Cellular/Molecular

Distinct γ2 Subunit Domains Mediate Clustering and Synaptic Function of Postsynaptic $GABA_A$ Receptors and Gephyrin

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Modulation of the concentration of postsynaptic $GABA_A$ receptors contributes to functional plasticity of inhibitory synapses. The γ2 subunit of $GABA_A$ receptor is specifically required for clustering of these receptors, for recruitment of the submembrane scaffold protein gephyrin to postsynaptic sites, and for postsynaptic function of GABAergic inhibitory synapses. To elucidate this mechanism, we here have mapped the γ2 subunit domains required for restoration of postsynaptic clustering and function of $GABA_A$ receptors in γ2 subunit mutant neurons. Transfection of $\gamma2^{-/-}$ neurons with the γ2 subunit but not the α2 subunit rescues postsynaptic clustering of $GABA_A$ receptors, results in recruitment of gephyrin to postsynaptic sites, and restores the amplitude and frequency of miniature inhibitory postsynaptic currents to wild-type levels. Analogous analyses of chimeric γ2/α2 subunit constructs indicate, unexpectedly, that the fourth transmembrane domain of the γ2 subunit is required and sufficient for postsynaptic clustering of $GABA_A$ receptors, whereas cytoplasmic γ2 subunit domains are dispensable. In contrast, both the major cytoplasmic loop and the fourth transmembrane domain of the γ2 subunit contribute to efficient recruitment of gephyrin to postsynaptic receptor clusters and are essential for restoration of miniature IPSCs. Our study points to a novel mechanism involved in targeting of $GABA_A$ receptors and gephyrin to inhibitory synapses.

Key words: **GABAergic; GAD; synapse; synaptogenesis; trafficking; clustering; IPSP; lipid raft; gephyrin; endocytic recycling; IPSC; miniature currents**

Introduction

Regulated trafficking and postsynaptic targeting of ligand-gated ion channels contribute to functional plasticity of synapses both during development and in the mature CNS (Moss and Smart, 2001; Bredt and Nicoll, 2003). $GABA_A$ receptors, the main receptors mediating neural inhibition, are heteropentameric chloride channels composed of several homologous subunits. Different receptor subtypes characterized by distinct subunit compositions are preferentially localized at postsynaptic or extrasynaptic sites where they mediate phasic or tonic inhibition, respectively (for review, see Fritschy and Brunig, 2003; Luscher and Keller, 2004). The postsynaptic receptor subtypes thus far identified invariably contain the γ2 subunit, typically in combination with α1–3 and ill-defined β subunits. The putative postsynaptic scaffold protein gephyrin is perfectly colocalized with γ2 subunit-containing $GABA_A$ receptors but does not seem to interact with these receptors biochemically. In contrast, gephyrin acts as a prototypical clustering protein for the closely related glycine receptors by direct interaction with the glycine receptor β subunit (Kneussel and Betz, 2000; Sola et al., 2004).

Gephyrin is required for proper localization of only a subset of postsynaptic $GABA_A$ receptors, notably those that contain the γ2 subunit together with the α2 or α3 subunit, but not those that contain the α1 subunit (Essrich et al., 1998; Baer et al., 1999; Kneussel et al., 1999, 2001; Fischer et al., 2000; Levi et al., 2004). Conversely, the γ2 subunit is essential for clustering of $GABA_A$ receptors and gephyrin, regardless of the type of α subunit present (Essrich et al., 1998; Baer et al., 1999; Schweizer et al., 2003). Importantly, the γ2 subunit and gephyrin are essentially dispensable for trafficking of nonsynaptic receptors to the plasma membrane (Gunther et al., 1995; Baer et al., 1999; Kneussel et al., 1999).

By analogy to glycine receptors, postsynaptic clustering of $GABA_A$ receptors is believed to involve specific interaction(s) of cytoplasmic receptor domains with components of a subsynaptic protein scaffold. Proteins implicated in trafficking of $GABA_A$ receptors by interaction with the γ2 subunit cytoplasmic domain include the trafficking factor $GABA_A$ receptor-associated protein (GABARAP) (Wang et al., 1999; Kneussel et al., 2000; Kittler et al., 2001) and the thioacyl transferase Golgi-specific DHHC zinc finger protein (GODZ) (Keller et al., 2004), but neither of these proteins is concentrated at synapses and their function in neurons has not been analyzed so far. Palmitoylation of the γ2 subunit major cytoplasmic loop, possibly by GODZ, contributes to normal expression and stability of $GABA_A$ receptors in the neural plasma membrane and at postsynaptic sites (Keller et al., 2004; Rathenberg et al., 2004).

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The molecular interactions that target $GABA_A$ receptors to synapses are unknown. To elucidate the role of the γ2 subunit, we have mapped the subunit domains that are involved. Unexpectedly, we find that the fourth transmembrane domain (TM4) but not the major cytoplasmic loop domain of the γ2 subunit is essential for clustering of $GABA_A$ receptors and gephyrin at inhibitory synapses. The cytoplasmic loop domain contributes to association of receptors with gephyrin and is essential for GABAergic synaptic function but its effect is evident only in the presence of TM4.

Materials and Methods

Generation of plasmid constructs. The mouse γ2S (γ2) subunit cDNA (Connolly et al., 1999a), including 51 nucleotides of untranslated leader and 33 nucleotides of 3′ untranslated mRNA, was cloned into pEGFP-N (Clontech, Palo Alto, CA), substituting the γ2 cDNA for enhanced green fluorescent protein (EGFP). An oligonucleotide (5′-CAAAA ACTAA TATCA GAAGA AGACC TAACT AGT-3′) encoding the nine amino acid 9E10 myc epitope and an adjacent *Spe*I site (QKLISQQDL-TS) was inserted between amino acids four and five of the mature γ2 polypeptide by site-directed mutagenesis. A GFP-tagged version of this ^{9E10}γ2 construct (GFPγ2) was constructed by PCR amplification of the EGFP open reading frame of pEGFP-N using *Spe*I site adaptor primers and insertion of this fragment into the *Spe*I site downstream of the 9E10 tag of ^{9E10}γ2. Toward construction of chimeric subunits containing portions of the γ2 and α2 subunit (Benson et al., 1998), the nucleotide sequences flanking the cytoplasmic loop region (amino acids 318–404) of the γ2 polypeptide in the ^{9E10}γ2 construct were subjected to site-directed mutagenesis to introduce silent *Eco*0109 I and *Eco*N I restriction sites. PCR-generated fragments derived from the α2 subunit cDNA that were homologous to the γ2 subunit domains to be swapped were amplified using adapter primers that contained the matching restriction sites and inserted into the 9E10-tagged γ2 subunit backbone, thereby replacing the corresponding γ2 subunit fragment. Thus, the 5′ untranslated sequences as well as the leader peptide and the 13 N-terminal amino acids including the epitope tag of the mature polypeptides are identical for all these 9E10-tagged subunit constructs. The constructs lacked $GABA_A$ receptor subunit-derived untranslated sequences save for 33 nucleotides in the construct containing the γ2 TM4 region. All constructs were verified by sequencing. The expression vectors for untagged α2, β2, and β3 subunits have been described (Malherbe et al., 1990; Benson et al., 1998).

Protein extracts and Western blot. Transfected human embryonic kidney (HEK) 293T cells (American Type Culture Collection, Manassas, VA) were extracted in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.2% Triton X-100, 1 μg/ml antipain, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, and 0.5 mM PMSF. The extracts were cleared by centrifugation (10,000 × *g*; 5 min), and the supernatant analyzed by SDS-PAGE (10%; 40 μg protein per lane). Proteins resolved in SDS gels were transferred to a nitrocellulose membrane using a semidry blotter (Bio-Rad, Hercules, CA), and the membrane was blocked with 5% nonfat dry milk in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Tween 20) and incubated overnight at 4°C with primary antibody (mouse anti-9E10, 1:50) in TBST containing 5% dry milk. The membrane was washed with 20 mM Tris-HCl, pH 7.5, 60 mM NaCl, 2 mM EDTA, 0.4% SDS, 0.4% Triton X-100, 0.4% deoxycholate, followed by four rinses in TBST, reblocked for 30 min at room temperature, incubated with donkey anti-mouse antibody conjugated to horseradish peroxidase (Amersham Biosciences, Piscataway, NJ) (1:5000 in dry milk/TBST; 2 hr at room temperature), and washed again. Antibody complexes were detected using ECL Plus (Amersham Biosciences).

Tissue culture and transfection. HEK 293T cells were maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum at 37°C in 5% CO_2. For protein expression assays, cells were seeded onto 60 mm dishes and allowed to reach 70% confluency. They were then transfected with a mixture of cDNAs containing the chimeric construct indicated (10 μg) and $GABA_A$ receptor α2 and β3 subunits (5 μg each), using the standard $CaPO_4$ transfection method (Chen and Okayama, 1987). For surface expression assays, cells were seeded onto poly-L-lysine-coated coverslips and analogously transfected with the chimeric constructs (4 μg each) alone or together with a mixture of $GABA_A$ receptor α2 and β3 subunits (4 μg each). The cells were harvested or analyzed by immunofluorescent staining 36–48 hr after addition of the DNA precipitate.

Cortical neurons were generated from γ2 subunit-deficient embryonic day 14.5 embryos generated by crossing of $\gamma2^{+/-}$ mice on a 129SvJ inbred background (Essrich et al., 1998). Cortical hemispheres were collected in PBS containing 5.5 mM glucose and treated with papain (0.5 mg/ml) and DNase I (10 μg/ml) (both from Sigma, St. Louis, MO) in PBS containing 1 mg/ml bovine serum albumin (Fraction V, Sigma) and 10 mM glucose for 15 min at room temperature. The cells were triturated with a fire-polished Pasteur pipette and plated on poly-L-lysine-coated glass coverslips (22 × 22 mm) at 4×10^4 cells per square centimeter in modified Eagle medium (MEM) (Invitrogen) containing 10% v/v fetal bovine serum (FBS) (Invitrogen) in an atmosphere of 10% CO_2. After 60 min, the medium was replaced with fresh MEM containing 10% v/v FBS. The genotype of cultures was determined using PCR as follows. Tail biopsies (3 mm) were incubated for 30 min at 55°C in 25 μl lysis buffer (200 mM NaCl, 5 mM EDTA, 0.2% SDS, 100 mM Tris-HCl, pH 8.5), and 1% of the supernatant was used for PCR using standard conditions with the primer 5′-CATCT CCATC GCTAA GAATG TTCGG GAAGT-3′ combined with either 5′-GCTGA CAAAA TAATG CAGGG TGCCA TACTC-3′ to amplify the wild-type γ2 locus or the primer 5′-ATGCT CCAGA CTGCC TTGGG AAAAG C-3′ to amplify the mutant γ2 locus. The 24-hr-old cultures that exhibited the desired genotypes were turned upside down onto a glial feeder layer in a Petri dish containing Neurobasal-A supplemented with B27 (Invitrogen), in an atmosphere of 10% CO_2. Feeder cells were prepared from cortices of newborn rat pups as described (Banker and Goslin, 1998). Neuron cultures were maintained without medium change for 18 d *in vitro* (DIV) and then transferred into new Petri dishes containing Neurobasal A/B27 supplemented with 1 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 100 μM 2-amino-5-phosphonovaleric acid (Sigma), with the cells facing up. They were transfected with epitope-tagged $GABA_A$ receptor subunit constructs (8 μg) using a $CaPO_4$ transfection kit (BD Biosciences, Palo Alto, CA). When testing constructs that failed to cluster, we cotransfected 100 ng pEGFP-C1 (BD Biosciences) to unambiguously identify transfected cells. The DNA precipitate (200 μl) was prepared according to the instructions of the kit manufacturer, allowed to precipitate for 15 min, and added to the cells for 45 min, and the coverslips were then returned to the original dishes containing conditioned medium. Neurons were processed for immunofluorescent analysis at 21 DIV.

Immunofluorescence analyses. For labeling of $GABA_A$ receptor subunits expressed in the plasma membrane of HEK 293T cells, the cells were washed three times with PBS, fixed in 4% paraformaldehyde in 150 mM sodium phosphate buffer, pH 7.4, for 12 min without permeabilization, and stained overnight at 4°C with rabbit anti-myc (1:1000; Medical and Biological Labs, Woburn, MA) and guinea pig anti-α2 (1:700; gift of J. M. Fritschy, University of Zurich, Switzerland). Neurons used for immunofluorescence studies were washed three times in PBS, fixed in 4% paraformaldehyde for 12 min, and permeabilized for 4 min with 0.2% Triton X-100 in PBS containing 10% donkey serum. After a brief wash in PBS, cells were incubated with primary antibody overnight at 4°C using the following dilutions: rabbit anti-myc (1:500), guinea pig anti-α2 (1:2000), gephyrin monoclonal antibody (mAb) 7a (1:500; gift of H. Betz, Max Planke Institute, Frankfurt, Germany), or mAb glutamic acid decarboxylase (GAD)-6 (1:75; Developmental Studies Hybridoma Bank, University of Iowa). For detection of primary antibodies, AlexaFluoro 488-conjugated goat anti-rabbit, AlexaFluoro647-conjugated goat anti-mouse or rabbit (Molecular Probes, Eugene, OR), or Cy3 donkey anti-mouse or guinea pig (Jackson ImmunoResearch, West Grove, PA) were used as appropriate. Fluorescent images were captured from a Zeiss Axiophot2 microscope equipped with a 40 × 1.3 numerical aperture objective and an ORCA-100 video camera linked to an OpenLab imaging system (Improvision, Lexington, MA). Digital gray scale images representing sequentially recorded fluorescences were pseudocolored in green, red, and blue, respectively. Images of cells that had been cotransfected with trace amounts of GFP were developed using AlexaFluoro 647

as a secondary antibody to reveal immunoreactivity of the chimeric constructs and then pseudocolored green to match the color of images that had been developed with AlexaFluoro 488. Images were adjusted for contrast using OpenLab and assembled into figure palettes using Adobe Photoshop.

Quantitation of immunofluorescent staining. For semiquantitative analyses of $GABA_A$ receptor clusters, digitized microscopic images were recorded from cells that were innervated by GABAergic axons as judged by GAD staining. Two properly innervated dendritic segments of 40 μm in length were selected from each of 13–18 different cells transfected in three or more independent experiments. Immunoreactive puncta stained for the 9E10 epitope were automatically selected using OpenLab imaging software. Receptor clusters were defined as immunofluorescent puncta within the region of interest that exceeded a fluorescence intensity threshold that was twofold greater than the diffuse fluorescence measured on the shaft of the same dendrite and fit a target size range of 0.2–2 μm in diameter. To determine the percentage of 9E10-immunoreactive puncta at synapses, the fraction of puncta that were maximally one pixel apart from punctate GAD immunoreactivity was declared to be postsynaptic. Puncta determined to be postsynaptic by this method were selected to compute the average size (area) of postsynaptic $GABA_A$ receptor clusters. Similarly, 9E10-immunoreactive puncta were selected in dendritic segments of 40 μm in length using the intensity and size limitations indicated above, and the fraction of puncta that exhibited at least one pixel overlap with punctate gephyrin immunoreactivity was defined to be colocalized with gephyrin (n = 13–26 cells per construct). Transfected neurons that did not show any punctate gephyrin immunoreactivity were excluded from the analysis to ensure that cells that were not viable or did not express gephyrin were excluded from analysis. Statistical comparisons were performed using ANOVA one-way comparison with Dunnett's post-test.

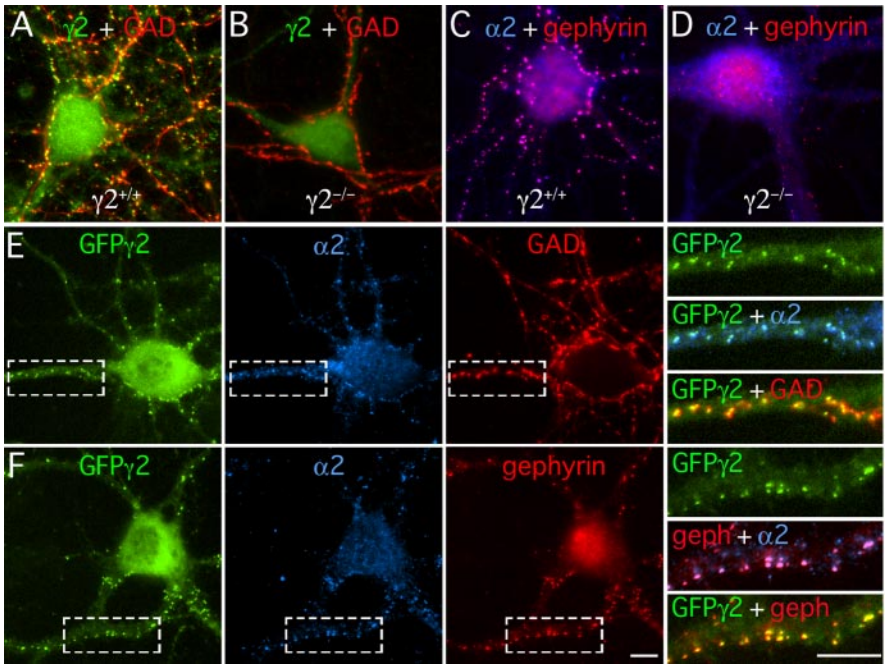

Figure 1. Restoration of postsynaptic $GABA_A$ receptors and gephyrin clusters in $\gamma2^{-/-}$ neurons by transfection of GFP-tagged $\gamma2$ subunit. *A–D*, Cortical neurons cultured from embryonic day 14.5 $\gamma2^{+/+}$ (*A, C*) or $\gamma2^{-/-}$ embryos (*B, D*) (20 DIV) were double stained with antibodies specific for the $\gamma2$ subunit (shown in green) and GAD (red) (*A, B*) or the $\alpha2$ subunit (blue) and gephyrin (red) (*C, D*); colocalization is shown in yellow (*A, B*) and pink (*C, D*), respectively. Note the dramatic loss of punctate staining for the $\gamma2$ and $\alpha2$ subunits as well as gephyrin in $\gamma2^{-/-}$ neurons, whereas presynaptic GAD staining is unchanged. *E, F*, Cortical neurons cultured from $\gamma2^{-/-}$ embryos were transfected at 18 DIV with GFP$\gamma2$. They were fixed, permeabilized, and processed for immunofluorescent staining at 21 DIV with an antiserum specific for the $GABA_A$ receptor $\alpha2$ subunit (blue) and antibodies specific for either GAD (red) (*E*), indicating GABAergic terminals, or gephyrin (red) (*F*), as indicated. Boxed dendritic segments are shown enlarged in the panels on the right for either GFP$\gamma2$ alone (green) or as merged images. Colocalization in the merged enlargement of the cell in *A* representing GFP$\gamma2$ (green) and the $\alpha2$ subunit (blue) is shown in cyan blue and colocalization of GFP$\gamma2$ and GAD (red) is shown in yellow. Colocalization in the merged enlargement of the cell in *B* between gephyrin and the $\alpha2$ subunit is shown in magenta, and colocalization of GFP$\gamma2$ and gephyrin is shown in yellow. Scale bars, 10 μm.

Electrophysiology. To determine the GABA dose–response curves of recombinant $GABA_A$ receptors, HEK 293T cells were passaged onto poly-L-lysine-coated glass coverslips (12 mm diameter) and transfected 24–36 hr later as described above using 200 ng of pEGFP-C1 (Clontech) and 1 μg each of the $\alpha2$ and $\beta3$ expression vectors (Benson et al., 1998) supplemented with 1 μg of chimeric construct as indicated in the figures. Transfected cells were identified by EGFP fluorescence 24–48 hr after transfection, and membrane currents were recorded in the whole-cell mode with the membrane potential clamped at −60 mV using a Multiclamp 700A amplifier (Axon Instruments, Foster City, CA). Borosilicate glass pipettes (Harvard Apparatus, Holliston, MA) were fire polished for a final resistance of 2–6 MΩ. The recording chamber was perfused continuously with a bath solution containing (in mM): 128 NaCl, 30 D-glucose, 25 HEPES, 5 KCl, 2 $CaCl_2$, and 1 $MgCl_2$ (adjusted to pH 7.4 using NaOH). The pipette solution contained (in mM): 147 KCl (or CsCl), 5 disodium phosphocreatine, 2 EGTA, 10 HEPES, 2 MgATP, and 0.3 Na_2GTP (pH 7.4, adjusted with KOH). GABA solutions were prepared daily from stock solution containing 100 mM GABA (Acros Organics, Geel, Belgium). Series resistances were typically 10–25 MΩ with a membrane resistance mostly in the range of 250–800 MΩ. Data were acquired using PCLAMP 8 software, sampled at 10 kHz, filtered at 1–2 kHz, and analyzed using CLAMPFIT 8/9 software (Axon Instruments). GABA-induced currents were normalized to fractions of the maximum current recorded in the same cell under the same conditions. Using SigmaPlot 8.0 (SPSS Inc., Chicago IL), dose–response values of each cell were fitted to the equation $I = I_{max}/[1 + (EC_{50}/A)^n]$, where A is the GABA concentration, EC_{50} the concentration of GABA eliciting a half-maximal current amplitude, I_{max} the maximal current amplitude, I the measured current amplitude, and n the Hill coefficient. Curves determined separately for 3–10 cells expressing the same subunit/chimera combination were averaged to yield the corresponding EC_{50} value. The EC_{50} values of different subunit/chimera combinations were compared using a two-tailed t test and are expressed as mean ± SE of measurement.

For analysis of miniature IPSCs (mIPSCs), cultured cortical neurons were transfected at 16 DIV using 8 μg of cDNA plasmid per construct and 100 ng of pEGFP-C1 as described above. We recorded mIPSCs selectively from GFP-positive pyramidal cells 24–48 hr later. Currents were recorded in the whole-cell voltage-clamp mode with the holding potential set at −70 mV, in the presence of 200 nM tetrodotoxin (Sigma) and 10 μM CNQX (Tocris Cookson, Ellisville, MO). The GABAergic nature of these events was confirmed by blocking with 40 μM bicuculline (Tocris Cookson). Miniature events were analyzed using MiniAnalysis software (Synaptosoft, Decatur, GA) and inspected visually for accuracy. The average amplitude and frequency of mini-events (n = 6–15 neurons per construct or genotype) were compared using a two-tailed Student's t test.

Results

The $\gamma2$ subunit is essential for clustering and postsynaptic targeting of $GABA_A$ receptors and gephyrin at inhibitory synapses, as shown previously in cultured neurons and brain sections from $\gamma2^{-/-}$ mice (Essrich et al., 1998; Schweizer et al., 2003). These results were replicated here in cultured cortical neurons under different culture conditions (Fig. 1 *A–D*). In particular, loss of the

$\gamma 2$ subunit in $\gamma 2^{-/-}$ neurons is associated with a dramatic loss of punctate $\alpha 2$ subunit and gephyrin immunoreactivity, whereas presynaptic GAD staining is unaffected. Consistent with lack of postsynaptic $GABA_A$ receptors, $\gamma 2^{-/-}$ cortical neurons exhibit a dramatically reduced frequency and amplitude of miniature IPSCs (Essrich et al. 1998); however, $\gamma 2^{-/-}$ neurons exhibit GABA-evoked benzodiazepine-insensitive whole-cell currents (Baer et al., 1999) and almost normal levels of GABA binding sites (Gunther et al., 1995), which indicates expression of functionally abnormal $GABA_A$ receptors containing α and β subunits in the plasma membrane (Gunther et al., 1995; Baer et al., 1999).

Transfection of cultured cortical $\gamma 2^{-/-}$ neurons at 18 DIV with GFP$\gamma 2$ resulted in efficient restoration of postsynaptic $GABA_A$ receptors, as evidenced by rescue of immunoreactive puncta for the $GABA_A$ receptor $\alpha 2$ subunit that are colocalized with clustered GFP$\gamma 2$ and juxtaposed to punctate immunoreactivity for presynaptic GAD, a marker for GABAergic terminals (Fig. 1*E*). Moreover, restoration of postsynaptic $GABA_A$ receptors was associated with rescue of punctate immunofluorescent staining for gephyrin (mAb 7a) (Fig. 1*F*). These results support the view that gephyrin is recruited to postsynaptic sites by $\gamma 2$ subunit-containing $GABA_A$ receptors, rather than vice versa. Moreover, the findings suggest that transfection of $\gamma 2^{-/-}$ neurons with $\gamma 2$ subunit-derived constructs provides a means to determine the subunit domains required for proper targeting of $GABA_A$ receptors and for recruitment of gephyrin to postsynaptic sites, without interference by endogenous $\gamma 2$ subunit.

Assembly of receptors containing chimeric subunits

Toward mapping the $\gamma 2$ subunit domains required for proper localization of $GABA_A$ receptors, we generated a series of chimeric constructs in which different extracellular, transmembrane, and intracellular domains of the $\gamma 2$ subunit were replaced with homologous domains derived from the $\alpha 2$ subunit (Fig. 2*A*,*B*). The $\alpha 2$ subunit was chosen for construction of $\gamma 2/\alpha 2$ chimeric subunits because it is normally expressed almost exclusively at postsynaptic sites yet strictly dependent on the $\gamma 2$ subunit and gephyrin for postsynaptic localization (Fig. 1*A–D*) (Essrich et al., 1998; Schweizer et al., 2003). To maximize insertion into the plasma membrane of recombinant subunit constructs, we relied on the 9E10 epitope rather than GFP as a tag to monitor expression of transfected constructs. In addition to the vector backbone, noncoding sequences at the 5′ end including the leader peptide, the first four amino acids, and the 9E10 epitope were kept the same in all constructs to minimize potential differences in expression levels. Save for maximally 32 nucleotides downstream of the translational stop signal, all $GABA_A$ receptor subunit-derived 3′ untranslated mRNA sequences were deleted to avoid sequences that might affect dendritic targeting of transcripts. We adopted a tripartite nomenclature to describe these constructs (for example, $^{9E10}\alpha$-γ-γ), with the first term indicating the subunit origin of the epitope-tagged extracellular domain together with the first three transmembrane domains, the second term indicating the major cytoplasmic loop domain between TM3 and TM4, and the third term indicating the origin of the TM4 domain and the short C-terminal tail (Fig. 2*B*). Proper expression of all constructs was assessed after cotransfection with $\alpha 2$ and $\beta 3$ subunits into HEK 293T cells. Western blot analyses of whole-cell extracts of transfected cells indicated that the chimeric constructs gave rise to stable polypeptides of the expected mobility when analyzed by SDS-PAGE (Fig. 2*C*). The translocation and integration of these constructs into the plasma membrane was then assessed using immunofluorescent staining of nonpermeabilized HEK 293T cells for the transfected constructs. When expressed alone, the $\gamma 2$ subunit was able to reach the cell surface efficiently (Fig. 3*A*) as expected (Connolly et al., 1999a). In contrast, only small amounts of the $\alpha 2$ subunit or of the chimeric constructs were able to reach the cell surface on their own (Fig. 3*B–G*); however, when coexpressed with $\alpha 2$ and $\beta 3$ subunits, all constructs efficiently reached the plasma membrane where they colocalized with immunoreactivity for the $\alpha 2$ subunit (Fig. 3*B′–G′*). Similar results were obtained when the $\beta 2$ subunit was substituted for the $\beta 3$ subunit (data not shown). Surface expression of α subunits is known to require coassembly with β subunits into heteromeric ion channels (Connolly et al., 1996) (and data not shown). Thus, the data indicate that all of the chimeric constructs assemble with $\alpha 2$ and/or $\beta 2/3$ subunits, thereby giving rise to heteromeric complexes that are efficiently inserted into the plasma membrane.

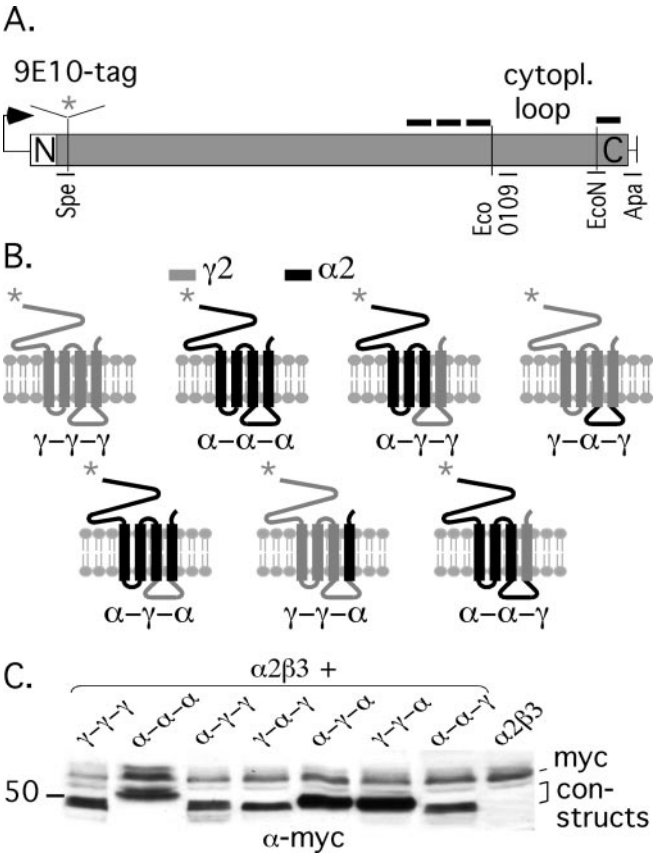

Figure 2. Schematic representation of chimeric subunit constructs and analysis of their expression after transfection into HEK 293T cells. *A*, Schematic representation of the myc 9E10 epitope-tagged $\gamma 2$ subunit with the position of silent restriction sites inserted flanking the large cytoplasmic loop domain. The $\gamma 2$ subunit translational open reading frame is shown as a gray box, with N and C termini indicated. The positions of the four transmembrane domains are indicated by short black lines above this box. The 9E10 epitope tag and an adjacent *Spe*I site are inserted between the fourth and fifth amino acid of the open reading frame. *B*, Schematic representation of chimeric constructs, with the $\gamma 2$ subunit-derived portion shown in gray and the $\alpha 2$ subunit-derived portion shown in black. The nomenclature for chimeric construct indicated underneath each drawing is explained in Results. *C*, Western blot analysis of chimeric constructs cotransfected with $\alpha 2$ and $\beta 3$ subunits into HEK 293T cells. Equal amounts of protein (40 μg) were loaded on the gel, and the blot was developed using an antiserum raised against the 9E10 myc epitope. The ubiquitous protein band running as a band of ~60 kDa represents endogenous myc and serves as a gel loading control. The constructs and subunits transfected in each lane are indicated.

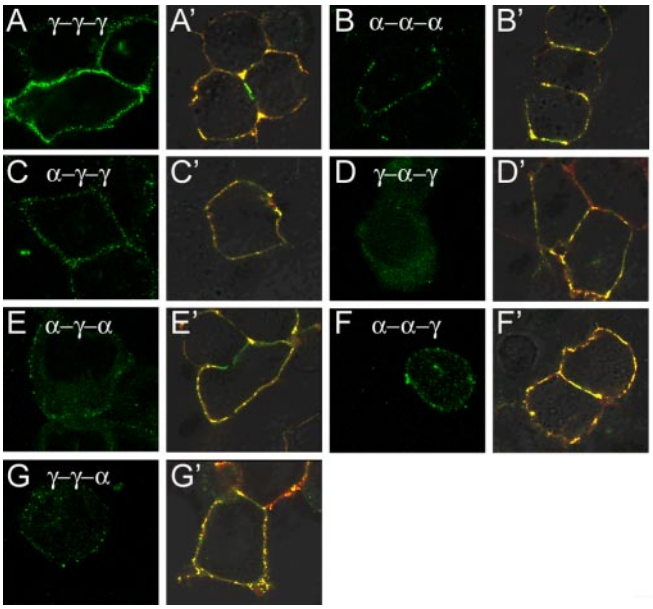

Figure 3. Analysis of surface expression of chimeric subunit constructs transfected into HEK 293T cells. The 9E10-tagged constructs indicated were transfected either alone (*A–G*) or together with $\alpha 2$ and $\beta 3$ subunits (*A′–G′*). Nonpermeabilized cells were stained with antibody specific for the 9E10-tagged construct indicated (*A–G*) or double labeled for this construct and the $\alpha 2$ subunit (*A′–G′*). Both antibodies are directed against N-terminal epitopes and selectively recognize subunits inserted into plasma membrane. The staining was developed with fluorescent secondary antibody for imaging. Note the efficient expression of the $\gamma 2$ subunit ($^{9E10}\gamma$-γ-γ) in the plasma membrane independent of whether it is expressed alone (*A*) or together with $\alpha 2$ and $\beta 3$ subunits (*A′*). In contrast, efficient incorporation of the $\alpha 2$ subunit (*B*, *B′*) or chimeric constructs (*C–G*, *C′–G′*) depends on coexpression of the $\alpha 2$ or $\beta 3$ subunit, or both.

Functional analysis of chimeric subunits

The GABA efficacy of receptors found in $\gamma 2^{-/-}$ neurons is significantly below that of wild-type receptors (Gunther et al., 1995), which is consistent with the reduced single-channel conductance of $\gamma 2$ subunit-deficient $GABA_A$ receptors found in $\gamma 2^{-/+}$ and $\gamma 2^{-/-}$ cortical and dorsal root ganglia neurons (Crestani et al., 1999; Lorez et al., 2000) and with results obtained by analysis of recombinant receptors composed of α and β subunits (Verdoorn et al., 1990; Angelotti and Macdonald, 1993). To determine whether the chimeric constructs could contribute to functional GABA-gated ion channels, we used whole-cell patch-clamp analyses of HEK 293T cells transfected either with $\alpha 2$ and $\beta 3$ subunits alone or together with different chimeric constructs. GABA dose–response curves were recorded to evaluate differences in the GABA efficacy of putative receptors (EC_{50} = GABA concentration resulting in half-maximal GABA-evoked currents) (Fig. 4*A*,*B*). On coexpression with $\alpha 2\beta 3$ subunits, the $^{9E10}\alpha$-γ-γ, $^{9E10}\gamma$-α-γ, $^{9E10}\alpha$-α-γ, and $^{9E10}\gamma$-γ-α constructs produced channels with GABA EC_{50} values significantly lower than the value observed for $\alpha 2\beta 3$ receptors [EC_{50} ($\alpha 2\beta 3$) = 48.9 ± 4.5 μM (SEM), ($\alpha 2\beta 3$ + $^{9E10}\alpha$-γ-γ) = 26.2 ± 1.9 μM, $p < 0.01$; ($\alpha 2\beta 3$ + $^{9E10}\gamma$-α-γ) = 21.0 ± 2.7 μM, $p < 0.001$; ($\alpha 2\beta 3$ + $^{9E10}\alpha$-α-γ) = 18.4 ± 1.6 μM, $p < 0.001$; ($\alpha 2\beta 3$ + $^{9E10}\gamma$-γ-α) = 16.7 ± 1.9 μM, $p < 0.01$] and comparable or even lower than the value for the $\alpha 2\beta 3$ + $^{9E10}\gamma$-γ-γ subunit combination (25.6 ± 3.9 μM). Similar results were obtained when the $\beta 2$ subunit was substituted for $\beta 3$ and regardless of whether the chimeric constructs were transfected at a 1:1 ratio with $\alpha 2$ and $\beta 3$ subunits or at a 10-fold excess (data not shown). In contrast, the $^{9E10}\alpha$-γ-α construct coexpressed with $\alpha 2$ and $\beta 3$ subunits produced less responsive receptors with an EC_{50} value significantly greater than that of $\alpha 2\beta 3$ receptors [EC_{50} ($\alpha 2\beta 3$+ $^{9E10}\alpha$-γ-α) = 107.9 ± 17.2; $p < 0.05$] (Fig. 4*B*). Together, the GABA dose–response curves of recombinant receptors containing α/γ subunit chimeric constructs confirm and extend the conclusions from immunofluorescent analyses and show that the $^{9E10}\alpha$-γ-γ, $^{9E10}\gamma$-α-γ, $^{9E10}\gamma$-γ-α, $^{9E10}\alpha$-α-γ, and $^{9E10}\alpha$-γ-α constructs can each assemble with α and β subunits and contribute to the formation of GABA-gated ion channels that are functionally distinct from channels produced by $\alpha 2$ and $\beta 3$ subunits alone.

Receptor domains required for postsynaptic localization

To determine the $\gamma 2$ subunit domains required for trafficking and accumulation of $GABA_A$ receptors at postsynaptic sites, each of the constructs was transfected into cultured cortical neurons (18 DIV) derived from $\gamma 2^{-/-}$ embryos, and the cultures were processed for immunofluorescence analyses 3 d later. Whereas immunoreactivity for the $^{9E10}\gamma$-γ-γ construct (anti-myc antiserum) was found to accumulate at membrane sites apposed to GAD-positive GABAergic terminals, essentially no punctate staining was evident for the $^{9E10}\alpha$-α-α construct, as expected (Fig. 5*A*,*B*). The $^{9E10}\alpha$-γ-γ construct revealed punctate staining apposed to GAD-positive terminals similar to $^{9E10}\gamma$-γ-γ, indicating that the extracellular domain and the first three transmembrane domains of the $\gamma 2$ subunit are dispensable for postsynaptic localization (Fig. 5*C*). Surprisingly, however, the $^{9E10}\alpha$-α-γ construct accumulated at postsynaptic sites similar to $^{9E10}\gamma$-γ-γ, suggesting that clustering and postsynaptic localization of $\gamma 2$ subunit-containing $GABA_A$ receptors can be mediated by the TM4 domain of the $\gamma 2$ subunit and that the cytoplasmic domain is dispensable (Fig. 5*D*). Consistent with this notion, the $^{9E10}\gamma$-α-γ construct was also clustered and localized to postsynaptic sites (Fig. 5*E*), whereas both the $^{9E10}\alpha$-γ-α and $^{9E10}\gamma$-γ-α constructs (Fig. 5*F*,*G*) failed to cluster and instead were diffusely distributed in dendrites.

To confirm our visual impression, the images of cells selected for proper GABAergic innervation (n = 13–18 cells for each construct) were subjected to semiquantitative analyses using automatic detection of immunofluorescent puncta above a set fluorescence intensity threshold (see Materials and Methods). The total number of clusters per dendritic segment of 40 μm detected for each of the $^{9E10}\alpha$-γ-γ, $^{9E10}\alpha$-α-γ, and $^{9E10}\gamma$-α-γ constructs was indistinguishable from values determined for the $\gamma 2$ subunit ($^{9E10}\gamma$-γ-γ) (Fig. 6*A*). Moreover, the percentage of these clusters that were apposed to presynaptic GAD immunoreactivity and the average size of these clusters were indistinguishable from corresponding values observed for the $\gamma 2$ subunit (Fig. 6*B*,*C*). In contrast, many fewer clusters were detected for the $^{9E10}\alpha$-α-α, $^{9E10}\alpha$-γ-α, and $^{9E10}\gamma$-γ-α constructs (Fig. 6*A*), and almost none of these clusters were apposed to GAD (Fig. 6*B*), indicating that they represented extrasynaptic receptors. Thus, the TM4 domain of the $\gamma 2$ subunit is required and sufficient to deliver $\alpha\beta\gamma 2$ receptors to dendritic sites apposed to GABAergic terminals. Conversely, the $\gamma 2$ subunit major cytoplasmic loop domain is neither sufficient nor required for postsynaptic clustering.

Recruitment of gephyrin to $GABA_A$ receptors

Postsynaptic $GABA_A$ receptors are invariably colocalized with the putative subsynaptic scaffold protein gephyrin; however, the function of gephyrin with respect to clustering and targeting of $GABA_A$ receptors remains ill-defined. In the case of the closely related glycine receptors, interaction of receptors and gephyrin is mediated by the major cytoplasmic loop domain of the receptor β subunit, and this interaction is believed to mediate clustering

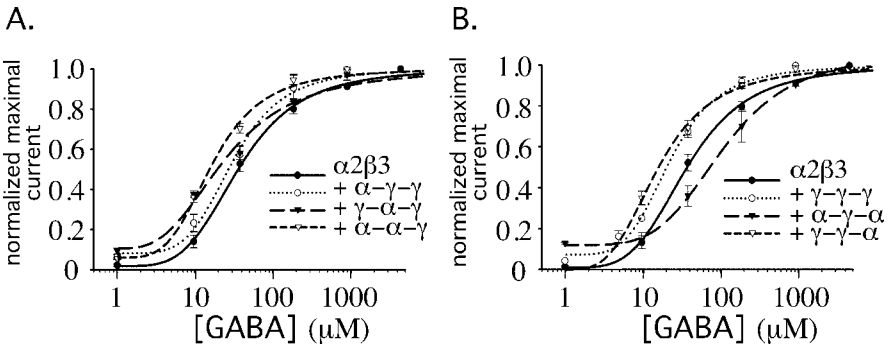

Figure 4. GABA dose–response curves of $GABA_A$ receptors containing chimeric subunits expressed in HEK 293T cells. *A*, *B*, The $\alpha 2$ and $\beta 3$ subunits were transfected either alone or in combination with the $^{9E10}\gamma$-γ-γ, $^{9E10}\alpha$-γ-α, or $^{9E10}\gamma$-γ-α constructs (*A*) or in combination with the $^{9E10}\alpha$-γ-γ, $^{9E10}\gamma$-α-γ, or $^{9E10}\alpha$-α-γ constructs (*B*). GABA-evoked whole-cell currents were normalized to the maximal responses obtained at 1–5 mM GABA application. For each concentration tested, the data were averaged from 3–11 cells.

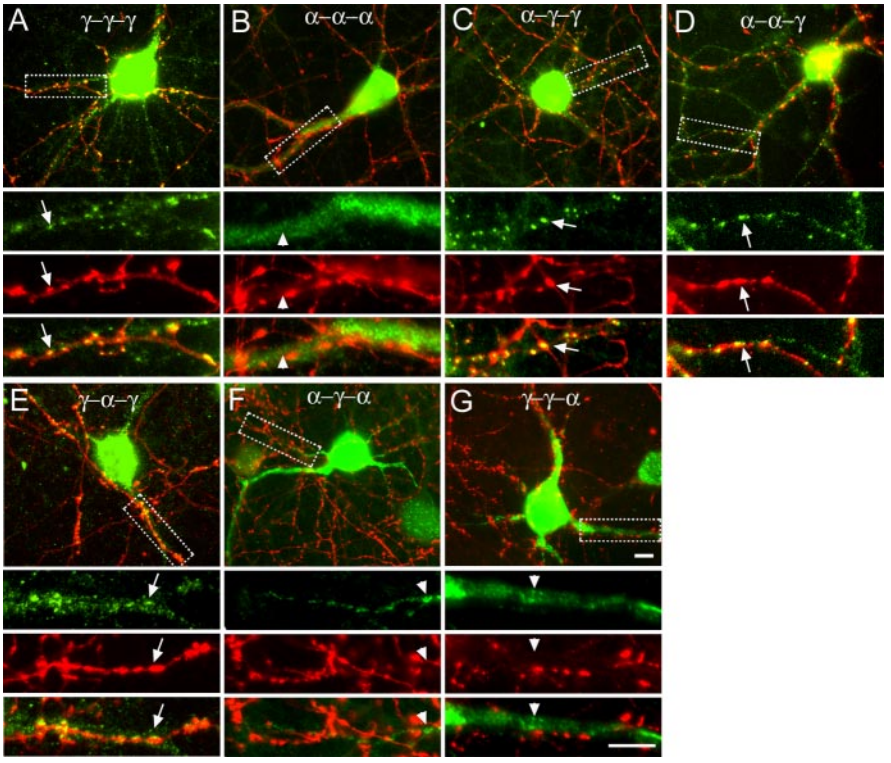

Figure 5. Restoration of postsynaptic $GABA_A$ receptor clusters in $\gamma 2^{-/-}$ neurons transfected with chimeric $\gamma 2/\alpha 2$ subunit constructs. *A–G*, Cortical neurons isolated from $\gamma 2^{-/-}$ embryos were transfected at 18 DIV with (9E10) epitope-tagged constructs indicated and processed for immunofluorescence analysis at 21 DIV for the 9E10 epitope (shown in green) and presynaptic GAD (red). Shown are merged images with colocalization represented in yellow. Boxed dendritic segments of the images are shown enlarged in separate panels below each image. Note the faithful formation of clusters revealed in the form of punctate staining for the $^{9E10}\gamma$-γ-γ (*A*), $^{9E10}\alpha$-γ-γ (*C*), $^{9E10}\alpha$-α-γ (*D*), and γ-α-γ (*E*) constructs that were closely apposed to presynaptic GAD, whereas the $^{9E10}\alpha$-α-α (*B*), $^{9E10}\alpha$-γ-α (*F*), and $^{9E10}\gamma$-γ-α (*G*) constructs failed to form clusters and showed no juxtaposition to GAD immunoreactivity. Arrows point to clusters apposed to GAD; arrowheads indicate 9E10 immunoreactivity that is diffuse or punctate but not apposed to GAD immunoreactivity. Scale bars, 10 μm.

and postsynaptic anchoring of glycine receptors in the postsynaptic membrane. To address whether the TM4 region or the cytoplasmic loop domain of the $GABA_A$ receptor $\gamma 2$ subunit or both might recruit gephyrin to GABAergic synapses, each of the constructs shown to induce postsynaptic clusters above ($^{9E10}\gamma$-γ-γ, $^{9E10}\alpha$-γ-γ, $^{9E10}\gamma$-α-γ, $^{9E10}\alpha$-α-γ) was analyzed with respect to its ability to induce colocalization with gephyrin (Fig. 7*A–D*). Indeed, after transfection into $\gamma 2^{-/-}$ cortical neurons, all of the constructs that formed postsynaptic clusters in Figure 5 also resulted in recruitment and clustering of gephyrin as evident by colocalization of punctate immunoreactivity for the transfected constructs (anti-myc antiserum) and endogenous gephyrin (mAb 7A); however, whereas the $^{9E10}\alpha$-γ-γ construct recruited gephyrin as effectively as the $\gamma 2$ subunit ($^{9E10}\gamma$-γ-γ) as determined by the percentage of 9E10-immunoreactive puncta colocalized with punctate gephyrin immunoreactivity [$^{9E10}\gamma$-γ-γ, 73.8 ± 4.1 (SEM); $^{9E10}\alpha$-γ-γ, 83.7 ± 3.9; $p < 0.05$], significantly lower fractions of the $^{9E10}\gamma$-α-γ and $^{9E10}\alpha$-α-γ clusters were colocalized with gephyrin compared with $^{9E10}\gamma$-γ-γ ($^{9E10}\gamma$-α-γ, 37.1 ± 6.1, $p < 0.01$; $^{9E10}\alpha$-α-γ, 30.8 ± 7.0, $p < 0.01$; $n = 13$–26 cells per construct) (Fig. 7*E*). Less efficient recruitment of gephyrin by the $^{9E10}\gamma$-α-γ and $^{9E10}\alpha$-α-γ constructs was reflected by a significant number of immunoreactive puncta for these constructs that were not colocalized with gephyrin immunoreactivity (Fig. 7*C*,*D*). Moreover, cells transfected with the $^{9E10}\gamma$-α-γ or $^{9E10}\alpha$-α-γ constructs more often than the $^{9E10}\gamma$-γ-γ and $^{9E10}\alpha$-γ-γ constructs failed to recruit gephyrin entirely (data not shown). Because transfected cells that lacked gephyrin immunoreactivity were excluded from quantitation, the low percentages of colocalization given for these constructs in Figure 7*E* are likely to be overestimated. Thus, both the TM4 and the major cytoplasmic loop region of the $\gamma 2$ subunit contribute to $GABA_A$ receptor-mediated recruitment of gephyrin to postsynaptic sites. Whereas the $\gamma 2$ subunit TM4 domain can contribute to recruitment of gephyrin independently of other $\gamma 2$ subunit sequences, the effect of the $\gamma 2$ subunit cytoplasmic loop domain is evident only in the presence of the TM4 domain.

The finding that the cytoplasmic portion of the $\gamma 2$ subunit was unable to induce clustering of $GABA_A$ receptors is surprising, given that postsynaptic targeting of glycine receptors, AMPA receptors, and NMDA receptors is mediated by cytoplasmic receptor domains. One possible explanation would be that constructs that contain the $\gamma 2$ subunit cytoplasmic domain in a heterologous context would interfere with synapse formation in a dominant-negative manner. To address this question we transfected the $^{9E10}\gamma$-γ-γ, $^{9E10}\alpha$-γ-α, and $^{9E10}\gamma$-γ-α constructs into wild-type neurons and addressed whether they would interfere with postsynaptic clustering of endogenous gephyrin (Fig. 8). The $\gamma 2$ subunit ($^{9E10}\gamma$-γ-γ) formed immunoreactive puncta that were colocalized with gephyrin clusters and juxtaposed to GABAergic sites as expected (Fig. 8*A*). Similar to observations made in $\gamma 2^{-/-}$ neurons, the

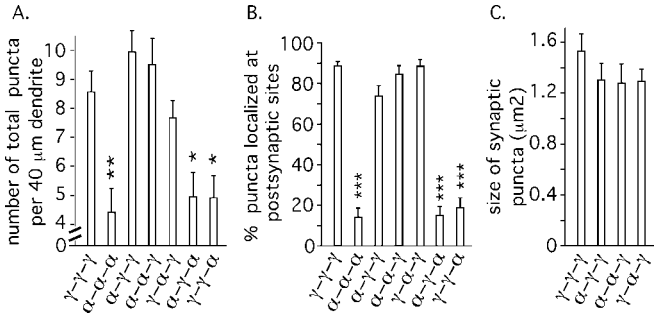

Figure 6. Quantitative analyses of postsynaptic clusters formed by chimeric constructs transfected into $\gamma2^{-/-}$ neurons. Cortical cultures isolated from $\gamma2^{-/-}$ embryos were transfected and double labeled for the 9E10 epitope tag of the transfected construct and for the GABAergic terminal marker GAD as shown in Figure 5. Immunoreactive puncta on dendritic segments that were innervated by a GABAergic axon as revealed by GAD immunoreactivity were quantified from digitized video images as described in Materials and Methods. *A*, The average number of 9E10 immunoreactive puncta per 40 μm dendritic segment determined for each of the chimeric constructs including $^{9E10}\alpha$-α-α was compared with the value determined for the $\gamma2$ subunit ($^{9E10}\gamma$-γ-γ). Note the similar number of puncta observed for the $^{9E10}\gamma$-γ-γ, $^{9E10}\alpha$-γ-γ, $^{9E10}\alpha$-α-γ, and $^{9E10}\gamma$-α-γ constructs ($n = 13, 15, 19, 16$ cells, respectively). In contrast, the number of puncta observed for the $^{9E10}\alpha$-γ-α ($n = 14$) and $^{9E10}\gamma$-γ-α ($n = 16$) constructs was similar to that observed for the $\alpha2$ subunit ($^{9E10}\alpha$-α-α; $n = 17$) and greatly reduced compared with the $\gamma2$ subunit ($^{9E10}\gamma$-γ-γ). *B*, For comparison of the number of clusters localized to postsynaptic sites, the fraction of puncta apposed to punctate GAD immunoreactivity was determined for each chimeric construct and compared with the value determined for $^{9E10}\gamma$-γ-γ. Note that the same constructs that showed a high propensity to cluster in *A* similar to the $\gamma2$ subunit are also indistinguishable from the $\gamma2$ subunit in that they result in a high percentage of clusters that are postsynaptic. In contrast, the percentage of immunoreactive puncta for the $^{9E10}\alpha$-γ-α and $^{9E10}\gamma$-γ-α constructs and the $\alpha2$ subunit ($^{9E10}\alpha$-α-α) are significantly reduced compared with the value for the $\gamma2$ subunit. *C*, The average size of postsynaptic clusters induced by the $^{9E10}\alpha$-γ-γ, $^{9E10}\alpha$-α-γ, and $^{9E10}\gamma$-α-γ constructs is indistinguishable from the value observed for the $\gamma2$ subunit ($^{9E10}\gamma$-γ-γ). Error bars represent SE. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

$^{9E10}\alpha$-γ-α and $^{9E10}\gamma$-γ-α constructs were diffusely expressed in dendrites of wild-type neurons, and they did not interfere with clustering and postsynaptic localization of endogenous gephyrin (Fig. 8*B*,*C*). Thus, failure of these constructs to cluster and to accumulate at postsynaptic sites is not caused by negative effects on mechanisms involved in synapse formation.

Rescue of inhibitory synaptic function

Efficient insertion of chimeric constructs into the plasma membrane of HEK 293T cells requires coexpression of α and β subunits (Fig. 3), suggesting that these constructs assemble into heteromeric complexes. To test whether chimeric constructs can assemble with endogenous α and β subunits and restore the function of GABAergic inhibitory synapses in $\gamma2^{-/-}$ neurons, we recorded mIPSCs from transfected pyramidal cell neurons. The frequency of mIPSCs recorded from $\gamma2^{-/-}$ compared with wild-type cortical neurons (17–22 DIV) was greatly reduced (Fig. 9*A*, *B*), as shown previously (Essrich et al., 1998). On transfection of $\gamma2^{-/-}$ neurons with the $\gamma2$ subunit ($^{9E10}\gamma$-γ-γ) or with the $^{9E10}\alpha$-γ-γ chimeric construct, the mIPSC frequency was restored to values similar to the wild-type control, consistent with restoration of function and postsynaptic localization of $GABA_A$ receptors. Consistent with normal immunoreactivity for GAD (Fig. 1*B*) and the vesicular inhibitory amino acid transporter (Schweizer e al., 2003) in $\gamma2^{-/-}$ neurons, efficient rescue of mIPSCs in transfected $\gamma2^{-/-}$ pyramidal cells confirmed that presynaptic function of GABAergic $\gamma2^{-/-}$ neurons was intact and that the functional deficit of GABAergic $\gamma2^{-/-}$ neurons was limited to the postsynaptic apparatus. Furthermore, the $^{9E10}\gamma$-γ-α and $^{9E10}\alpha$-γ-α constructs both failed to restore mIPSCs, consistent with the notion that the $\gamma2$ subunit TM4 is required for postsynaptic clustering of $GABA_A$ receptors and gephyrin.

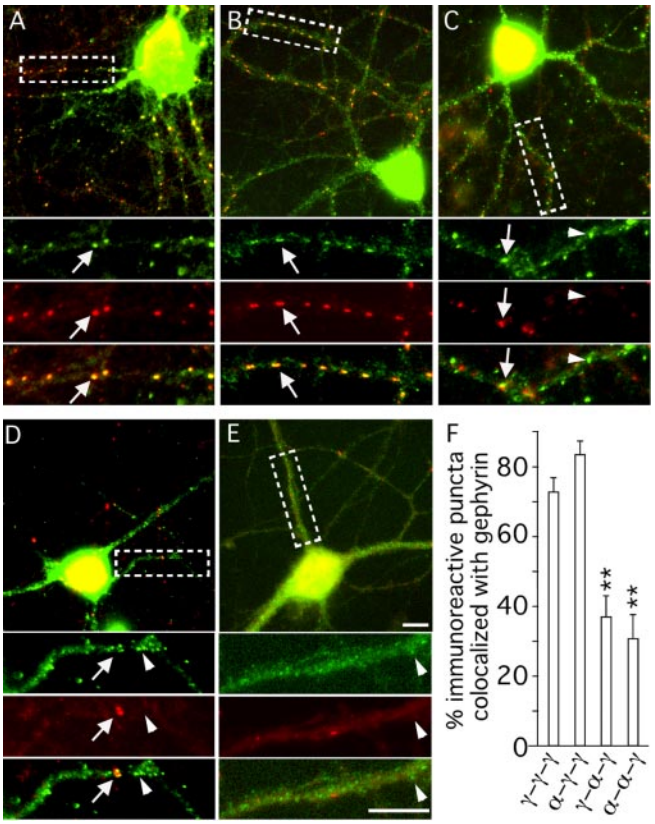

Figure 7. Recruitment of gephyrin to $GABA_A$ receptor clusters. The $\gamma2$ subunit (*A*) and the three other $\gamma2$ subunit TM4-containing chimeric constructs shown in Figure 5 to form clusters (*B*, $^{9E10}\alpha$-γ-γ; *C*, $^{9E10}\gamma$-α-γ; *D*, $^{9E10}\alpha$-α-γ) were transfected into $\gamma2^{-/-}$ neurons and analyzed for colocalization of punctate 9E10 immunoreactivity (green) with endogenous gephyrin (red). In addition, analysis of the diffusely expressed construct $^{9E10}\alpha$-γ-α (*E*), which is not concentrated at synapses (Fig. 5*F*), is shown for comparison. Boxed dendritic segments are shown enlarged in color-separated and merged panels underneath each image with arrows pointing to colocalized clusters (yellow) and arrowheads indicating punctate 9E10 immunoreactivity that was not colocalized (green). Note the similar high degree of colocalization seen for the $\gamma2$ subunit (*A*) and for the $^{9E10}\alpha$-γ-γ construct (*B*) with only few 9E10-immunoreactive puncta that were not colocalized with gephyrin (green puncta in the merged enlargement). In contrast, only a fraction of the 9E10-immunoreactive puncta that were formed by the $^{9E10}\gamma$-α-γ and $^{9E10}\alpha$-α-γ constructs were colocalized with gephyrin (*C*, *D*). This visual impression was confirmed by quantitative analyses (*F*). The percentage of $^{9E10}\alpha$-γ-γ puncta colocalized with gephyrin was similar to values seen with the $\gamma2$ subunit. In contrast, the percentage of $^{9E10}\gamma$-α-γ or $^{9E10}\alpha$-α-γ puncta colocalized was significantly reduced compared with the $\gamma2$ subunit, consistent with the notion that $GABA_A$ receptors can form clusters in the absence of gephyrin. Colocalization of $^{9E10}\alpha$-γ-α and gephyrin (*E*) could not be quantified because expression of this construct was mostly diffuse. Scale bars, 10 μm.

Unexpectedly, however, the $^{9E10}\gamma$-α-γ and $^{9E10}\alpha$-α-γ constructs failed to restore mIPSCs on transfection into $\gamma2^{-/-}$ neurons, although they formed clusters at postsynaptic sites similar to the $^{9E10}\gamma$-γ-γ and $^{9E10}\alpha$-γ-γ constructs. The very low mIPSC frequency observed for the $^{9E10}\gamma$-α-γ and $^{9E10}\alpha$-α-γ constructs expressed in $\gamma2^{-/-}$ neurons was similar to that recorded from untransfected $\gamma2^{-/-}$ neurons (Fig. 9*A*,*B*). Moreover, the low mIPSC frequency of $^{9E10}\gamma$-α-γ or $^{9E10}\alpha$-α-γ transfected cells was mirrored in a significantly lower amplitude of rare miniature currents detected compared with values seen with the $^{9E10}\gamma$-γ-γ construct ($^{9E10}\gamma$-γ-γ, 34.4 ± 3.1 pA, $n = 10$ cells showing minis; $^{9E10}\gamma$-α-γ, 16.4 ± 1.7 pA, $n = 8$, $p < 0.001$; $^{9E10}\alpha$-α-γ, 16.2 ± 1.0

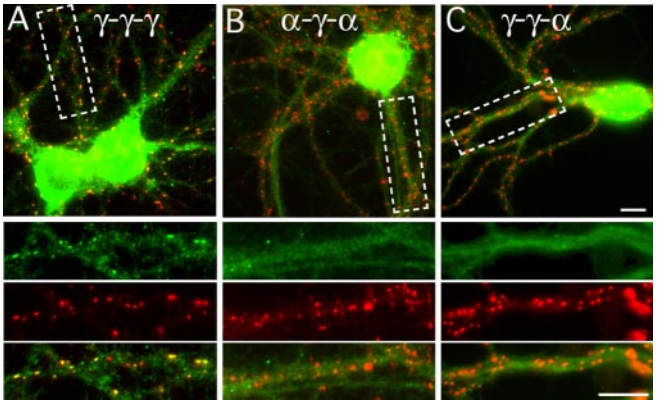

Figure 8. Assessment of dysfunctional constructs in wild-type neurons. The ^{9E10}γ-γ-γ, ^{9E10}α-γ-α, and ^{9E10}γ-γ-α constructs were transfected into wild-type neurons and double labeled for the 9E10 epitope and endogenous gephyrin to test for negative effects on postsynaptic differentiation. *A*, The ^{9E10}γ-γ-γ construct formed clusters colocalized with gephyrin as expected. The ^{9E10}α-γ-α (*B*) and ^{9E10}γ-γ-α (*C*) constructs failed to cluster, but endogenous gephyrin clusters remained unaffected, indicating that failure of these constructs to accumulate at synapses was not associated with dominant-negative effects on synapse formation. Scale bars, 10 μm.

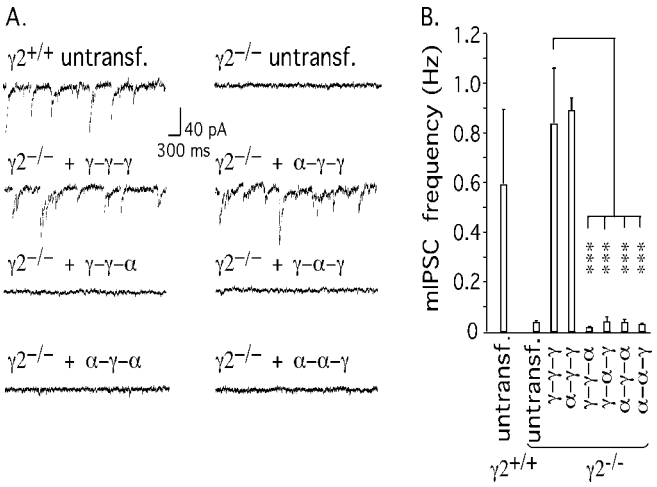

Figure 9. Functional rescue of inhibitory synapses in $\gamma2^{-/-}$ neurons requires both the major intracellular loop and the fourth transmembrane domain of the γ2 subunit. *A*, Representative traces are shown of mIPSCs recorded from $\gamma2^{+/+}$ and $\gamma2^{-/-}$ neurons and of $\gamma2^{-/-}$ neurons transfected with the γ2 subunit (^{9E10}γ-γ-γ) or chimeric subunits, as indicated. *B*, Summary of data showing mIPSC frequencies confirming that transfection of either ^{9E10}γ-γ-γ or ^{9E10}α-γ-γ restored the function of inhibitory synaptic transmission in $\gamma2^{-/-}$ neurons to values similar to those found in wild-type neurons. Note that none of the ^{9E10}γ-γ-α, ^{9E10}γ-α-γ, ^{9E10}α-γ-α, and ^{9E10}α-α-γ constructs were able to restore mIPSCs of $\gamma2^{-/-}$ neurons, indicating that both the γ2 subunit cytoplasmic loop and TM4 domain are required for restoration of synaptic function in $\gamma2^{-/-}$ neurons. Error bars represent SE. ***$p < 0.001$.

pA, $n = 3$, $p < 0.001$; Student's *t* test). Not surprisingly, the number of $\gamma2^{-/-}$ neurons transfected with the ^{9E10}γ-γ-α or ^{9E10}α-γ-α constructs that revealed any mIPSCs at all was greatly reduced compared with ^{9E10}γ-γ-γ transfected $\gamma2^{-/-}$ neurons (data not shown). The amplitudes of rare synaptic events detected in a minority of the ^{9E10}γ-α-γ- or ^{9E10}α-α-γ-transfected cells did not differ significantly from the amplitude of mIPSCs sporadically detected in a subset of untransfected $\gamma2^{-/-}$ neurons (13.5 ± 2.5 pA; $n = 5$; $p > 0.3$ for comparison with both ^{9E10}γ-α-γ and ^{9E10}α-α-γ), confirming that these two constructs did not rescue synaptic function. Thus, reminiscent of the structural prerequisites for efficient recruitment of gephyrin, restoration of mIPSCs requires both the γ2 subunit TM4 and cytoplasmic loop domains. Conversely, the data suggest that postsynaptic accumulation of GABA receptors alone is not sufficient to ensure synaptic function.

Discussion

The γ2 subunit mediates postsynaptic clustering of $GABA_A$ receptors and gephyrin (Essrich et al., 1998; Schweizer et al., 2003), but little is known about the mechanism involved. Although gephyrin is concentrated at inhibitory synapses, it does not appear to interact directly with $GABA_A$ receptors and is required for postsynaptic clustering of only a subset of postsynaptic $GABA_A$ receptor subtypes (Meyer et al., 1995; Fischer et al., 2000; Kneussel et al., 2001; Levi et al., 2004). Moreover, known proteins so far shown to interact with the γ2 subunit are not specifically localized to postsynaptic sites and therefore are unlikely to contribute to maintenance of $GABA_A$ receptors at synapses. We now show that targeting of $GABA_A$ receptors to postsynaptic dendritic sites occurs by a mechanism that is mostly independent of γ2 subunit intracellular domains and gephyrin but requires the γ2 subunit C-terminal sequence containing the fourth transmembrane domain. Thus, the mechanism involved in proper localization of $GABA_A$ receptors appears to be fundamentally different from presumed mechanisms mediating postsynaptic targeting of glycine and glutamate receptors.

The γ2 subunit cytoplasmic loop can interact with itself and homologous domains of other $GABA_A$ receptor subunits *in vitro*, prompting speculation that such interactions might contribute to higher-order aggregates of $GABA_A$ receptors (Nymann-Andersen et al., 2002); however, the data presented here from transfected neurons indicate that γ2 subunit cytoplasmic sequences are dispensable for clustering of $GABA_A$ receptors and therefore do not support such a mechanism. In contrast, we found that the γ2 subunit major cytoplasmic loop is essential for recruitment of gephyrin to $GABA_A$ receptor clusters and is required for restoration of synaptic activity in $\gamma2^{-/-}$ neurons. Clustering of $GABA_A$ receptors at postsynaptic sites therefore can be dissociated from recruitment of gephyrin and from restoration of synaptic activity. Moreover, recruitment of gephyrin to $GABA_A$ receptor clusters correlates with restoration of synaptic function.

One candidate mechanism that might deliver $GABA_A$ receptors to synapses independently of γ2 subunit cytoplasmic sequences would involve selective association of the γ2 subunit TM4 domain with cholesterol/sphingolipid-rich vesicles or microdomains of the plasma membrane (lipid rafts). Indeed, such a mechanism is implicated in postsynaptic localization of nicotinic acetylcholine (nACh), $GABA_A$, and AMPA receptors (Bruses et al., 2001; Hering et al., 2003; Pediconi et al., 2004). For the nACh receptor, which is structurally closely related to $GABA_A$ receptors, the TM4 domain of α and γ subunits is believed to interact directly with the lipid bilayer (Blanton and Cohen, 1994; de Almeida et al., 2004; Pediconi et al., 2004). Interestingly, the few amino acid residues that are uniquely present in the TM4 domain of the $GABA_A$ receptor γ2 subunit but not the α1,2 and β2,3 subunits, map to two separate surfaces of the presumed TM4 α-helix (Fig. 10*A*,*B*), suggesting that they might contribute to unique interaction domains between the γ2 subunit and the lipid bilayer. Alternatively, the γ2 subunit TM4 domain might contribute to subunit-specific interaction(s) with integral membrane proteins localized at inhibitory synapses. A recent report by Graf et al. (2004) indicates that neuroligin-2 recruited to postsynaptic sites by neurexin expressed on GABAergic terminals provides an

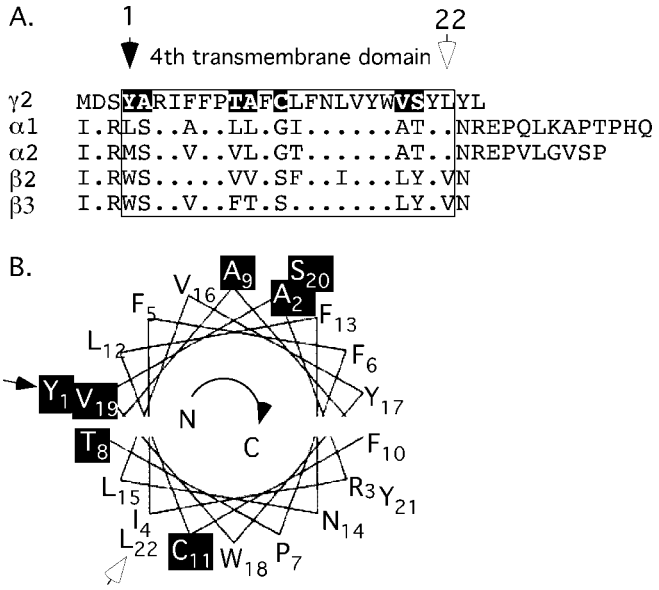

Figure 10. Structural features of the γ2 TM4 domain. *A*, Alignment of the C-terminal domains of major $GABA_A$ receptor subunits known to constitute postsynaptic $GABA_A$ receptors reveals seven amino acids that are uniquely present in the γ2 subunit TM4 domain (shown as white text on black background). *B*, A helical wheel representation of the putative α-helix of the γ2 subunit TM4 domain predicts that amino acids uniquely present in the γ2 subunit TM4 domain map to two narrow faces of the putative TM4 α-helix, suggesting potential contact sites that are uniquely present in this subunit. Amino acids in *B* are denoted in single-letter code with numeric subscripts indicating the position of the amino acid in the transmembrane domain, as shown in *A*. Amino acids that are unique for the γ2 subunit TM4 domain are shown as white text on black background.

essential signal that induces postsynaptic aggregation of $GABA_A$ receptors and gephyrin. Neuroligin-2 therefore controls proper apposition of presynaptic and postsynaptic elements of inhibitory synapses. Current evidence, however, suggests that interaction between neuroligin-2 and $GABA_A$ receptors or gephyrin is indirect at best (Graf et al., 2004).

A recent report by van Rijnsoever et al. (2005) suggests that postsynaptic clusters of $GABA_A$ receptors identified by immunofluorescence techniques are located mainly intracellularly in a submembrane endocytic compartment, rather than concentrated in the postsynaptic plasma membrane. Thus, consistent with our data, the γ2 subunit cytoplasmic loop domain might be required for efficient insertion of endocytosed receptors into the plasma membrane although it is dispensable for delivery of receptors to subsynaptic dendritic sites. In further agreement with this interpretation, studies in heterologous cells by Connolly et al. (1999a,b) suggest that the γ2 subunit plays a critical role in intracellular trafficking of endocytosed $GABA_A$ receptors.

The γ2 subunit domains required for restoration of functional inhibitory synapses are the same as those required for maximal recruitment of gephyrin. Rather than anchoring or trapping of receptors in the postsynaptic membrane, an important function of gephyrin therefore might be to ensure the stability of a localized endocytic recycling apparatus that is tailored for postsynaptic $GABA_A$ receptors. Reversible modification of the γ2 subunit cytoplasmic loop domain by phosphorylation (Brandon et al., 2001; Wang et al., 2003) and palmitoylation (Keller et al., 2004; Rathenberg et al., 2004) might contribute to dynamic modulation of this machinery.

Physical interaction of the γ2 subunit cytoplasmic domain with the phosphatase calcineurin is implicated in NMDA receptor-dependent functional plasticity of inhibitory synapses (Lu et al., 2000; Wang et al., 2003). Moreover, the γ2 subunit cytoplasmic loop domain mediates interaction with the $GABA_A$ receptor trafficking factor GABARAP (Wang et al., 1999) and the palmitoyltransferase GODZ (Keller et al., 2004). Although neither of these proteins has been detected consistently at synapses, palmitoylation of cysteines in the γ2 subunit cytoplasmic loop domain appears to ensure the stability of $GABA_A$ receptors at the cell surface and at synapses (Keller et al., 2004; Rathenberg et al., 2004). Furthermore, the γ2 subunit contains a binding site for the clathrin adaptor AP2, and clathrin-mediated endocytosis of $GABA_A$ receptors has been shown to limit the amplitude of mIPSCs in cultured neurons (Kittler et al., 2000). Together with the findings presented here, these independent lines of evidence support the interpretation that the γ2 subunit cytoplasmic domain contributes to normal endocytosis and recycling of $GABA_A$ receptors and thereby maintains the steady-state receptor concentration in the postsynaptic plasma membrane.

The finding that maximal recruitment of gephyrin to postsynaptic sites requires the γ2 subunit cytoplasmic portion does not necessarily imply that there is a direct interaction between the γ2 subunit and gephyrin. Indeed, the partial recruitment of gephyrin to receptors containing $^{9E10}\gamma$-α-γ or $^{9E10}\alpha$-α-γ chimeric constructs (Fig. 7) suggests that α and β subunits can also contribute to association of gephyrin with $GABA_A$ receptors. Because gephyrin is absent in affinity-purified $GABA_A$ receptor preparations (Meyer et al., 1995), all of these interactions appear to be sensitive to solubilization of $GABA_A$ receptors by detergent and/or treatment with reducing agents that hydrolyze palmitoylated cysteine residues of the γ2 subunit. Solving the roles of gephyrin and the γ2 subunit in modulating inhibitory synaptic function will thus continue to pose a major challenge.

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