequences of mouse α1 GlyR (gi|118130520_58-1407), mouse α3 GlyR (gi|20300935_32-1474), and rat ρ1 GABA_A/C (gi|8393398_130-1554) were aligned according to the ClustalW algorithm using the default settings shown in Ref. 17. Additionally, the generated multiple sequence alignment was manually adjusted to coincide with the alignment reported in previous work (18, 19). The visualization of the alignment was performed with GenDoc (20). Molecular modeling was performed by standard procedures using MODELLER6.2 (21). All five subunits of the pentamer were modeled simultaneously. The obtained models were improved by 200 steps of conjugated gradient energy minimization using the Powell algorithm in Sybyl7.3 (22). The quality of the models was verified by WHAT_CHECK (23) and DaliLite (24). The visualization of the structures was performed with DS Viewer Pro6.0 (25). Hybrid complexes of α1, α3, and ρ1 were generated by replacing side chains of nonconserved TM4 amino acids in the original models using the Sybyl7.3 program package. Noncovalent interactions were improved by 200 steps of conjugated gradient energy minimization using the Powell algorithm.

**Molecular Biology and Cloning—**Truncated GlyRα3 and GABA_A/C ρ1 were generated by introduction of an early STOP codon at the corresponding amino acid position to the GlyRα1 oscillator truncation localized in the TM3–4 loop. The GlyRα3 was truncated at position L330X and GABA_A/C ρ1 at position Q370X (referring in both cases to numbering of the mature protein). Corresponding tail constructs were generated with no overlapping sequence and represent the lacking portion of both truncated GlyRα3 and ρ1 with a large portion of the TM3–4 loop, TM4, and the C terminus. Site-directed mutagenesis was used to exchange the TM4 domains between α1 and α3 GlyRs. Large overlapping primers representing the specific α1 or α3 TM4 sequence were used in an overlap extension PCR to amplify extended TM4 domains. These amplimers were cut at subunit-specific restriction sites and ligated afterward into the appropriate tail construct. Chimera between α1 and α3 were generated using the sequence identity of TM3 and therefore the existence of shared restriction sites. All clones were verified by sequencing and encoded on a pRK5 plasmid under the control of a CMV promoter that allows eukaryotic cell expression.

**Membrane Preparation and Biotinylation—**Following 24–48 h post-transfection of various cDNA constructs into HEK293 cells using the calcium precipitation method, cells were harvested for cell lysates, membrane preparations, or used directly for cell surface protein detection with a biotinylation assay. For membrane preparation, the cells were collected in PBS, pH 7.4, centrifuged, and transferred into a potassium buffer containing 10 mM K_H2PO4, pH 7.4, 250 mM EDTA, 250 mM EGTA, and protease inhibitor mixture tablets (Roche Applied Science). Cells were homogenized with a glass homogenizer followed by very short sonification and centrifugation at 25,000 × g for 20 min. The pellet was again homogenized, and the centrifugation step was repeated. Pellets were resuspended in buffer B (25 mM K_H2PO4, pH 7.4, 200 mM KCl, 250 mM EDTA, 250 mM EGTA, and protease inhibitor mixture tablets; Roche Applied Science) and stored at −80 °C. Biotinylation experiments were performed as described by Unterer et al. (26).

**Immunocytochemistry—**Transfected HEK293 cells were fixed using 4% paraformaldehyde with 4% sucrose. For intracellular detection of receptor proteins, cells were permeabilized with 0.1% Triton X-100, blocked with goat serum, and stained with the GlyR pan-α antibody Mab4a (Synaptic Systems, Göttingen, Germany). All tail constructs were tagged with a Myc epitope and stained using either the c-Myc monoclonal (9E10)
or c-Myc polyclonal (C19) antibody (Santa Cruz Biotechnology, Dallas, TX). For detection of the ρ1 subunit, a polyclonal antibody (kindly provided by R. Enz (27)) was used. This antibody can only be used for immunocytochemistry; however, it does not stain the ρ1 protein in a Western blot. GlyRα1 or -α3 variants were detected with the pan-α antibody Mab4a in immunostainings following Western blotting. pDsRed-ER and pDsRed-PM vectors were co-transfected for sub-compartmen-
tal localization of α3 and ρ1 variants. Both vectors express fusion proteins of the red fluorescent protein DsRed and a domain of the ER marker calreticulin (pDsRed-ER) or GAP-43, a membrane marker protein.

**Electrophysiological Recordings**—Whole-cell currents were recorded using a HEKA EPC9 amplifier (HEKA Electronics, Lambrecht, Germany) controlled by Pulse software (HEKA Electronics) on a personal computer. Recording pipettes were pulled from borosilicate glass (World Precision Instruments, Berlin, Germany) using a Sutter P-97 horizontal puller. Ligand application using a U-tube gave a time resolution of 20–30 ms. The external buffer consisted of NaCl 137 mM, KCl 5.4 mM, CaCl2 1.8 mM, MgCl2 1.0 mM, Hepes 5.0 mM, pH adjusted to 7.2 with NaOH; the internal buffer was CsCl 120 mM, N(Et)4Cl 20 mM, CaCl2 1.0 mM, MgCl2 2.0 mM, EGTA 11 mM, Hepes 10 mM, pH adjusted to 7.2 with CsOH. Current responses could be measured at a room temperature of 21–23 °C, the holding potential was −60 mV. For each construct, the mean maximum current ($I_{\text{max}}$) at a saturating glycine concentration (3 mM) was calculated from all cells that were used for analysis. In recordings with the absence of ρ1 subunit domains, the ligand GABA was applied at a concentration of 500 μM.

**Data Analysis**—For desensitization analysis, whole-cell current traces were transferred to Microcal Origin (Microcal Software, Inc.), and the decaying current phase was analyzed using a single exponential function plus a constant as shown in Equation 1,

$$I_{\text{obs}} = I_{1} * e^{-t/\tau_{1}} + I_{\text{const}} \quad (\text{Eq. 1})$$

where $I_{\text{obs}}$ is the observed total current amplitude; $I_{1}$ is the fraction of current desensitizing with time constant $\tau_{1}$; and $I_{\text{const}}$ is the amplitude of the nondesensitizing current fraction. For all constructs, a single exponential decay plus a constant term were sufficient to describe desensitization behavior. Functional constants of the co-expressed subunits were compared using one-way analysis of variance (Microsoft Origin) followed by Dunnett’s post hoc test. A probability of error of $p < 0.05$ was considered significant.

**RESULTS**

**Generation of Individual Receptor Domains and Chimeric Constructs**—Recent experiments have shown that in vitro functional reconstitution of a truncated GlyRα1 (α1trc) receptor, as present in the mouse mutant oscillator, can be achieved by co-expression of the missing C-terminal GlyR (α1_tail) portion (13). It was demonstrated that two parts of the receptor behave as independent folding domains and are capable of assembling into functional pentameric receptors (Fig. 1A). To test for inter-subunit compatibility of truncated N-terminal domains with C-terminal domains derived from other GlyR subunits or even from other members of the CLR family, we cloned the respective truncated and tail constructs with no overlapping sequences for HEK293 cell expression of the following: 1) GlyRα1 (α1trc, α1_tail); 2) GlyRα3 (α3trc, α3_tail); and 3) the ρ1 (ρ1trc, ρ1_tail) subunit, a member of the GABA_A/C receptor family. The GABA_A/C ρ1 subunit as a non-glycine but also an inhibitory Cl⁻-permeable receptor isoform of the CLR family was selected, as this subunit was able to form functional homomers in vitro. A variety of different intra-

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**FIGURE 1. Constructs used to investigate receptor rescue efficiency and functionality.** A, concept of functional rescue by co-expression of independent portions. N-terminal (NT) truncated constructs include the extracellular ligand-binding domain followed by three transmembrane domains (including the connecting loops). C-terminal constructs comprise part of the intracellular TM3–4 loop (ICD) with the white circle representing an alternative splicing cassette present in GlyRα1 and GlyRα3, and TM4 as well as the extracellular C terminus. B, schematic representations of receptor portions (truncated N-terminal domain = trc, C-terminal complementation constructs = tail) and combinations for co-expression studies. Color-coded GlyR (α1, light gray, and α3L and α3K white) and GABA_A/C (ρ1, dark gray) receptor subunits. α3 refers to the respective long splice variant (GlyR3L) except as otherwise noted. Black boxes represent TMs. C-terminal tail constructs include a Myc tag (myc) for detection purposes. TM4 belongs to the same subunit unless noted elsewhere.
FIGURE 2. Subcellular localization of independently as well as co-expressed receptor portions. A–D, immunocytochemical stainings of transfected permeabilized HEK293 cells expressing GlyRα3 and GABA<sub>A/C</sub> p1 constructs. A, GlyRα3 WT and the truncated N-terminal part of α3 (α3trc) were stained with Mab4a and a secondary antibody g<sub>r</sub>-Alexa488. The C-terminal part (tail of α3 and p1) was stained with a c-Myc antibody and g<sub>m</sub>-Alexa488 (A and B). B, wild type p1 and p1trc were stained with a specific polyclonal antibody against the N-terminal portion of p1, and g<sub>a</sub>-Alexa488 was used as secondary antibody. Cells were co-transfected either with pDsRed-PM or pDsRed-ER, both encoding fusion proteins of the red fluorescent protein DsRed and part of the ER marker calreticulin or GAP-43, a membrane marker protein. Notably, the C-terminal constructs and the truncated N-terminal domains were retained intracellularly when expressed as single domains compared with the corresponding WTs. C and D, co-expression of both α3 and p1 domains co-expressed with pDsRed-PM (GAP-43). Co-localizations of both receptor domains from α3 and p1 are marked with white arrowheads (enlarged upper right panels in C and D). Same antibodies used as in A and B, except α3_tall was stained with a polyclonal c-Myc antibody and g<sub>a</sub>-Cy3. Middle and lower enlarged right panels in C and D represent a co-staining of the membrane marker GAP-43 with truncated and tail domains of α3 and p1. Note that the tails domains are shown in false-color (cyan) for better discrimination. **White bar corresponds to 15 μm.**

subunit and intersubunit combinations of independently expressed domains was tested (Fig. 1B). Additional tail constructs were generated with an exchange of the TM4 domain between α1 and α3 (Fig. 1B) as follows: (i) α1(TM4_α3) contains the TM3–4 loop of α1 and TM4 of α3; (ii) α3(TM4_α1) contains TM3–4 loop of α3 and TM4 of α1. These chimeric tail constructs were generated to analyze the importance of the TM4 domain complementation, because TM4 has been recently shown to play a crucial role for receptor assembly (Fig. 1B) (28). For detection as well as for the analysis of correct membrane integration, a Myc tag was added to the N terminus of the tail constructs.

Subcellular Localization and Expression Levels of Individual Receptor Domains—Subcellular localization of the truncated N-terminal receptor domains of α3trc and p1trc was investigated using single expression as well as co-expression with the appropriate C-terminal portion. Single domains of α3 or p1 and the corresponding wild types were co-expressed with pDsRed-ER (encoding a fusion protein of DsRed and calreticulin as an ER marker) or pDsRed-PM (fusion protein of DsRed and GAP-43 for membrane expression) to distinguish between intracellular and surface receptor proteins.

The monoclonal antibody Mab4a was used for detection of the GlyR truncated domain α3trc, and the polyclonal anti-p1 antibody was used to stain the GABA<sub>A/C</sub> p1 truncated N-terminal domain (p1trc). Detection of the tail constructs was performed using a monoclonal or a polyclonal c-Myc antibody depending on the origin of the antibody against trc variants. Because of the intracellular location of the Myc epitope attached to the C-terminal tail variants, all stainings were carried out after permeabilization of transfected HEK293 cells.

For both the GlyRα3L as well as the GABA<sub>A/C</sub> p1 wild type (WT) receptors, staining throughout the cell body could be observed with expected enhanced fluorescence at the cell membrane (Fig. 2, A and B). The truncated N-terminal domains α3trc and p1trc, in contrast, showed a high intracellular fluorescence with no detectable accumulation at the plasma membrane, indicating a disturbed subcellular trafficking of truncated variants (Fig. 2, A and B, middle panels). The same pattern of distribution was observed in previous studies on α1trc (13).
The exclusively expressed tail constructs (α3_tail and ρ1_tail) displayed a strong cytoplasmic staining, presumably due to retention of most of the protein within the endoplasmic reticulum (Fig. 2, A and B, lower right panels). Thus, no robust localization at the plasma membrane could be observed for any of the single expressed receptor domains of GlyRα3 and ρ1.

Upon co-expression of the truncated α3 and ρ1 together with α3_tail or ρ1_tail domains, however, co-localization as well as translocation to the plasma membrane of the complementary fragments were observed (Fig. 2, C and D, merged picture with insets demonstrating close proximity to the membrane marker GAP-43). These findings could be corroborated using Western blotting of whole-cell lysates (Fig. 3A) and specific cell surface localization using biotinylation of protein as a tool for separation from intracellular polypeptides (Fig. 3, B–D). In contrast to α3 variants, ρ1_tail expression was highly decreased in whole-cell as well as surface expressions (Fig. 3, C and D).

**Differences in Rescue Efficiency between Intra- and Intersubunit Rescue Combinations**—The GlyRα1 is composed of independent folding domains able to restore functionality from nonfunctional subunit domains (13). Indeed, co-expression of α1trc with α1_tail resulted in glycine-gated currents of up to 1.4 ± 0.2 nA, which corresponds to about 50% rescue efficiency compared with Cl– currents recorded from α1 WT expression in vitro (3.0 ± 0.6 nA, Fig. 4, A and B, and Table 1). Co-expression of α3 and ρ1 domains restored functionality of glycine-gated α3 and GABA-gated ρ1 channels with efficiencies of 16% for α3 and 7.5% for ρ1 compared with the appropriate WT currents (Fig. 4, A and B, and Table 1). For test of intersubunit compatibility of domains for functional ion channel rescue, α1trc was co-expressed with α3_tail or ρ1_tail and α3trc together with α1_tail (Fig. 4A). Interestingly, the α3trc was rescued by the α1_tail (0.26 ± 0.1 nA with 8% of α3 WT), but restoration of α1trc did not occur in a co-expression with the α3_tail nor with the ρ1_tail (Fig. 4C). Thus, these data show that...
Domain Architecture of GlyRα1 and GlyRα3

FIGURE 4. Functional complementation of GlyRα subunits and the GABA\textsubscript{A\_receptor} variant ρ1. A, representative traces of intrafamiliar functional rescue experiments of α1 + α1\_tail, α2 + α3\_tail, p1 + p1\_tail, and interfamiliar domain co-expressions of p1 variants with α3 and vice versa, also α1 with p1 (ratio 1:5 of Xtrc:tail with X either α1, α2, or p1). Glycine (500 μM or 3 mM) was applied for 2 s or 10 s as indicated. When p1 trc was expressed, GABA-induced (500 μM and 3 mM) GABAergic currents were detected. B, relative maximal current amplitudes (I\textsubscript{max}) from intrafamiliar rescue experiments are shown. The mean I\textsubscript{max} values of the wild type subunits α1, α3L, and ρ1 was set to 100%. Note, the reduced rescue efficacy for α3 and ρ1 complementation from independent domains is compared with α1. C, relative I\textsubscript{max} values from interfamiliar rescue experiments (trc and tail domain from different GlyR subunits or GlyRα1 together with p1) compared with intrafamiliar rescue (trc and tail domains originate always from the same subunit). The mean current amplitudes of the most efficient rescue of α1 were set to 100%. Interestingly, the α3trc is rescued by an α1\_tail but α1trc together with α3\_tail does result in nonfunctionality.

TABLE 1

| Whole-cell maximal currents for various co-expressed GlyR and GABA\textsubscript{A\_receptor} domains |
|-------------------------------------------------|
| HEK293 cells expressing different GlyR or GABA\textsubscript{A\_receptor} variants without or with co-expressed tail domains were patched following 48 h post-transfection; n = number of cells recorded; two different agonist concentrations were applied (500 μM glycine or 500 μM and 3 mM GABA). * indicates cells responded out of cells recorded; and the I\textsubscript{max} values were calculated from cells that responded only. |

| expressed domains | number of cells | I\textsubscript{max} [pA] | number of cells | I\textsubscript{max} [pA] |
|-------------------|----------------|----------------|----------------|----------------|
| 500 μM glycine    |                |                |                |                |
| α1 WT             | 16             | 3040 ± 591     | 12             | 2646 ± 495     |
| α1 trc            | 8              | 0 ± 0          | 8              | 0 ± 0          |
| α1 trc + α1 tail  | 20             | 1433 ± 218     | 30             | 991 ± 148      |
| α1 trc + α3 tail  | 6              | 0 ± 0          | 6              | 0 ± 0          |
| α1 trc + p1 tail  | 7              | 0 ± 0          | 7              | 0 ± 0          |
| α1 trc + p1 (TM4\_α3) | 10           | 813 ± 150     | 10             | 0 ± 0          |
| α3L WT            | 16             | 2140 ± 333     | 28             | 3134 ± 320     |
| α3 trc            | 8              | 0 ± 0          | 14             | 0 ± 0          |
| α3 trc + α3 tail  | 4 / 19*        | 71 ± 20*       | 16 / 13*       | 494 ± 140      |
| α3 trc + α1 tail  | 5              | 248 ± 122      | 13             | 250 ± 32       |
| α3 trc + α3 (TM4\_α1) | 28           | 3725 ± 436     | 28             | 3725 ± 436     |
| α3 trc + α1 (TM4\_α3) | 8              | 961 ± 385      | 8              | 961 ± 385      |

| 3 mM GABA         |                |                |                |                |
| p1 WT             | 15             | 1451 ± 743     |                |                |
| p1 trc            | 5              | 0 ± 0          | 5              | 0 ± 0          |
| p1 trc + p1 tail  | 3 / 14*        | 109 ± 40*      | 6 / 15*        | 111 ± 45*      |
| p1 trc + α1 tail  | 4              | 0 ± 0          |                |                |

an interfamiliar functional complementation of independent folding domains seems to be impossible.

To determine the reason for α1trc nonrescue by the α3 tail in contrast to α3trc rescue with the α1\_tail, we compared the corresponding amino acid sequences. The tail domain is composed of most of the TM3–4 loop, the region with highest variability among all CLRs, TM4, and the C terminus. The TM3–4 loops of α1 and α3 have been shown to be important for assembly, desensitization, interactions with Gβγ proteins, and phosphorylation (6, 29, 30). TM4 contains six variations in the amino acid composition between α1 and α3 with three nonconservative exchanges (α1Gly-424 to α3Cys-434, α1Met-427 to α3Leu-437, and α1Met-434 to α3Ile-444) (Fig. 5).

To verify whether differences in the TM3–4 loop or TM4 are the underlying cause for the lack of functional rescue of α1trc with the α3\_tail, we used molecular modeling of the TM domains to determine surface contacts of TM4 to TM1–3 based on the structural information of the GluCl (Fig. 6A). TM1 and TM3 exhibit identical amino acids at the interface in α1 and α3 that is recognized by TM4 (Fig. 6B, dotted line). The side chains of residues α1Ile-270 localized at the interface in TM1 and of α1Gly-284 in TM2 twisted outward from the contact interface of α3TM4 to α1TM1–3 (Fig. 6B). The in silico analysis suggested that this interface, which is composed of identical amino acids in α3 and α1, can bind both the TM4 of α1 and α3.
FIGURE 5. Sequence alignment of the transmembrane domains of the GlyR and GluClR. TM1, TM2, TM3, and TM4 of mouse α1 GlyR (Glrα1, gi|118130520_58–1407), mouse α3 GlyR (Glrα3, gi|120300935_32–1474), and rat α1 GABA<sub>A</sub>C (Gabrr1, gi|8393398_130–1554) were aligned with the glutamate-gated chloride channel (GluCl<sub>1</sub>, 3RIF). Amino acids that are conserved and semi-conserved residues in all aligned sequences are indicated by asterisks, colons, and dots, respectively. Locations of alternative splice cassettes in α1 and α3 are marked by gray boxes.

FIGURE 6. Molecular model of the transmembrane domains (TM1–4). TM4 is shown in orange ribbon representation. A, TM1–3 are shown in van der Waals representation and are colored in white. The interface that is recognized by TM4 is colored in dark gray. Comparing α1 and α3, the different amino acids in TM4 are shown as sticks. B, view from the intracellular region. Comparing the TM1–3 of α1 and α3, the different amino acids are shown in space-fill representation. The dashed line represents the contact surface of TM4 toward the other TM domains.
Domain Architecture of GlyRα1 and GlyRα3

A typical traces recorded from transfected HEK293 cells expressing truncated α1 or α3 with tail domains that carry either the TM3–4 loop sequences of α1 together with TM4 of α3 (α1(TM4_α3)) or TM3–4 loop sequences of α3 together with TM4 of α1 (α3(TM4_α1)). B, bars diagram representing absolute glycine-induced current values of various domain co-expressions (e.g. α1trc with α3(TM4_α3) or α3(TM4_α1)). The same tail constructs α1(TM4_α3) and α3(TM4_α1) were used for functional rescue experiments together with α3trc.

and is therefore most likely not responsible for the nonfunctionality between α1trc and α3_tail.

To test the predictions of these models, we constructed chimeric tail variants of α1 and α3 with a TM3–4 loop of α1 and TM4 of α3 (α1(TM4_α3)) and vice versa with the TM3–4 loop of α3 and TM4 of α1 (α3(TM4_α1)) (Fig. 1B). The co-expression of α3trc together with α1(TM4_α3) gave 1.0 ± 0.1 nA (32% of α3 WT), thereby increasing rescue efficiency as compared with α1_tail (Fig. 7, A and B), arguing for an effect of TM4 in domain-domain communication. When α3trc was co-expressed with the construct α3(TM4_α1), current responses similar to WT α3L were observed. Interestingly, this rescue was more efficient than α3trc + α3_tail. However, the α1trc was not rescued by an α3_tail. Likewise, providing a tail construct with TM4 of α1 (α3(TM4_α1)) did not result in generation of functional ion channels. If the tail domain contained the TM3–4 loop of α1 with TM4 of α3, functionality was restored similar to the α1 rescue (α1trc + α1(TM4_α3) 0.8 ± 0.15 nA compared with α1trc + α1_tail 1.0 ± 0.15 nA; Fig. 7, A and B).

Thus, the presence of a TM3–4 loop of the same GlyR subunit as the co-expressed truncated N-terminal receptor portion seems to improve GlyR domain-domain communication. Although our models demonstrated no differences at the contact sites of TM4 between α1 and α3 toward other TMs of the same subunit, TM4 of α3 led to an increase in \( I_{\text{max}} \) values upon co-expression with α3trc.

**Chimeric Complementation of GlyRα1 and GlyRα3**—The α3 tail does not coassemble with α1trc. Chimeric approaches between various types of CLRs, however, have demonstrated that exchanges of the TM3–4 loop between different members of the CLR family do indeed result in functional receptors. Even more, ion channel properties determined by the TM3–4 loop sequence were restored and transferable to other subunits (31, 32). We generated chimeric constructs of α1 and α3 domains that have been used for domain complementation experiments before to analyze the function of both domains in a continuous polypeptide chain (Fig. 8A). The ICD of α1 and α3 harbors alternative splicing cassettes that vary in length (8 amino acid residues in α1 and 15 amino acid residues in α3, Fig. 5) (5, 33). Different from α1 splice variants, the long α3 receptor variant α3L differs to the short variant α3K in desensitization (5).

Generally, the subunit switch was located at the transition from TM3 to the TM3–4 loop (Fig. 8A). Chimeras between α1 and α3 were generated with the alternative 15-amino acid residue cassette of α3 intact (α1-α3L) or missing (α1-α3K, Fig. 8A). In addition, we also enclosed a chimeric receptor where the transition was located at the position homologous to the end of the oscillator GlyRα1 (upper bar, Fig. 8A, refer to construct α1rescue). In line with the domains analyzed so far, we also constructed chimeras with TM4 and the C terminus of α1 (α1-α3L-α1 and α1-α3K-α1) constructs are α1 with the TM3–4 loop sequence of α3L or α3K (Fig. 8A).

All chimera were expressed at the outer cell surface of transfected nonpermeabilized HEK293 cells (Fig. 8B). Whole-cell recordings in the presence of saturating glycine concentrations (3 mM) revealed functional ion channels for all α1-α3 chimera with \( I_{\text{max}} \) values not significantly different from α1 or α3 WT (Fig. 8B and Table 2).

Traces of α3K, α3L, and the α1α3 chimeras were analyzed for desensitization kinetics (Fig. 8D) due to the fact that the subunit switch lies within the subdomain determining differences in desensitization in GlyRα3. Lack of the spliced insert in α3K resulted in desensitization time constants of 1.5 ± 0.2 s similar to previously published data (5, 7). The desensitization properties of α3K and α3L are restored in the α1α3 chimera (Fig. 8D and Table 2). The α1rescue construct harboring nine residues of the 15-amino acid insert present in α3L ended up with a large fraction of nondesensitizing currents (α1rescue 75.2% compared with α3L 91.3%). These data show that not all residues of the 15-amino acid splice cassette are required to generate non-desensitizing ion channels.

**Desensitization in Domain Complementation Studies**—Here, we used α3trc and co-expressed this domain with various α3 tail to analyze whether desensitization is restored in an ion channel assembled from independent domain co-expressions. Tail constructs with the complete splice cassette (α3L_tail), a half-splice cassette (α3_tail), and lack of the insert (α3K_tail) were used (Fig. 9A). The expression level of the tail constructs together with α3trc was verified in a biotinylation assay.
Although all GlyR domains are expressed in total protein samples, the surface expression varied considerably (Fig. 9B). The $\alpha_3$trc and $\alpha_3$_tail expressions in the plasma membrane were markedly reduced compared with the other constructs. This finding might also explain the low rescue efficiency observed in whole-cell recordings for the $\alpha_3$trc $\alpha_1$WT (Fig. 9B, lane 2) co-expression. The expression of the $\alpha_3$K_tail and $\alpha_3$L_tail was higher, leaving the low cell surface expression of $\alpha_3$trc unaffected (Fig. 9B). Functional ion channels were generated by all domain co-expressions of $\alpha_3$trc with various $\alpha_3$_tails generating $I_{\text{max}}$ reductions of 8–43% of $\alpha_3$L WT that might be due to the low expression levels of $\alpha_3$trc (Fig. 9, B and C). Again, $\alpha_1$trc was insufficiently rescued by either the $\alpha_3$K_tail or the $\alpha_3$L_tail (Fig. 9C) arguing for poor interaction between the N-terminal domain of GlyR$\alpha_1$ with the C-terminal domain of GlyR$\alpha_3$ in a co-expression approach. The fast desensitization of $\alpha_3$K was completely abolished by domain co-expressions. All restored $\alpha_3$ ion channels showed nondesensitizing current responses with time constants similar to $\alpha_3$L (Table 2). Likewise, the fraction of nondesensitizing currents was
**TABLE 2**

Desensitization properties of various co-expressed GlyRα3 domains

HEK293 cells expressing different GlyRα3 variants without or with co-expressed tail domains were patched following 48 h post-transfection; n = number of cells recorded; agonist concentration was applied 3 mM glycine; *, n = number of cells responded out of all cells recorded; #, only one cell. p values refer to the appropriate WT values as follows: *, p < 0.05; ***, p < 0.001.

| Expressed domains | No. of cells | 3 mM Gly | 3 mM Gly |
|-------------------|--------------|----------|----------|
|                   | n            | I_{max} (pA) ± S.E. | Nondesensitizing current fraction (%) ± S.E. | \( t\)_{desens} (s) ± S.E. |
| \( \alpha3\text{L WT} \) | 14           | 3200 ± 540 | 91.3 ± 1.4 | 1.4 ± 0.3 |
| \( \alpha3\text{K WT} \) | 12           | 2293 ± 616 | 59.9 ± 1.8*** | 1.1 ± 0.1 |
|           | 14           | 3436 ± 748 | 82.4 ± 4.5 | 0.9 ± 0.1 |
| \( \alpha3\text{K tail} \) | 8            | 958 ± 197  | 67.6 ± 5.8*** | 1.1 ± 0.2 |
|           | 2/19*        | 67 ± 8*    | 89*       | 2.8* |
|           | 17           | 403 ± 196  | 86.7 ± 2.3 | 1.8 ± 0.2*** |
| Chimera        |              |           |          |
| \( \alpha1 \) | 8            | 3899 ± 499 | 69.0 ± 4.8 | 1.8 ± 0.4 |
| \( \alpha3 \text{K} \) | 10          | 3641 ± 507 | 52.6 ± 9.8 | 1.5 ± 0.2 |
| \( \alpha1\text{rescue} \) | 7           | 3621 ± 776 | 75.2 ± 6.6 | 1.8 ± 0.2 |
| \( \alpha1\text{-}\alpha3L \) | 10          | 2593 ± 478 | 76.0 ± 6.5 | 1.3 ± 0.2 |
| \( \alpha1\text{-}\alpha3K \) | 11          | 5430 ± 1084 | 41.9 ± 4.3 | 1.3 ± 0.2 |
| \( \alpha3\text{-}\alpha3L \) | 8           | 2636 ± 690  | 63.9 ± 10.2 | 2.2 ± 0.2 |
| \( \alpha1\text{-}\alpha3K\text{-}\alpha1 \) | 14          | 5091 ± 1050 | 21.2 ± 1.3* | 0.7 ± 0.1* |

**FIGURE 9.** Functional complementation disturbs desensitization properties determined by the \( \alpha3 \) splice cassette. A, GlyRα3 variants used in this study. Amino acid residues of the alternatively spliced 15-residue segment (positions 358-372) region are highlighted in gray. Flanking sequences of the \( \alpha3\text{L tail} \) constructs are aligned C-terminal to the splice cassette. B, biotinylation assay of transfected HEK293 cells (ratio 1:5 of Xtrc:tail with X either \( \alpha1 \), \( \alpha3 \), or \( \rho1 \) to represent the domain distribution used for electrophysiological recordings, see C and D) with indicated receptor constructs (numbers refer to distinct combinations, see right box). Comparison of surface and total expression of co-transfected GlyRα3 variants. All constructs were detected using the pan-\( \alpha \) Mab4a antibody. Tail constructs were stained with the monoclonal c-Myc antibody 9E10. GlyRα3 WT served as a positive control. Size markers are indicated. Black arrows point to the specific GlyR protein bands (48 kDa for the full-length \( \alpha3\text{L WT} \) and 33 kDa for the truncated \( \alpha3\text{trc} \) or \( \alpha3\text{K trc} \); white arrows mark the Myc-tagged tail variants (apparent molecular mass of 16 kDa). C, maximum current responses (means ± S.E.) of transfected HEK293 cells with indicated truncated and tail constructs as well as \( \alpha3\text{L WT} \) \( (\text{I}_{\text{max}} \text{ values see Table 2}) \). The constructs are indicated at the bottom of the bars. Differences between \( \alpha3\text{L WT} \) and functional receptors generated from independent domain expressions were tested for significance using one-way analysis of variance. D, fraction of nondesensitizing currents (means ± S.E.) observed for \( \alpha3\text{L WT} \) and \( \alpha3\text{trc} \) co-expressed with tail constructs harboring or lacking the splice cassette important for desensitization of \( \alpha3 \) (for values see Table 2). Representative current traces of \( \alpha3\text{trc} \) and various \( \alpha3\text{L tail} \) constructs used for calculation of desensitization behavior are shown on the right. Value # was calculated only from one recorded trace; out of 19 cells recorded (\( \alpha1\text{trc} + \alpha3\text{L tail} \)) only two responded to the agonist glycine, see also Table 2. Significance was determined using an one-way analysis of variance combined with Dunnett’s post hoc \( t \) test, with values shown as follows: ***, p ≤ 0.001; ns = not significant.
not altered between α3L WT and domain complementations (Fig. 9D and Table 2).

Hence, desensitization of GlyRα3 is not only determined by the presence or absence of the alternative splicing cassette; an interaction of the TM3–4 loop subdomain around the 15-residue cassette with other GlyR domains is required to enable ion channel desensitization, which is most likely disturbed by the domain co-expression approach.

**DISCUSSION**

Ligand-gated ion channels are composed of independent subunits. Each subunit is divided into distinct domains. A recent study on the GlyRα1 normally leading to death of homozygous mice at the end of the 3rd postnatal week revealed that co-expression with the lacking portion of α1 restored the expression of the truncated GlyR protein in neurons and ion channel function in vitro in transfected cell lines (13). Hence, GlyRα1 receptors are composed of independent folding domains generating functional ion channels from nonfunctional subunit fragments. Similarly, co-expression of single domains of the muscarinic m3 acetylcholine receptor or the GluN1/GluN2A receptor resulted in functional rescue of single nonfunctional domains (34, 35).

In the inhibitory GlyR, the mouse mutant oscillator serves as a model system for human hyperkplexia (12, 36), because similar truncations have been found in human patients that suffer from this neuromotor disorder. The observed phenotype of these patients is most probably due to a disturbed expression of affected α1 subunits (14, 16). Truncations of proteins have also been detected in other channelopathies, such as cystic fibrosis or a special form of epilepsy (GEFS+ ) (37, 38).

To test whether the in vitro rescue of the oscillator defect is transferable to other CLRs, we introduced corresponding truncations into the highly homologous subunit GlyRα3 and another closely related Cl− channel, the ρ1 GABA_A/C receptor. The oscillator truncation in GlyRα1 is localized within the large ICD in an alternative splicing cassette (39), giving rise to two splice α1 variants in wild type mice that do not differ in the extent of desensitization and other functional properties. Independent of alternative splicing, the large ICD is of highest variability among CLRs and carries important motifs for phosphorylation, interaction with cytoskeletal proteins or other interaction partners (40–42).

For GlyRα1, amino acid residues have been identified that are essential for proper assembly and receptor pentamerization (9, 28, 43). The functional rescue shown for GlyRα1 was highly dependent in efficiency on the basic stretch 318RRKRR, which is essential for surface expression. GlyRα3 contains the same basic subdomain at the N terminus of the ICD. This motif is unaffected by the truncation corresponding to α1 oscillator.

The functional rescue of truncated GlyRα3 and GABA_A/C ρ1 in the TM3–4 loop showed decreased rescue efficiencies of 16 and 7.5% compared with WT activities. An unfamiliar cross of α1 with ρ1 and vice versa never led to the formation of functional ion channels indicating that these domains are incapable of coassembling into heteromeric GlyR-GABA_C receptors. The subtle changes of intersubunit interactions appear sufficient to prevent efficient assembly of mixed GlyR/GABA_C receptor channels.

Interestingly, our findings of intrafamiliar rescue between GlyRα1 and GlyRα3 led to functional reconstitution only in one direction with truncated α3 together with an α1 complementation domain. Why is the α3_tail not able to complement α1trc? First, α1trc might hinder transport of α3_tail to the cell surface. Second, the position of the truncation in the α3_tail lies within an alternatively spliced 15-residue motif critical for receptor desensitization (5, 6). Third, a different positioning of TM4 in α3 disables communication with TM1–3 of α1 (40). Consistent with these assumptions, we have detected a low expression of the α3_tail upon co-expression with α1trc. The α1trc construct harbors the membrane-directing motif 318RRKRR and incorporates well into the plasma membrane alone (13) and in co-expression with α3_tails.

The truncations corresponding to the oscillator mutation in α1 and α3 lie in alternative splicing cassettes. In α1, the truncation does not hinder the interaction of front and tail fragments to restore functionality. A recent study showed that upon co-expression of GlyRα1 domains, an N-terminal truncation of more than 49 residues in the TM3–4 loop of GlyRα1 resulted in nonfunctional channels (26).

In the α3 sequence, the 15-amino acid insert determines differences in desensitization of both α3 variants (5). The truncation does result in two α3 domains both harboring half of the alternative splicing cassette. This disruption might disable communication with the α1trc.

Structural information of the TM3–4 loop that might explain intrasubunit interactions is scarce, except for the short MA-stretch N-terminal to TM4 (44, 45). Sequence comparison and modeling indicate that the TM1s in α1 and α3 are almost identical. TM4 carries six different amino acid residues, three of which are nonconserved between α1 and α3. An exchange of these residues between α1 and α3 has been proposed to be responsible for disparities in agonist efficacies mediated by diverse interactions with the surrounding lipids. These variations in glycine efficacy of α1 to α3 are due to different orientations of TM4 to their corresponding TM3 domains (31, 40). Aromatic residues in TM4 that are important for pentamerization of GlyR complexes are conserved between α1 and α3, e.g. in α1Phe–425, Phe–429, Phe–432, Phe–435, Tyr–436, Trp–437, and Tyr–440 (28). Thus, a defect in pentamerization is most likely not the main determinant for lack of functional reconstitution from co-expressed GlyR domains.

The contact surface of TM4 toward TM1–3 is identical between α1 and α3. The two residues, which are different between α1 and α3, are not directed toward TM4. Using tail variants with exchanged TM4s of α1 or α3 to generate a domain co-expression with subunit-specific TM4s generated functional ion channels for α3trc with both α1_tails either containing a TM4 of α1 or α3. Hence, TM4 is interchangeable without loss of function. Rather, the disruption of the TM3–4 loop sequence within the alternative splicing cassette of the α3 results in a defective subdomain important for domain-domain communication.

This alternative splicing cassette with additional 15 amino acids in α3 resulting from an unused alternative splice acceptor site has been shown to determine desensitization of the two resulting α3 variants (5, 6). Three hydroxylated amino acids
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within the insert of 15 residues in the TM3–4 loop of α3 have been intensively studied as crucial determinants of desensitization. Furthermore, the insert seems to stabilize a spatial structure of the domain (6, 7). Therefore, the truncation localized within the α3 insert might disturb desensitization. In our complementation experiments, no differences in desensitization time constants were observed.

Does this necessarily mean that desensitization behavior in general cannot be transferred between subunits? In contrast to domain co-expression, disruption of the 15-residue insert in chimera of α1 and α3 gave rise to large fractions of desensitzing currents. The presence of the insert led to nondoensitizing currents. These data demonstrate that the chimera between α1 and α3 results in functional ion channels similar to previous findings on the exchange of the full-length TM3–4 loop plus TM4 between both GlyR subunits (31). Hence, in a continuous polypeptide chain, desensitization can be transferred from α3 to α1 constructs via a TM3–4 loop switch between these subunits. In contrast, desensitization is not transferable in domain co-expression experiments, although functionality of the receptor was restored. The disrupted α3 insert localized at the N terminus of the tail domain was described to stabilize the secondary structure of the GlyRα3 TM3–4 loop (6). The N-terminal location of the 15-residue insert might disable the formation of secondary structures and requires neighboring residues that facilitate a structural stabilization of the domain necessary for receptor conformations and re-orientation of TM3 during desensitization.

Thus, receptor desensitization was identified as a useful measure for the quality of ion channel assembly as it allows us to distinguish between continuous polypeptides and receptors assembled from subunit fragments. Overall, these data provide further evidence that desensitization and channel opening are separate elemental processes that are governed by distinct and structurally well defined domains of the receptor protein.

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