GABA<sub>A</sub> Receptor Alpha Subunits Play a Direct Role in Synaptic versus Extrasynaptic Targeting

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* Running title: Synaptic and extrasynaptic targeting of GABA<sub>A</sub>-Rs

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Background: GABA<sub>A</sub> receptor γ2 and δ subunits are thought to be responsible for synaptic and extrasynaptic targeting.

Results: We demonstrate here that α2 and α6 subunits can target δ/γ2 chimeras to synaptic and extrasynaptic sites.

Conclusion: The α subunits play a direct role in GABA<sub>A</sub> receptor targeting.

Significance: Different subunits of GABA<sub>A</sub> receptors encode intrinsic signals to control subcellular targeting.

GABA<sub>A</sub> receptors (GABA<sub>A</sub>-Rs) are localized at both synaptic and extrasynaptic sites, mediating phasic and tonic inhibition, respectively. Previous studies suggest an important role of γ2 and δ subunits in synaptic versus extrasynaptic targeting of GABA<sub>A</sub>-Rs. Here, we demonstrate differential function of α2 and α6 subunits in guiding the localization of GABA<sub>A</sub>-Rs. To study the targeting of specific subtypes of GABA<sub>A</sub>-Rs, we used a molecularly engineered GABAergic synapse model to precisely control the GABA<sub>A</sub>-R subunit composition. We found that in neuron-HEK cell heterosynapses, GABAergic events mediated by α2β3γ2 receptors were very fast (rise time ~2 ms), whereas events mediated by α6β3δ receptors were very slow (rise time ~20 ms). Such an order of magnitude difference in rise time could not be attributed to the minute differences in receptor kinetics. Interestingly, synaptic events mediated by α6β3 or α6β3γ2 receptors were significantly slower than those mediated by α2β3 or α2β3γ2 receptors, suggesting a differential role of α subunit in receptor targeting. This was confirmed by differential targeting of the same δ-γ2 chimeric subunits to synaptic or extrasynaptic sites, depending on whether it was co-assembled with the α2 or α6 subunit. In addition, insertion of a gephyrin-binding site into the intracellular domain of α6 and δ subunits brought α6β3δ receptors closer to synaptic sites. Therefore, the α subunits, together with the γ2 and δ subunits, play a critical role in governing synaptic versus extrasynaptic targeting of GABA<sub>A</sub>-Rs, possibly through differential interactions with gephyrin.

Neural inhibition in the brain is mostly mediated by GABA<sub>A</sub> receptors (GABA<sub>A</sub>-Rs). To date, nineteen isoforms of GABA<sub>A</sub>-R subunits have been identified: α1-6, β1-3, γ1-3, δ, ε, θ, π, and ρ1-3 (1,2). Most GABA<sub>A</sub>-Rs expressed in the brain are composed of two α, two β, and one γ subunit, of which the γ subunit can be substituted by δ, ε, θ, or π (3,4).
There are two forms of GABAergic inhibition: phasic and tonic (5,6). Phasic inhibition is mediated by postsynaptically clustered GABA$_A$-Rs composed of $\alpha1$-$3$, $\beta2$-$3$ and $\gamma2$ subunits. Tonic inhibition is mediated by extrasynaptic GABA$_A$-Rs typically composed of $\alpha4$-$6$ (and possibly $\alpha1$), $\beta$ and $\delta$ subunits (7-9), as well as $\alpha5\beta2$ subunits (10-12). Blocking tonic inhibition significantly enhanced neuronal excitability (5,13-15). Malfunction of tonic inhibition is implicated in epilepsy, abnormal cognition and memory, sleep disorders, anxiety, depression, schizophrenia, and alcohol addiction (16-23).

Although the mechanisms for synaptic receptor targeting have been extensively studied, little is known about the molecular mechanisms specifying extrasynaptic targeting of $\delta$ subunit-containing GABA$_A$-Rs. Neurons deficient of $\alpha1$ or $\alpha2$ or $\alpha3$ subunit showed diminished postsynaptic GABA$_A$-R clusters in different subcellular localizations (24-27). The $\gamma2$ subunit, and particularly its intracellular loop (IL) and the fourth transmembrane domain (TM4), plays a critical role in synaptic clustering of GABA$_A$-Rs (28-31). In contrast, the $\delta$ subunit-containing GABA$_A$-Rs are mainly localized at extrasynaptic membranes (7,8). Thus, the $\gamma2$ and $\delta$ subunits have been thought to be involved in the synaptic versus extrasynaptic targeting of GABA$_A$-Rs. However, the mostly extrasynaptic $\alpha5\beta\gamma2$ and punctated $\alpha1\beta\delta$ GABA$_A$-Rs suggest that $\gamma2$ and $\delta$ subunits cannot be solely responsible for guiding GABA$_A$-R targeting (9,10,12).

Here, we employed a molecularly engineered synapse model to investigate the mechanism of $\delta$-GABA$_A$-R targeting. We demonstrated that in neuron-HEK cell synapses, distinct subunit combinations control GABA$_A$-R targeting. Electrophysiological as well as immunoelectronmicroscopic results indicated that in HEK cells, $\alpha2\beta3\gamma2$ receptors cluster at synaptic sites, while $\alpha6\beta3\delta$ receptors mainly localize at extrasynaptic membranes. Interestingly, when paired with the same chimeric $\delta$-$\gamma2$ subunit, different $\alpha$ subunits ($\alpha2$ versus $\alpha6$) dictated synaptic versus extrasynaptic targeting of the corresponding GABA$_A$-Rs. We also showed that molecularly engineered interaction with gephyrin recruited modified $\alpha6\beta\delta$ receptors to synaptic sites. Thus, GABA$_A$-R targeting is controlled by specific subunit composition and the ability to interact with gephyrin.

**EXPERIMENTAL PROCEDURES**

**Cell culture and transfection** - Astrocytes were cultured from cortical tissue of neonatal rat pups (postnatal day 3-5) as described before (32,33). Briefly, cells dissociated from cortices were plated in 25 cm$^2$ flasks for up to a week, during which time astrocytes grew to confluence while non-astrocytic cells were removed by rigorous shaking. The flat astroglial cells were then trypsinized and replated on poly-D-lysine (0.1 mM) coated coverslips to serve as a supporting substrate for cocultured neurons. Hypothalamic cultures were prepared from Sprague-Dawley rat day 18 embryos (of either sex) as described previously (34). The medial hypothalamus was dissected out, cut into small cubes, and digested in 0.05% trypsin-EDTA solution with 50 units/ml DNaseI at 37˚C for 25 minutes. After digestion, the tissue blocks were dissociated into single neurons by gentle trituration and plated on poly-D-lysine coated coverslips covered by a monolayer of astrocytes at the density of 4000-8000 cells/cm$^2$. The neuronal culture medium contained 500 ml MEM (Invitrogen, Eugene, OR), 5% fetal bovine serum (HyClone, Logan, UT), 10 ml B-27 supplement (Invitrogen), 100 mg NaHCO$_3$, 20 mM D-glucose, 0.5 mM L-glutamine, and 25 units/ml penicillin/streptomycin.

Neurons were transfected with a modified Ca$^{2+}$-phosphate transfection protocol (35). Immunocytochemistry was performed in neurons 24 - 48 hours after transfection.

Human embryonic kidney (HEK) 293T cells were maintained in DMEM supplemented with 10% FBS and 25 units/ml penicillin/streptomycin. HEK 293T cells were also transfected with the Ca$^{2+}$-phosphate transfection method and cocultured with hypothalamic neurons as described previously (34). In general, 24 hours after transfection, HEK cells were dissociated with 0.05% trypsin-EDTA and plated on top of one-week-old hypothalamic cultures. The cells were utilized for electrophysiological recordings or immunocytochemistry after 2-3 days of coculture. Each experiment was repeated in at least three independent batches of cultures.

**Plasmid constructs** - The CMV-based expression vectors for the GABA$_A$ receptor $\alpha2$, $\beta3$, and $\gamma2$ subunits were previously described (30). The $\gamma2$ subunit construct contained the myc 9E10...
epitope between the 4th and 5th amino acid of the mature polypeptide. The cDNAs encoding the rat GABA<sub>A</sub>-R α6 and δ subunits were cloned into the expression vector pCMVneo (36). The δ/γ2 chimeras (M1i and M2e) were constructed using the splice overhang extension method (37). The rat chimera δ/γ2ILT4M was generated with a two-step strategy to swap the fragment containing the large intracellular loop (IL), the TM4 and the C-terminus of the δ subunit with the corresponding one of γ2S (γ2ILT4M). In the first step, cmyc-γ2S in pCDNA3.1 was used as template in a PCR reaction to amplify a 447 bp fragment. In the second step this fragment was combined with methylated full-length cmyc-δ (in pCDNA3.1) as a template, using the Invitrogen GeneTailor™ Site-Directed Mutagenesis System. The δ/γ2ILT4M chimeric subunit was sequenced and the full-length chimeric open reading frame fragment was amplified by PCR using specific primers containing 5'-Nhe1 and 3'-Xho1 sites. The coding sequence of the α6 subunit IL (amino acids 306-400) was amplified from pCMVneo-α6 and inserted into pcDNA3.1 between the two EcoRI sites just outside the coding region of the α6 subunit IL (amino acids 306-400). For the α2/α6 chimeric subunit, the α2 subunit IL coding sequence was amplified from the α2 construct and inserted between the two EcoRI sites just outside the coding region of the α6 subunit IL (amino acids 306-400). For the α2/α6 chimeric subunit, the α2 subunit IL coding sequence was amplified from the α2 construct using HindIII and ApaI restriction site-containing primers and inserted into pCDNA3.1+ between HindIII and ApaI sites. An EcoRI site was engineered just upstream of the IL coding region through synonymous mutagenesis at amino acid 303. The EcoRI and XbaI sites around amino acid 16 and 17 were eliminated in the same way to ensure successful insertion of the α6 subunit IL coding sequence. The coding sequence of the α6 subunit IL was amplified and inserted between the engineered EcoRII site and an XbaI site just downstream the α2 subunit IL coding region. For both constructions, the sequences outside the ILs were not changed.

δCαS and α6CαS chimeras were constructed in pCMVneo by insertion of the 18-amino acid gephyrin-binding site (GBS) of the glycine receptor β subunit (38) into the IL of the δ subunit (after amino acid 341 of the mature polypeptide), and the IL of α6 subunit after amino acid 340. Two DNA fragments, one including coding sequences of the target protein from the N-terminus to the insertion site, the other including that from the insertion site to the C-terminus, were amplified from pCMVneo-α6 and pCMVneo-δ. Both fragments also contained partial and overlapping insertion sequences and were fused into one fragment by PCR. The resulting fragment was inserted back into pCMVneo vector.

The murine HA-tagged NL2A expression vector (pNicNLG-2, referred to as NL2 in this manuscript) was obtained from Dr. P. Scheiffele (University of Basel) (39). The HA tag was inserted between the signal peptide and the C-terminus of the mature protein. The gephyrin-GFP construct encodes human gephyrin with GFP fused to the C-terminal of gephyrin (40). The collybistin constructs encode two isoforms of human collybistin: CB3SH3+/hPEM2SH3+ containing the SH3 domain and CB3SH3-/hPEM2SH3- lacking the SH3 domain (41).

Electrophysiology - Whole-cell recordings were performed in voltage clamp mode by using Multiclamp 700A amplifier (Molecular Devices, Palo Alto, CA) as described before (42). Patch pipettes were pulled from borosilicate glass and fire polished to resistance of 3-6 MΩ. The recording chamber was continuously perfused with a bath solution containing 128 mM NaCl, 30 mM glucose, 25 mM HEPES, 5 mM KCl, 2 mM CaCl<sub>2</sub>, and 1 mM MgCl<sub>2</sub> (pH 7.3, adjusted with NaOH, ~320 mM Osm.). The pipette solution contained 135 mM KCl, 10 mM HEPES, 2 mM EGTA, 10 mM Tris-phosphocreatine, 4 mM MgATP, 0.5 mM Na<sub>2</sub>GTP (pH 7.3, adjusted with KOH, ~300 mM Osm). Data were acquired using the pCLAMP 9 software (Molecular Devices), sampled at 5 kHz and filtered at 1 kHz, and analyzed with Clampfit 9.0 (Molecular Devices). Drugs were applied through a fast drug application system (VC-6; Warner Instruments, Hamden, CT) to assess the pharmacological properties of the reconstituted GABA<sub>A</sub>-Rs, as indicated by the rapid rise phase of whole-cell GABA and THIP currents in the pharmacological study (Fig. 1). Spontaneous IPSCs were analyzed by MiniAnalysis software (Synaptosoft). The 20 - 80% rising time and the weighted time constant (τ<sub>weighted</sub> = (τ1 x A1 + τ2 x A2)/(A1 + A2)) of the IPSCs were analyzed to compare the kinetics of the events. Pooled data were presented as mean ± standard error of the mean.
(S.E.M.), and n represents the number of the cells recorded. One-way ANOVA was employed to analyze multiple groups of data, followed by Bonferroni’s pairwise comparison.

Ultrafast GABA application and out-side-out patch recording were employed to assess the onset kinetics of GABA$_A$-Rs composed of different subunits. The ultrafast drug application system (ALA Inc, Long Island, USA) consists of solution reservoirs, manual switching valves, a solenoid-driven four-way pinch valve, and two tubes (inner-diameter 500 µm) orientated at 50° for rapid solution exchange (43,44). One tube contains normal bath solution and the other contains 10 mM GABA to maximally activate GABA$_A$-Rs. The solution exchange rate was estimated to be within 1 msec (20-80% rise time), using an open tip electrode to detect the junction potential caused by different salt concentrations (75 mM versus 150 mM NaCl). Typically 6 pulses of GABA were applied to each patch. The duration of GABA application was sufficient (200 – 500 ms) to reach the peak current value. Data was sampled at 10 kHz and low-pass filtered at 4 kHz (8-pole Bessel filter). Individual traces were aligned and averaged, and the 20-80% rising time was analyzed with Minianalysis software.

**Drugs** - GABA, tetrodotoxin (TTX), and 3α,21-dihydroxy-5α-pregnan-20-one (THDOC) were obtained from Sigma (St. Louis, MO). Bicuculline methobromide (BIC), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP) hydrochloride, were purchased from Tocris (Ellisville, MO). CNQX and THDOC were initially dissolved as concentrated stock solutions in dimethyl sulfoxide (DMSO) and diluted to appropriate concentrations in the bath solution. The final DMSO concentration was lower than 0.1%. Other drugs were first dissolved in deionized water and freshly diluted to the final concentration in bath solution. The δ-subunit in αββδ-transfected neurons were labeled before permeabilization (Fig. 4A), while the rest of the stainings were conducted after permeabilization (Fig. 4B; Fig. 7C,G,H,I). The following primary antibodies were used: rabbit-anti-Myc-tag (1:200; Millipore, Billerica, MA), rabbit-anti-δ-Nterm antibody (1:500; PhosphoSolutions, Aurora, CO), mouse-anti-GAD6 (1:100; Developmental Studies Hybridoma Bank), and mouse-anti-gephyrin mAb7a (1:500; Synaptic Systems, Goettingen, Germany). Secondary antibodies were: Alexa Fluor 647 goat-anti-mouse, Alexa Fluor 546 goat-anti-rabbit, and Alexa Fluor 488 donkey-anti-rabbit (1:300, Molecular Probes, Eugene, OR).

For the electronmicroscopy experiments, HEK cells were co-transfected with: 1) NL2, αδ, β3, and δ; 2) NL2, α2, β3, and γ2-GFP and cocultured with hypothalamic neurons for two days. The cells were briefly fixed with 4% paraformaldehyde + 0.05% glutaraldehyde (10 min at room temperature followed by 20 min in 4°C), quenched in 0.15% glycine for 10 minutes, and incubated in blocking solution (3% normal goat serum plus 2% normal donkey serum in bath solution) for 1 hour at 4°C. Primary antibodies were diluted in blocking solution [rabbit-anti-δ-Nterm (1:100); rabbit-anti-GFP (1:200, Invitrogen)] and applied to the samples at 4°C overnight. The cells were then incubated with secondary antibodies [1.4-nm Nanogold goat-anti-rabbit (1:50; Nanoprobes, Yaphank, NY)] for 1 hour at room temperature, fixed with 1% glutaraldehyde for 20 minutes, and processed with the HQ Silver Enhancement Kit (Nanoprobes, Yaphank, NY) according to the instructions of the manual. After developing with the silver enhancer, the cells were submersed in 2% glutaraldehyde, scraped off from the coverslips and centrifuged at 8000 RCF for 10 min to collect the cells. The pellets were further fixed with 2% glutaraldehyde for 1 hr at room temperature before EM processing. The cell pellets were post-fixed in 1% OsO$_4$ for 1 hr. The cells were then dehydrated in a serial of graded ethanol solutions and embedded in Eponite 12. Thin sections (80 nm) were cut with a Leica UC6 ultramicrotome, contrasted with uranyl acetate and lead citrate, and examined in a TEM JEOL JEM 1200 EXII (Peabody, MA) at 80 kV. Hetero-synapses were identified by nerve terminals (filled with synaptic vesicles) apposing to HEK cells that showed immunogold puncta on the plasma membrane.

**Immunocytochemistry and Immuno-Electron Microscopy** - For immunofluorescent stainings, cells were fixed with 4% paraformaldehyde for 12 minutes, and permeabilized with 0.1% TritonX-100 in the blocking solution (PBS with 3% normal goat serum + 2% normal donkey serum) for 30 minutes at room temperature. The cells were incubated with the primary antibodies at 4°C overnight, followed by the secondary antibodies at room temperature for 1 hour. All antibodies were diluted with the blocking solution. The δ-subunit in α6β3δ-transfected neurons were labeled before permeabilization (Fig. 4A), while the rest of the stainings were conducted after permeabilization (Fig. 4B; Fig. 7C,G,H,I). The following primary antibodies were used: rabbit-anti-Myc-tag (1:200; Millipore, Billerica, MA), rabbit-anti-δ-Nterm antibody (1:500; PhosphoSolutions, Aurora, CO), mouse-anti-GAD6 (1:100; Developmental Studies Hybridoma Bank), and mouse-anti-gephyrin mAb7a (1:500; Synaptic Systems, Goettingen, Germany). Secondary antibodies were: Alexa Fluor 647 goat-anti-mouse, Alexa Fluor 546 goat-anti-rabbit, and Alexa Fluor 488 donkey-anti-rabbit (1:300, Molecular Probes, Eugene, OR).

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The edge of synapses was fixed in 1% OsO$_4$ for 1 hr. The cells were then incubated with secondary antibodies [1.4-nm Nanogold goat-anti-rabbit (1:50; Nanoprobes, Yaphank, NY)] for 1 hour at room temperature, fixed with 1% glutaraldehyde for 20 minutes, and processed with the HQ Silver Enhancement Kit (Nanoprobes, Yaphank, NY) according to the instructions of the manual. After developing with the silver enhancer, the cells were submersed in 2% glutaraldehyde, scraped off from the coverslips and centrifuged at 8000 RCF for 10 min to collect the cells. The pellets were further fixed with 2% glutaraldehyde for 1 hr at room temperature before EM processing. The cell pellets were post-fixed in 1% OsO$_4$ for 1 hr. The cells were then dehydrated in a serial of graded ethanol solutions and embedded in Eponite 12. Thin sections (80 nm) were cut with a Leica UC6 ultramicrotome, contrasted with uranyl acetate and lead citrate, and examined in a TEM JEOL JEM 1200 EXII (Peabody, MA) at 80 kV. Hetero-synapses were identified by nerve terminals (filled with synaptic vesicles) apposing to HEK cells that showed immunogold puncta on the plasma membrane. The edge of synapses was
defined as the point where the plasma membrane of nerve terminals starts to diverge from HEK cell membrane. The localization of silver-enhanced gold labeling of GABA<sub>AX</sub>-Rs were characterized into three categories: 1) synaptic, inside a synapse and more than 30 nm away from the edges; 2) persynaptic, less than 30 nm away from the synaptic edges; 3) extrasynaptic, outside synapses and over 30 nm away from the edges of synapses (8).

**Co-immunoprecipitation** - HEK cells were transfected with either δ or δ<sub>GK</sub> together with gephrin-GFP. Gephyrin-GFP single transfection served as the control. Rabbit-anti-δ<sub>GK</sub>-Nterm was used for the immuno-precipitation, and rabbit-anti-GFP was used in the immuno-blotting to detect the gephrin-GFP.

**RESULTS**

**Distinct pharmacological properties of heterologously expressed GABA<sub>AX</sub>-Rs** - Neurons express a broad spectrum of GABA<sub>AX</sub>-Rs composed of different subunits, making it difficult to identify the critical factors important for the targeting of a specific receptor subtype. We therefore employed our recently established hetero-synapse system to investigate the targeting of different subtypes of GABA<sub>AX</sub>-Rs (34). When HEK cells were transfected with GABA<sub>AX</sub>-R subunits and a cell adhesion molecule neuroligin-2 (NL2), then cocultured with neurons, both spontaneous and action potential-evoked GABAergic events were detected (34). With this system, we can precisely control the subunit composition of GABA<sub>AX</sub>-Rs and their potential interacting proteins to investigate the targeting mechanism of GABA<sub>AX</sub>-Rs.

The αδβδα receptors were selected in this study because they are known to be present mainly at extrasynaptic sites. We first demonstrated that both αδβδα and αββγ2-Rs were efficiently expressed on the plasma membranes of HEK cells, as shown by the surface immunostaining of the δ and γ2 subunits (Fig. 1A). We then examined the pharmacological characteristics of the reconstituted GABA<sub>AX</sub>-Rs in HEK cells. Bath application of GABA (100 µM) evoked a significant current in HEK cells expressing αδβδα receptors (Fig. 1B). Pre-application of the neurosteroid THDOC (100 nM) for 30 seconds significantly potentiated the GABA-evoked peak current (Fig. 1C, I<sub>GABA</sub> = 417 ± 89 pA; I<sub>THDOC+GABA</sub> = 801 ± 169 pA; p < 0.001, n = 16, paired t-test). THIP (100 µM) induced a larger whole-cell current than that induced by 100 µM GABA (Fig. 1D, I<sub>GABA</sub> = 422 ± 84 pA; I<sub>THIP</sub> = 854 ± 151 pA; p < 0.001, n = 17, paired t-test). These data are consistent with previous studies on neurosteroid modulation and THIP activation of δ subunit-containing GABA<sub>AX</sub>-Rs (45,46). In contrast, THDOC (100 nM) negatively regulated the GABA current mediated by αβγδ2 receptors (Fig. 1E and F, I<sub>GABA</sub> = 1348 ± 195 pA; I<sub>THDOC+GABA</sub> = 1019 ± 173 pA; p < 0.001, n = 13, paired t-test), and THIP was a very weak agonist for αβγδ2 receptors (Fig. 1G; I<sub>THIP</sub> = 114 ± 24 pA; p < 0.001, n = 13, paired t-test). Therefore, THDOC and THIP showed distinct pharmacological effects on αδβδα and αβγδ2 receptors.

**Distinct kinetic properties of GABAergic events mediated by different subtypes of GABA<sub>AX</sub>-Rs** - We previously demonstrated that NL2-transfected HEK cells receive GABAergic innervation from surrounding neurons in the HEK cell-neuron coculture system (34). Orthogonal views of Z-stack confocal images showed GABAergic terminals labeled by GAD staining (green) wrapped around a transfected HEK cell (Fig. 2A). Interestingly, GAD puncta were found not only at the bottom of the HEK cell, where the initial contact with neurons took place, but also on side and top surfaces of HEK cells. This observation suggests that following initial contact with transfected HEK cells, neuronal axons have ramified to innervate large portions of the cell surface.

We next employed patch clamp recordings to examine synaptic events in HEK cells expressing different subtypes of GABA<sub>AX</sub>-Rs. We found that GABA (100 µM) induced large whole-cell currents in HEK cells expressing αβδα, αδβα, α 2βγ2, αδβγ2, or αδβδα receptors (Fig. 2B; αβδα, 1158 ± 277 pA, n = 16; αδβα, 1192 ± 295 pA, n= 33; αβγ2, 1348 ± 195 pA, n = 13; αδβγ2, 793 ± 172 pA, n = 9; αδβδα, 417 ± 89 pA, n = 16). The large GABA currents coincided with the observation of synaptic-like events recorded from the cocultured HEK cells expressing these receptor subtypes (Fig. 2C-G). All synaptic events in HEK cells were abolished by bicuculline (BIC, 20 µM, data not shown), indicating that they were GABAergic inhibitory postsynaptic currents (IPSCs) (34). In contrast, HEK cells expressing αβδδ subunits showed small whole-cell currents after bath application of 100 µM GABA (Fig. 2B; αβδδ, 45 ±
IPSCs mediated by α2β3 receptors showed rapid rise and exponential decay phases, whereas IPSCs mediated by α6β3 receptors showed slower rise and decay phases (Fig. 2 C and D). Thus, in the absence of the γ2 subunit the α2β3 or α6β3 receptors could form functional postsynaptic structures with NL2 in HEK cells. Intriguingly, compared to the rapid rising IPSCs mediated by α2β3γ2 receptors (Fig. 2E), α6β3γ2 receptor-mediated IPSCs were also slow (Fig. 2F), indicating a difference between the α2 and α6 subunits.

The IPSCs mediated by α6β3δ receptors showed an even slower rise phase than α6β3γ2 IPSCs (Fig. 2G). The slow rise phase of α6β3δ IPSCs was not simply due to asynchronous release of GABA from many release sites, because even in the presence of tetrodotoxin (TTX, 0.5 µM), which blocks action potentials, single quantal events still showed a very slow rise phase (Fig. 2H). In the presence of the neurosteroid THDOC (100 nM), the amplitude of α6β3δ IPSCs was significantly increased, confirming that these events were mediated by δ subunit-containing receptors (Fig. 2I, median IPSC amplitude: control, 42.6 ± 7.5 pA, n = 4; THDOC, 92.5 ± 19.2 pA, n = 4; p < 0.05). THDOC also slightly enhanced the sIPSC frequency from 0.6 ± 0.2 Hz (n = 4) to 0.8 ± 0.3 Hz (n = 4, p > 0.09, not reaching statistical significance, paired t-test). Furthermore, a tonic GABA current (~50 pA) was evident in HEK cells expressing α6β3δ receptors, as illustrated by the decreased baseline conductance and noise level in the presence of BIC (20 µM; Fig. 2J).

We next quantitatively compared the kinetics of IPSCs mediated by different GABA<sub>3</sub>-Rs in HEK cells with those of IPSCs recorded from neurons (Fig. 3A-D). The IPSCs recorded from α2β3γ2-expressing HEK cells showed a slightly yet significantly slower rise phase compared to neuronal IPSCs (Fig. 3 A, B, E, & F; α2β3γ2 T<sub>20-80%Rise</sub> = 1.7 ± 0.2 ms, n=16; neuron T<sub>20-80%Rise</sub> = 1.0 ± 0.1 ms, n=9; p < 0.05). Meanwhile, α2β3γ2 IPSCs had a typical two-exponential decay phase with a weighted time constant (τ<sub>weighted</sub>) significantly faster than that of neuronal IPSCs (Fig. 3G; neuron τ<sub>weighted</sub> = 52.2 ± 5.4 ms, n= 9; α2β3γ2 τ<sub>weighted</sub> = 29.2 ± 2.9 ms, n=16; p < 0.001). Coexpression of gephyrin with α2β3γ2-Rs in HEK cells did not change the kinetics of IPSCs (T<sub>20-80%Rise</sub> = 1.9 ± 0.3 ms, n=9, p > 0.5, two-tailed t-test), suggesting that gephyrin may be dispensable for the formation of hetero-synapses, or HEK cells have low levels of endogenous gephyrin (see Suppl. Fig. 1). In contrast to the rapid rise phase of α2β3γ2 IPSCs, the rise time of α6β3δ IPSCs was an order of magnitude slower than that of neuronal IPSCs (Fig. 3 D, E, F & G; T<sub>20-80%Rise</sub> = 21.9 ± 2.6 ms; τ<sub>weighted</sub> = 140.6 ± 12.8 ms; n = 13; p < 0.001). The very slow rise phase was consistent with slow IPSCs previously observed in cerebellar granule cells, which are mediated by α6 subunit-containing GABA<sub>3</sub>-Rs localized far from GABA release sites (47). Our data suggest that α6β3δ receptors assume an extrasynaptic localization, while α2β3γ2 receptors cluster at synaptic sites in the hetero-synapse model.

Interestingly, the rise phase of α6β3γ2 IPSCs was significantly faster than that of α6β3δ IPSCs, yet significantly slower than that of α2β3γ2 IPSCs (Fig. 3 C, E & F; T<sub>20-80%Rise</sub> α6β3δ = 3.7 ± 0.4 ms, n=9; α6β3γ2 vs. α6β3δ, p < 0.001; α6β3γ2 vs. α2β3γ2, p < 0.001; Bonferroni’s Multiple Comparison Test). In addition, IPSCs mediated by α6β3 receptors showed significantly slower rise phase than the events mediated by α2β3 receptors (T<sub>20-80%Rise</sub>: α6β3, 5.1 ± 1.0, n=8; α2β3, 2.2 ± 0.3 ms, n=11; ** p < 0.01, two-tailed t-test). Notably, there is no significant difference between the rise phase of IPSCs mediated by α2β3 and α2β3γ2 receptors (p > 0.1), nor between α6β3 and α6β3γ2 receptors (p > 0.1). These results indicate that distinct α subunits play a significant role in shaping GABAergic responses.

Rapid onset kinetics of GABA<sub>3</sub>-Rs composed of different subunits – We wondered whether the onset kinetics of different receptors might explain such drastic difference in the IPSC rise phases. To answer this question, we employed a high-speed solution exchange system to apply GABA (10 mM) to outside-out patches excised from transfected HEK cells (Fig. 4A). Ultrafast GABA application was achieved by starting GABA perfusion and stopping bath solution simultaneously. We found that α2β3γ2-Rs were activated rapidly upon GABA application (Fig. 4B,G; T<sub>20-80%Rise</sub> α2β3γ2 = 1.0 ± 0.2 ms, n = 8), faster than the rise phase of α2β3γ2-mediated IPSCs in HEK cells but comparable to neuronal IPSCs. This result suggests that GABA<sub>3</sub>-Rs in HEK cells are not clustered as tightly as in neuronal cells. The rise phase of α6β3γ2-Rs was indistinguishable from that of α2β3γ2-Rs (Fig. 4B, G; T<sub>20-80%Rise</sub> α6β3γ2 = 1.0 ± 0.2 ms, n = 8; p > 0.9). However, the rise phase of
αδβ3δ-mediated GABA currents was significantly slower than that of αβγ2- or αδβγ2-receptors (Fig. 4 B, G; T<sub>20-80%Rise</sub>αδβ3δ = 2.3 ± 0.3 ms, n = 10; p < 0.01 for both comparison, one-way ANOVA followed by Bonferroni’s pairwise comparison), yet it was still an order of magnitude faster than that of αδβ3δ-IPSCs in HEK cells. Since the difference in receptor kinetics is too small to explain the drastic ten-fold difference between the rise phase of αβ3γ2 and αδβ3δ IPSCs, the slow αδβ3δ-IPSCs is likely a result of the extrasynaptic localization of αδβ3δ receptors.

**Ultrastructural localization of GABA<sub>1</sub>-Rs** - We further carried out immuno-electronmicroscopic studies to reveal the ultrastructural localization of αδβ3δ and αβγ2 receptors in neuron-HEK cell cocultures. HEK cells expressing αδβ3δ or αβγ2 receptors were identified by silver-enhanced gold particles immunolabeling the δ or α subunit. Nerve terminals containing synaptic vesicles were found in close contact with HEK cells. Importantly, gold particles immunopositive for α subunit were localized mostly at extrasynaptic membranes, whereas γ2-positive particles were mainly at synaptic cleft (Fig. 5A and B). To quantify the detailed receptor localization, 7 randomly selected sections with a total of 34 synapses and 55 gold particles labeling δ-receptors were analyzed. The majority of particles (80%) were localized at extrasynaptic membranes, while only 12.7% and 7.3% were localized persynaptically or synaptically (Fig. 5C ). For comparison, 6 sections containing 18 synapses from αβγ2-expressing HEK cells were assessed. Among 81 γ2-immunoreactive particles analyzed, 63% were synaptic and 16% persynaptic, with the remaining 21% being extrasynaptic (Fig. 5C). The immuno-EM results confirmed that the αδβ3δ GABA<sub>1</sub>-Rs are preferentially localized at extrasynaptic membranes in the hetero-synapse model. Together with the kinetics analysis (Fig. 2 - Fig. 4), the IPSC rise phase seems to be a faithful indicator of receptor localization in our hetero-synapse model: fast rise phase indicates synaptic localization while slow rise phase indicates extrasynaptic or persynaptic localization.

**α2 and α6 subunits directly target chimeric receptors to synaptic and extrasynaptic sites** - Given the significant difference in the rise phase of αβ3 vs. αδβ3 IPSCs, and αβ3γ2 vs. αβ3γ2 IPSCs (Fig. 2 - 3), we hypothesized that α2 and α6 subunits play a distinctive role in receptor targeting. To test this hypothesis, a series of δγ2 chimeras were co-expressed with either α2 or α6 subunit, together with β3 subunit and NL2 in HEK cells. Fig. 6A illustrates the domain compositions of the δγ2 chimeras. Interestingly, when different δγ2 chimeras were combined with α2 and β3 subunits, they all mediated fast rising IPSCs (Fig. 6B, black traces). In contrast, when combined with α6 and β3 subunits, these chimeras all yielded slow IPSCs (Fig. 6B, grey traces). Importantly, for each individual δγ2 chimera, the IPSC rise phase was always slower when it was co-assembled with the α6 compared to the α2 subunit (Fig. 6C, Suppl. Table 1). Similarly, the decay phase of the IPSCs mediated by each δγ2 chimera was always slower when co-expressed with the α6 subunit (Fig. 6D). Fig. 6E shows large GABA-induced whole-cell currents in HEK cells expressing all different chimeric receptors.

The onset kinetics of receptors containing δγ2ILTM4 and δγ2 M1i were analyzed as well (Fig. 4 C, G). Interestingly, different α subunits (α2 vs. α6) did not change the receptor onset kinetics (αβ3δ/γ2ILTM4, T<sub>20-80%Rise</sub> = 1.8 ± 0.3 ms, n=8; αδβ3δ/γ2ILTM4, T<sub>20-80%Rise</sub> = 1.5 ± 0.2 ms, n=8; p>0.3; α2β3δ/γ2 M1i, T<sub>20-80%Rise</sub> = 1.3 ± 0.2 ms, n=8; αδβ3δ/γ2 M1i, T<sub>20-80%Rise</sub> = 1.5 ± 0.2 ms, n=9; p>0.5). Therefore, the difference in IPSC rise phase mediated by these chimeric receptors shown in Fig. 6 was likely caused by distinct receptor localization. Thus, the same δγ2 chimera can be targeted to either synaptic sites if coassembled with the α2 subunit, or targeted to extrasynaptic sites if coassembled with the α6 subunit. These results suggest that different α subunits play a direct role in guiding receptor localization.

We then explored the structural differences between α2 and α6 subunits that might contribute to differential receptor targeting. The intracellular loop of the α2 subunit has been found to interact with gephyrin, a scaffolding protein at inhibitory synapses to stabilize GABA<sub>1</sub>-R clusters (48). To test the role of the intracellular loop in receptor targeting, we swapped the intracellular loop of α2 and α6 subunits, generating chimeras α2α6IL and α6α2IL (Fig. 7A). The α6α2IL chimera was capable to form functional GABA<sub>1</sub>-Rs efficiently with either β3γ2 or β3δ subunits, as indicated by large GABA-induced whole-cell currents in transfected HEK cells (Fig. 7B; α6α2ILβ3γ2, IgABA = 1224.0 ± 277.5 pA, n = 11; α6α2ILβ3δ, IgABA = 1385.0 ± 246.7 pA, n = 29). By contrast, the α2α6IL subunit-containing receptors
showed very small GABA-induced current when expressed in HEK cells (Fig. 7B; α2α6ILβ3γ2, IgABA = 217.1 ± 68.6 pA, n = 22; α2α6IL δ, IgABA = 9.4 ± 2.8 pA, n = 14), making it impossible to assay postsynaptic events mediated by these receptors. We therefore focused on the α6α6IL construct.

The IPSCs mediated by α6α6ILβ3γ2 and α6α6IL δ receptors were compared with those mediated by receptors containing the wildtype α6 subunit (α6β3γ2 and α6β3δ). Unexpectedly, the α6α6ILβ3γ2 IPSCs showed slower rise and decay phases than wildtype α6β3γ2 IPSCs (Fig. 7F-G; α6α6ILβ3γ2, T20-80%Rise = 6.6 ± 0.7 ms, τweighted = 80.7 ± 9.6 ms, n = 10; α6β3γ2 T20-80%Rise = 3.7 ± 0.4 ms, p < 0.05, τweighted = 50.6 ± 5.3 ms, p < 0.05). As for the α6α6IL δ IPSCs, the rise phase was not different from that of α6β3δ IPSCs (Fig. 7K; α6α6ILδ, 16.8 ± 2.0 ms, n = 13; α6β3δ 21.9 ± 2.6 ms, n = 13; p < 0.05). Our results showed that substitution of the α2 IL in the α6 subunit did not generate faster IPSCs, suggesting that the α2 IL domain alone did not direct GABA-A-Rs to synaptic sites.

The onset kinetics of α6α6IL-containing receptors was similar to those of receptors containing the wildtype α6 subunit (Fig. 4 D, E, G). The 20-80% rise time of GABA-induced currents was 1.5 ± 0.2 ms for the α6α6ILβ3γ2-Rs (n = 9, p > 0.1 compared to α6β3γ2-Rs), and 2.2 ± 0.2 ms for the α6α6IL δ-Rs (n = 9, p > 0.8 compared to α6β3δ-Rs).

Recruiting α6β3δ GABA-A-Rs to synaptic sites through forced interaction with gephyrin - Gephyrin is known to cluster at inhibitory synapses, where it stabilizes synaptic GABA-A-Rs (28,40,49-54). We wondered whether enhancing gephyrin interaction with α6β3δ receptors can target them to synaptic sites. Therefore, we modified the intracellular loop of α6 and δ subunits by insertion of a gephyrin-binding site (GBS) derived from the glycine receptor β subunit, generating α6GBS and δGBS chimeras (Fig. 8A). The interaction between δGBS and gephyrin was demonstrated by co-IP assay (Fig. 8B). The δGBS and α6GBS subunits were also co-expressed with gephyrin-GFP in HEK cells to examine their co-localization. Gephyrin-GFP tends to form large intracellular aggregates when overexpressed in HEK cells (Fig. 8C). Both α6β3δGBS and α6δGBS δ receptors colocalized with gephyrin aggregates, while the wildtype α6β3δ receptors did not (Fig. 8C). Thus, our newly constructed δGBS and α6GBS subunits interact with gephyrin as predicted.

The whole cell GABA current in cells expressing α6β3δGBS receptors was similar to that of α6β3δ receptor-expressing cells, while α6GBSβ3δ and α6GBSδGBS receptors showed a very small GABA-induced current (Fig. 8D; IgABA; α6β3δ, 417 ± 89 pA, n = 16; α6β3δGBS, 504 ± 113 pA, n = 20; α6β3δGBS, 100 ± 32 pA, n = 6, p < 0.05; α6GBSβ3δGBS, 47 ± 14 pA, n = 6, p < 0.001). Thus, we focused on analyzing the IPSCs by α6β3δGBS GABA-A-Rs. As shown in Fig. 8E-F, α6β3δGBS receptor-mediated IPSCs had significantly faster rise phase compared to those mediated by α6β3δ receptors (α6β3δGBS, T20-80%Rise = 13.9 ± 1.4 ms, n = 8; α6β3δ, T20-80%Rise = 21.9 ± 2.6 ms, n = 13, p < 0.05). Interestingly, coexpression of gephyrin with α6β3δGBS-Rs did not shorten the IPSC rise time (Fig. 8F; α6δGBS+gephyrin, T20-80%Rise = 12.1 ± 1.5 ms, n = 10, p > 0.4 comparing to α6β3δGBS). Similarly, further addition of collybistin (CB3SH3, or CB3SH3+, (41)) resulted in sIPSCs with rising time similar to that of α6β3δGBS alone (Fig. 8F; α6β3δGBS+gephyrin +CB3SH3, vs. α6β3δGBS, p > 0.3; α6β3δGBS+gephyrin +CB3SH3+, vs. α6β3δGBS, p > 0.6). These results suggest that insertion of the gephyrin binding domain brought the α6β3δGBS-receptors closer to postsynaptic sites, possibly through the interaction with endogenous gephyrin in HEK cells (Suppl. Fig. 1). Our kinetics analysis revealed that the α6β3δGBS-Rs (coexpressed with gephyrin and CB3SH3) responded to GABA application at a rate similar to α6β3δ-Rs (Fig. 4 F, G; α6β3δGBS, T20-80%Rise = 2.0 ± 0.3 ms, n = 7, p > 0.4), indicating that the insertion of GBS did not change the receptor kinetics.

The targeting of α6GBS and δGBS subunits was further analyzed in neurons cotransfected with α6GBS, β3 and δGBS subunits. Transfected neurons were double immuno-labeled to visualize the colocalization of the δ subunit and gephyrin, or the δ subunit and GAD. As control, neurons transfected with α6β3δ or α2β3γ2 were also examined. We found that δ subunit-containing receptors were diffusely localized throughout the neuronal membrane surface, without obvious enrichment at synaptic sites as opposed to GAD-labeled presynaptic terminals (Fig. 9A). By contrast, the immunostaining of the γ2 subunit revealed punctate labeling along the dendrites, with many clusters juxtaposed to GAD puncta (Fig. 9B). Intriguingly, neurons...
DISCUSSION

In this study, we demonstrate that different subtypes of GABA<sub>A</sub>-Rs are distinctly targeted to synaptic and extrasynaptic sites in neuron-HEK cell hetero-synapses. With this unique synapse model, we found that α2 and α6 subunits target the same δ/γ2 chimeric subunit to synaptic and extrasynaptic sites, respectively, suggesting a direct role of α subunits in GABA<sub>A</sub>-R targeting. Furthermore, forced interaction of the α6 or δ subunit with gephyrin can recruit normally extrasynaptic α6β3δ receptors closer to synaptic sites, suggesting that gephyrin can stabilize any interactive GABA<sub>A</sub>-Rs at synaptic sites. Figure 10 is a schematic diagram illustrating the relative subcellular localizations of different subtypes of GABA<sub>A</sub>-Rs investigated in this study. Importantly, the intermediate rise and decay phases of α6β3- and α6β3γ2-IPSCs suggest that these receptors are most likely localized at persynaptic sites, different from the synaptic α2β3γ2 or extrasynaptic α6β3δ receptors. Such distinct IPSC events with graded changes of rise and decay phases are difficult to distinguish in neurons, underscoring the advantage of our model synapses in pinpointing the precise targeting mechanisms of specific subtype receptors.

Molecularly engineered synapses as a model system to study receptor targeting - The heterococulture system is often used to study synaptogenesis induced by cell adhesion molecules, such as neuroligins, SynCAM, netrin-G ligand, and LRRTM (39,55-61). We have previously shown that functional GABAergic synapses can be formed in HEK 293T cells by coexpressing NL2 and α2β3γ2 GABA<sub>A</sub>-Rs (34). Here, we further developed the hetero-synapses as a model system to study GABA<sub>A</sub>-R targeting. The advantage of this system is the precise control of the expression of specific receptor subtypes, avoiding the complexity of GABA<sub>A</sub>-Rs in neurons. For example, if a neuron contains both α2β3γ2 and α6β3γ2 receptors, it will be difficult to know whether recorded IPSCs are mediated by α2β3γ2 or α6β3γ2 receptors, or both. Our model synapses offer clear distinction between synaptic events mediated by α2β3γ2 and α6β3γ2 receptors (Fig. 3), providing an important research tool for future studies on different subtypes of receptors.

Furthermore, we have recently demonstrated that such model system is useful for the screening of human disease-related gene mutations by coexpressing GABA<sub>A</sub>-Rs with wildtype or mutant NL2 identified from schizophrenia patients (62). Our previous and current studies suggest that molecularly engineered hetero-synapses are a versatile model system that can be used to study not only synaptogenesis but also receptor targeting and functional deficits of gene mutations.

α subunits are sufficient to target GABA<sub>A</sub>-Rs - Previous studies suggest that γ2 subunit-containing GABA<sub>A</sub>-Rs are mainly concentrated at postsynaptic sites (28-30), whereas δ subunit-containing GABA<sub>A</sub>-Rs are mostly distributed in extrasynaptic membranes (2,5,7,8,31,63). Based on the present analyses of δ/γ2 chimeras, it seems that there is no single domain in the δ subunit responsible for the slow IPSC kinetics, because the rise phases became increasingly slower with chimeras containing a greater portion of the δ subunit (Fig. 6B). As for the role of different α subunits, recent studies found that targeted deletion of α1, α2, or α3 subunit abolishes γ2-containing postsynaptic receptor clusters in selective subcellular regions (24-27). On the other hand, deletion of α4, α5, or α6 subunit greatly reduced tonic currents, suggesting an extrasynaptic localization (64-66). These knockout experiments suggest that the α subunit is required for functional assembly of synaptic (α1-3) and extrasynaptic (α4-6) GABA<sub>A</sub>-Rs, but they did not address whether the α subunit is involved in receptor targeting.

In the present work, we directly investigated the role of α2 and α6 subunits in GABA<sub>A</sub>-R targeting. We first observed slower rise phase of α6β3γ2-IPSCs than that of α2β3γ2-IPSCs. Similarly, α6β3-IPSCs were also slower than α2β3-IPSCs, a clear indication of differential functions of the two α subunits. The direct role of α subunit in receptor targeting was discovered by co-assembling with a series of δ/γ2 chimeras. We demonstrated that when combined with the α2 subunit, the δ/γ2 chimeras always mediated fast IPSCs, similar to that mediated by synaptic γ2-containing receptors; but when combined with the α6 subunit, the same δ/γ2
chimeras always mediated very slow IPSCs, reminiscent of that by extrasynaptic δ-containing receptors (Fig. 6). Because the α2 and α6 subunits do not affect the onset kinetics of GABA_{A}-Rs (Fig. 4), the drastic difference in IPSC rise phase likely reflects the difference in receptor localization. Thus, the same δ/γ2 chimera can be targeted to either synaptic or extrasynaptic membrane, depending on which α subunit it is co-assembled with. These experiments suggest that different α subunits directly play a targeting role in guiding GABA_{A}-Rs to synaptic vs. extrasynaptic sites.

**Gephyrin and GABA_{A}-R targeting** - Synaptic GABA_{A}-Rs are thought to be first inserted to extrasynaptic membranes and then laterally diffuse into postsynaptic sites, where they are stabilized by the scaffolding protein complex (40,50,67,68). Both knockout and knockdown of gephyrin expression disrupted the clustering of a major subset of synaptic GABA_{A}-Rs and result in decreased GABAergic neurotransmission (28,40,50,69,70). On the other hand, not all GABA_{A}-R clusters are dependent on gephyrin overexpression (72). We showed that the interaction between GABA_{A}-Rs and result in decreased GABAergic neurotransmission (28,40,50,69,70). On the other hand, not all GABA_{A}-R clusters are dependent on gephyrin overexpression (72). We showed that the interaction between GABA_{A}-Rs and gephyrin is much weaker than that between GABA_{A}-Rs and gephyrin (70,71). For example, α1-containing receptors in pyramidal neurons are likely stabilized by dystrophin–glycoprotein complex (27).

The α1-3 subunits, but not the α6 subunit, have been shown to directly bind with gephyrin through their large intracellular loop (IL) (48,52,53). By swapping the IL domain between α2 and α6 subunits, we generated α2α6IL and α6α2IL chimeras to test their targeting role. However, the α6α2ILβ3γ2-IPSCs did not show faster kinetics, but rather slightly slower than the IPSCs mediated by α6-containing receptors (Fig. 7). Thus, the α2 IL domain alone is not sufficient for the synaptic targeting of GABA_{A}-Rs. In agreement with our finding, a recent study showed that the interaction between GABA_{A}-R α2 subunit and gephyrin is much weaker than that between GlyR β subunit and gephyrin (54).

We hypothesized that the extrasynaptic localization of αδβδ receptors is due to the lack of interaction with gephyrin. To test this hypothesis, we inserted a high affinity gephyrin-binding site into the IL domain of α6 and δ subunits to force an interaction with gephyrin (38,72). We showed that α6βδEGBS IPSCs in HEK cells (with or without gephyrin overexpression) have faster kinetic properties than α6βδ IPSCs, suggesting that the δEGBS subunit-containing receptors are localized closer to synaptic sites than native δ subunit-containing receptors (Fig. 8). Furthermore, immunostaining in neurons demonstrated that αδEGBSβδEGBS receptors form clusters that colocalized with gephyrin and GAD at synapses (Fig. 9). These results suggest that forced interaction with gephyrin is capable of bringing extrasynaptic αδβδ receptors close to synaptic sites.

To our surprise, gephyrin or collybistin coexpression was not required for the δEGBS-Rs to mediate faster IPSC events (Fig. 8F). We hypothesize that endogenous gephyrin in HEK cells is sufficient to interact with δEGBS and target the receptors closer to synaptic membranes. Indeed, we found that a subpopulation of HEK cells expressed a high level of gephyrin while the rest showed a low level of expression. Interestingly, the HEK cells expressing high levels of gephyrin usually showed compact chromatin structures as revealed by DAPI staining (Suppl. Fig. 1). Since gephyrin is a microtubule-binding protein, we suspect that such high level of expression might indicate a potential role of gephyrin during cell division, which is worth of future study but beyond the scope of this work.

Besides GABA_{A}-Rs, recent studies suggest that collybistin and NL2 also interact with gephyrin (1). NL2 has been suggested to interact with gephyrin and collybistin to target GABA_{A}-Rs to perisomatic membranes (73). NL2 overexpression may also change GABA_{A}-R subunit composition as shown in cerebellar granule cells (74). Collybistin can facilitate gephyrin localization to submembrane sites (75) and increase synaptic GABA_{A}-R accumulation (76). Collybistin deficiency results in region-specific loss of gephyrin and a subset of GABA_{A}-Rs, as well as altered synaptic plasticity and increased levels of anxiety (77,78). Moreover, collybistin and gephyrin may form a complex that is particularly important for interaction with the δ subunit (79). In the present study, we have coexpressed collybistin (CB3SH3- or CB3SH3-) with αδβδEGBS and gephyrin as well as NL2 in HEK cells. Interestingly, GABA current amplitudes were increased by collybistin (data not shown), but the IPSC kinetics were not changed. This may suggest that collybistin contributes to GABA_{A}-R trafficking to the membrane surface but does not affect receptor localization.

**Conclusion** - Our studies suggest that different GABA_{A}-R subunits encode intrinsic targeting information, and the subcellular localization of a particular subtype of receptor is determined by the integral effect of not only the γ2 and δ subunits, but
also different α subunits (e.g. α2 versus α6 subunit). Thus, α subunits not only are required for the assembly of functional receptors but also carry a direct targeting signal for subcellular localization.

Our hetero-synapse system provides a unique model for further studying the targeting mechanisms of GABA_A receptors with a variety of subunit partnership.

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REFERENCES
FIGURE LEGENDS

FIGURE 1. Recombinant GABA<sub>A</sub>-Rs with distinct pharmacological properties. A, Comparable expression level of α2β3γ2 and αδβδ-receptors on HEK cell membranes, revealed by surface staining without permeabilization. B-D, Pharmacological responses of HEK 293T cells coexpressing α6, β3 and δ subunits. B, Representative trace showing the whole-cell GABA (100 μM) current in a HEK 293T cell transfected with αδβδ. C, Positive modulation of GABA-induced current by a neurosteroid THDOC (100 nM). D, THIP (100 μM) acts as a super agonist on αδβδ GABA<sub>A</sub>-Rs. E-G, Pharmacological responses of HEK 293T cells coexpressing α2, β3 and γ2 subunits. E, Representative trace showing whole-cell GABA (100 μM) current in a α2β3γ2 transfected HEK 293T cell. F, GABA induces a smaller whole-cell current in the presence of THDOC (100 nM). G, THIP acts as a partial agonist for the α2β3γ2-GABA<sub>A</sub>-Rs.

FIGURE 2. GABA<sub>A</sub> receptors with distinct subunit combinations mediate IPSCs in HEK cells. A, 3-D reconstruction of Z-stack confocal images showing the GABAergic nerve terminals (green) on the surface of a NL2-transfected HEK cell (blue). Scale bar 20 μm. B, Whole-cell currents (mean ± S.E.M.) induced by 100 μM GABA in HEK cells expressing α2β3, αδβδ, α2β3γ2, α2β3δ, and αδβδ receptors. C-G, Representative traces showing the IPSCs recorded in HEK cells coexpressing NL2 with (C) α2β3, (D) αδβδ, (E) α2β3γ2, (F) αδβδγ2, or (G) αδβδ GABA<sub>A</sub> receptors. H, Miniature IPSCs recorded from a HEK cell expressing NL2 and αδβδ receptors in the presence of TTX (0.5 μM). Lower panels show the expanded views of the boxed IPSCs from the top traces. I, THDOC (100 nM) increases the amplitude of IPSCs in HEK cells coexpressing αδβδ and NL2. J, Application of BIC (20 μM) reduces the baseline current and the noise level, revealing the tonic current in HEK cells expressing NL2 and αδβδ receptors.

FIGURE 3. Quantitative analysis of the kinetics of IPSCs recorded from HEK293T cells transfected with NL2 and different sets of GABA<sub>A</sub>-R subunits. A, Average trace of IPSCs in a neuron. B-D, Average traces of IPSCs mediated by (B) α2β3γ2, (C) αδβδγ2, or (D) αδβδ receptors. E, Scaled Overlay of IPSCs from A-D, showing the difference in rising and decay phases. F-G, Pooled kinetics data of the IPSCs recorded from neurons and HEK cells expressing α2β3γ2, αδβδγ2, and αδβδ receptors. (F) 20-80% rising time and (G) Weighted time constant (t<sub>weighted</sub>) of sIPSCs. *p < 0.05, ***p < 0.001 (One-way ANOVA followed by Bonferroni’s pairwise comparison).

FIGURE 4. Onset kinetics of recombinant GABA<sub>A</sub>-Rs. A, Schematic diagram illustrating the fast drug application system. Fast GABA application was achieve by starting the GABA perfusion and stopping the flow of bath solution instantaneously. B-F, Representative traces of GABA-induced currents on out-side-out patches excised from transfected HEK cells. B, The rise time of αδβδ-Rs was slower than that of α2β3γ2- and αδβδγ2-Rs. C, No significant difference in the onset kinetics when substituting the α2 with α6 subunit in δ/γ2IL-TM4 chimeric receptors. D-E, Insertion of α2IL into the α6 subunit (α6<sub>α2IL</sub>) did not change the onset kinetics of α6-containing receptors. F, Insertion of the gephyrin-binding site (GBS) into the δ subunit (δ<sub>GBS</sub>) did not change the onset kinetics of the αδβδ receptors. G, Polled data showing the 20-80% rise time of each recombinant GABA<sub>A</sub>-R. ***p < 0.001 (one-way ANOVA followed by Bonferroni’s pairwise comparison).

FIGURE 5. Ultrastructural localization of αδβδ and α2β3γ2-GABA<sub>A</sub>-Rs in the hetero-reconstituted synapses. A-B, Transfected HEK cells in close contact with nerve terminals. The nuclei of HEK cells are cyan-shaded. Nerve terminals containing synaptic vesicles are shown in magenta. A, αδβδ receptors were predominantly localized at extrasynaptic or perisynaptic membranes. Arrows: Silver-enhanced gold particles labeling the δ subunit-containing receptors. B, α2β3γ2 receptors were mainly localized at synaptic sites. Arrowheads: immunogold labeling of the GFP-γ2 subunit. C, Pie graphs showing the percentage of synaptic, perisynaptic, and extrasynaptic labeling in HEK cells expressing αδβδ- or α2β3γ2-receptors.
FIGURE 6. Different α subunits contribute to the distinct kinetics of sIPSCs. A, Schematic representation of a series of δ/γ2 chimeric subunits. IL, intracellular loop; TM, transmembrane domain. B, Average traces of sIPSCs recorded from representative HEK cells expressing γ2 subunits, δ subunits or δ/γ2 chimeras together with either α2 and β3 or α6 and β3 subunits and NL2. C, Pooled data (mean ± S.E.M.) showing the 20 - 80% rise time of IPSCs in HEK cells expressing different subunit combinations. Each chimera showed significantly faster rise phase when paired up with the α2 subunit, comparing to when the α6 subunit was their assembly partner. **p < 0.01, ***p < 0.001, two-tailed t-test. D, Average weighted time constant (τ_weighted) of sIPSCs mediated by different subunit combinations (mean ± S.E.M.). E, GABA-induced current (100 µM) mediated by δ/γ2 chimera-containing receptors in HEK cells.

FIGURE 7. The α2 subunit intracellular domain was not sufficient to determine synaptic receptor targeting. A, schematic diagram indicating the structure of α2α6IL and α6δ2IL chimeras. B, whole cell current induced by 100 µM GABA in HEK cells expressing α2α6ILβ3γ2, α2α6ILβ3δ, α6δ2ILβ3γ2, and α6δ2ILβ3δ receptors. C-D, averaged sIPSC traces recorded from HEK cells expressing α6β3γ2 or α6δ2ILβ3γ2 receptors. E, scaled overlay of α6β3γ2 or α6δ2ILβ3γ2 receptor-mediated IPSCs. F-G, pooled data (mean ± S.E.M.) showing the comparison of the 20-80% rising and τ_weighted of α6β3γ2 and α6δ2ILβ3γ2 receptor-mediated IPSCs. H-I, representative traces showing the averaged sIPSC events from HEK cells expressing (H) α6β3δ and (I) α6δ2ILβ3δ receptors. J, scaled overlay of α6β3δ or α6δ2ILβ3δ receptor-mediated IPSCs. K-L, pooled data comparing the 20-80% rising and τ_weighted of α6β3δ and α6δ2ILβ3δ receptor-mediated IPSCs.

FIGURE 8. The interaction with gephyrin targets α6β3δ-containing GABA_A-Rs to synaptic sites of reconstituted synapses. A, Schematic representation of the δ_GBS and α6δ_GBS chimeras. B, GFP-gephyrin was co-precipitated with the δ_GBS chimera. C, α6β3δ_GBS and α6δ2ILβ3δ receptors were found to colocalize with gephyrin-GFP in big intracellular aggregates when coexpressed in HEK cells, demonstrating the interaction between δ_GBS and α6δ2ILβ3δ subunits and gephyrin; α6β3δ receptors showed no colocalization with gephyrin-GFP. Scale bar, 10 µm. D, Whole cell GABA current in HEK cells expressing the δ_GBS and/or α6_GBS chimeras. E, Sample traces of sIPSCs mediated by α6β3δ or α6β3δ_GBS receptors. The events were scaled to the same amplitude and aligned according to the initial rise time. F, Pooled data of sIPSC rise time in different groups. The α6β3δ_GBS receptor-mediated sIPSCs showed a rise phase significantly faster than that of α6β3δ receptors. Coexpression of gephyrin or gephyrin plus collybistin did not further change the IPSC rise time. *p < 0.05, **p < 0.01, ***p < 0.001 (One-way ANOVA followed by Bonferonni’s pair wise comparison).

FIGURE 9. Incorporation of the gephyrin binding site induces clustering of α6β3δ-receptors at postsynaptic sites in neurons. A, Hypothalamic neurons cotransfected with α6, β3 and δ subunits were double immunolabeled for the δ subunit and GAD. Immunoreactivity for the δ subunit was diffusely localized on the neuronal surface. B, Neurons were cotransfected with α2, β3 and γ2 subunits, followed by GAD and surface γ2 double staining. The γ2 subunit-containing receptors formed puncta along the dendrites, some of which were apposed to GAD puncta. C-D, Neurons transfected with α6_GBS, β3 and δ_GBS subunits were double immunolabeled for the δ_GBS subunit and gephyrin (C) or δ_GBS and GAD (D). The δ_GBS subunit-containing receptors formed clusters in neurons. A portion of the δ_GBS subunit-containing receptors were colocalized with gephyrin clusters (right panels in C) or with GAD puncta (right panels in D), suggesting a synaptic localization.

FIGURE 10. Model for synaptic vs. extrasynaptic GABA_A-R targeting. The α2β3 and α2β3γ2 GABA_A-Rs are clustered at synaptic sites, mediating fast IPSCs. The α6β3δ GABA_A-Rs are localized at extrasynaptic sites, mediating very slow IPSCs, which may be partly due to a lack of binding with gephyrin. Importantly, the α6β3 and α6β3γ2 GABA_A-Rs are likely localized at perisynaptic sites, resulting in IPSC kinetics in-between that of α2β3γ2 and α6β3δ GABA_A-Rs. The α6_GBSβ3δ_GBS GABA_A-Rs are brought closer to synaptic sites.
Fig. 2

A Top View  Side View

B

C α2β3  D α6β3

E α2β3γ2  F α6β3γ2

G α6β3δ  H TTX

I THDOC  J BIC

*Figure 2: Analysis of GABA<sub>A</sub>-Rs targeting. (A) Top and side views of a cell with GAD expression. (B) GABA<sub>A</sub>-P<sub>A</sub> for different GABA<sub>A</sub>-Rs (α2β3, α6β3, α2β3γ2, α6β3γ2, α2β3δ, α6β3δ, α6β3δ, α6β3γ2). (C) Examples of current recordings for α2β3 and α6β3. (D) Examples for α2β3γ2 and α6β3γ2. (E) Examples for α6β3δ. (F) TTX and THDOC treatment effects. (G) BIC application.
Fig. 3

A. Neuron

B. α2β3γ2

C. α6β3γ2

D. α6β3δ

E. Scaled Overlay

F. 20%-80% Rising (ms)

G. τ Weighted (ms)
Fig. 4

A. Schematic diagram showing GABA binding and O-S-O patch.

B. Graph showing GABA response with different αβ3γ2, α6β3γ2, and α6β3δ.

C. Graph showing GABA response with α2β3δ/γ2ILTM4 and α6β3δ/γ2ILTM4.

D. Graph showing GABA response with α6β3γ2 and α6α2ILβ3γ2.

E. Graph showing GABA response with α6β3δ and α6α2ILβ3δ.

F. Graph showing GABA response with α6β3δ and α6β3δGBS.

G. Bar graph showing T_{20-80% Rise} with *** and ns annotations.
Fig. 5

A. δ Neuron

B. γ2 Neuron

C. δ

7.3% Synaptic
12.7% Perisynaptic
80.0% Extrasynaptic

C. γ2

21.0% Synaptic
16.0% Perisynaptic
63.0% Extrasynaptic
Fig. 7
Fig. 9

A δ/GAD

B γ2/GAD

C δ_{GBS}/Geph

D δ_{GBS}/GAD

10 µM
Fig. 10

![Image of a synaptic and extrasynaptic targeting of GABA$_A$-Rs]
GABA<sub>A</sub> receptor alpha subunits play a direct role in synaptic versus extrasynaptic targeting
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