γ-Aminobutyric Acid Type A (GABA$_{A}$) Receptor α Subunits Play a Direct Role in Synaptic Versus Extrasynaptic Targeting*

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Xia Wu, Zheng Wu, Gang Ning, Yao Guo, Rashid Ali, Robert L. Macdonald, Angel L. De Blas, Bernhard Luscher, and Gong Chen

From the Departments of Biology and Biochemistry and Molecular Biology, Huck Institutes of Life Sciences, Pennsylvania State University, University Park, Pennsylvania 16802, the Department of Physiology and Neurobiology, University of Connecticut, Storrs, Connecticut 06269, and the Department of Neurology, Vanderbilt University Medical Center, Nashville, Tennessee 37212

GABA$_{A}$ receptors (GABA$_{A}$-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$_{A}$-Rs. Here, we demonstrate differential function of α2 and α6 subunits in guiding the localization of GABA$_{A}$-Rs. To study the targeting of specific subtypes of GABA$_{A}$-Rs, we used a molecularly engineered GABAergic synapse model to precisely control the GABA$_{A}$-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$_{A}$-Rs, possibly through differential interactions with gephyrin.

Neural inhibition in the brain is mostly mediated by GABA$_{A}$ receptors (GABA$_{A}$-Rs). To date, 19 isoforms of GABA$_{A}$-R subunits have been identified as follows: α1–6, β1–3, γ1–3, δ, ε, θ, π, and ρ1–3 (1, 2). Most GABA$_{A}$-Rs expressed in the brain are composed of two α, two β, and one γ subunits, 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 α1–3, β2–3, and γ2 subunits. Tonic inhibition is mediated by extrasynaptic GABA$_{A}$-Rs typically composed of α4/6 (and possibly α1), β and δ subunits (7–9), as well as α5βγ2 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 δ subunit-containing GABA$_{A}$-Rs. Neurons deficient in the α1 or α2 or α3 subunits showed diminished postsynaptic GABA$_{A}$-R clusters in different subcellular localizations (24–27). The γ2 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 δ subunit-containing GABA$_{A}$-Rs are mainly localized at extrasynaptic membranes (7, 8). Thus, the γ2 and δ subunits have been thought to be involved in the synaptic versus extrasynaptic targeting of GABA$_{A}$-Rs. However, the mostly extrasynaptic α5βγ2 and punctated α1βδ GABA$_{A}$-Rs suggest that γ2 and δ 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 δ-GABA$_{A}$-R targeting. We
demonstrated that in neuron-HEK cell synapses, distinct subunit combinations control GABA_A-R targeting. Electrophysiological as well as immunoelectron microscopic results indicated that in HEK cells, α2β3γ2 receptors cluster at synaptic sites, whereas α6β3δ receptors mainly localize at extrasynaptic membranes. Interestingly, when paired with the same chimeric δ-γ2 subunit, different α subunits (α2 versus α6) dictated synaptic versus extrasynaptic targeting of the corresponding GABA_A-Rs. 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 the cortical tissue of neonatal rat pups (postnatal day 3–5) as described before (32, 33). Briefly, cells dissociated from cortices were plated in 25-cm² flasks for up to a week, during which time astrocytes grew to confluence, whereas nonastrocytic 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 co-cultured 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 1-mm³ pieces, and inserted into the C terminus of the mature polypeptide. The cDNAs encoding the rat GABA_A-R CB3SH3 subunits, whereas the CB3SH3 subunits were cloned into the expression vector pCMVneo (36). The δ/γ2 chimera (M1I and M2e) were constructed using the splice overhang extension method (37). The rat chimeras δ/γ2ILTM4 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 a template in a PCR 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 was amplified by PCR using specific primers containing 5’-Nhel and 3’-Xhol sites and inserted into pCDNA 3.1 to exclude possible modifications of pCDNA3.1 during the previous steps, followed by sequencing.

To construct the α6_2IL chimera, the α6 subunit coding sequence was amplified by PCR from pCMVneo-α6 and inserted into pcDNA3.1+ between BamHI and XbaI sites. The coding sequence of the α2 subunit IL (amino acids 307–391 of the mature polypeptide) 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_6IL chimera, the rat α2 subunit coding sequence was amplified from the α2 construct using HindIII and Apal restriction site-containing primers and inserted into pCDNA3.1+ between HindIII and Apal 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 EcoRI site and an XbaI site just downstream from the α2 subunit IL-coding region. For both constructions, the sequences outside the ILs were not changed.

δ_GBS and α6_GBS 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 the 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 and 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 pCMV neo vector.

The murine HA-tagged NL2A expression vector (pNiceNLG-2, referred to as NL2 in this work) was obtained from Dr. P. Scheiffele (University of Basel) (39). The HA tag was inserted between the signal peptide and the N terminus of the mature protein. The gephyrin-GFP construct encodes human gephyrin with GFP fused to the C terminus of gephyrin (40). The collybistin constructs encode two isoforms of human collybistin: CB3_3e +/hPEM2_3e+, containing the SH3 domain and CB3_3e−/hPEM2_3e− 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-pol-
ished to a resistance of 3–6 megohms. 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 CaCl₂, and 1 mm MgCl₂ (pH 7.3, adjusted with NaOH, ~320 mosM). The pipette solution contained 135 mm KCl, 10 mm HEPES, 2 mm EGTA, 10 mm Tris-phosphocreatine, 4 mm MgATP, 0.5 mm Na₂GTP (pH 7.3, adjusted with KOH, ~300 mosM). Data were acquired using the pCLAMP9 software (Molecular Devices), sampled at 5 kHz, 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 GABA₄-Rs, as indicated by the rapid rise phase of whole-cell GABA and THIP currents in the pharmacological study (Fig. 1). Spontaneous IPSCs were recorded with normal bath perfusion. Spontaneous events were analyzed by MiniAnalysis software (Synaptosoft). The 20–80% rising time and the weight time constant (τ_weighted = (τ₁ × A₁ + τ₂ × A₂)/(A₁ + A₂)) of the IPSCs were analyzed to compare the kinetics of the events. Pooled data were presented as means ± S.E., 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 outside-out patch recording were employed to assess the onset kinetics of GABA₄-Rs composed of different subunits. The ultrafast drug application system (ALA Inc., Long Island, NY) consists of solution reservoirs, manual switching valves, a solenoid-driven four-way pinch valve, and two tubes (inner diameter 500 μm) oriented at 50° for rapid solution exchange (43, 44). One tube contains normal bath solution and the other contains 10 mm GABA to maximally activate GABA₄-Rs. The solution exchange rate was estimated to be within 1 ms (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, six 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 were 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, and 3α,21-dihydroxy-5α-pregnan-20-one (THDOC) were obtained from Sigma. Bicuculline methobromide, 6-cyano-7-nitroquinoxaline-2,3-dione, and THIP hydrochloride were purchased from Tocris (Ellisville, MO). 6-Cyano-7-nitroquinoxaline-2,3-dione 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 immediately before the experiments.

Immunochemistry and Immunoelectron Microscopy—For immunofluorescent stainings, cells were fixed with 4% paraformaldehyde for 12 min and permeabilized with 0.1% Triton X-100 in the blocking solution (PBS with 3% normal goat serum + 2% normal donkey serum) for 30 min 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 h. All antibodies were diluted with the blocking solution. The δ subunit in αδβδ-δ-transfected neurons was labeled before permeabilization (Fig. 4A), and the rest of the stainings were conducted after permeabilization (Figs. 4B and 7, C and G–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 as follows: 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 electron microscopy experiments, HEK cells were co-transfected with the following: 1) NL2, α6, β3, and δ; 2) NL2, α2, β3, and γ2-GFP and co-cultured with hypothalamic neurons for 2 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 min, and incubated in blocking solution (3% normal goat serum plus 2% normal donkey serum in bath solution) for 1 h 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; Nanoprobe, Yaphank, NY)) for 1 h at room temperature, fixed with 1% glutaraldehyde for 20 min, and processed with the HQ silver enhancement kit (Nanoprobe, Yaphank, NY) according to the instructions. After developing with the silver enhancer, the cells were submerged in 2% glutaraldehyde, scraped off from the coverslips, and centrifuged at 8000 relative centrifugal force for 10 min to collect the cells. The pellets were further fixed with 2% glutaraldehyde for 1 h at room temperature before EM processing. The cell pellets were post-fixed in 1% OsO₄ for 1 h. 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, contracted 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 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 GABA₄-Rs was characterized into three categories as follows: 1) synaptic, inside a synapse, and more than 30 nm away from the edges; 2) perisynaptic, less than 30 nm away from the synaptic edges; and 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-GHS together with gephyrin-GFP. Gephyrin-GFP single transfection served as the control. Rabbit anti-δ-Nterm was used for the immunoprecipitation, and rabbit anti-GFP was used in the immunoblotting to detect the gephyrin-GFP.
RESULTS

Distinct Pharmacological Properties of Heterologously Expressed GABA<sub>A</sub>-Rs—Neurons express a broad spectrum of GABA<sub>A</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>A</sub>-Rs (34). When HEK cells were co-cultured 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>A</sub>-Rs and their potential interacting proteins to investigate the targeting mechanism of GABA<sub>A</sub>-Rs.

The α6β3δ receptors were selected in this study because they are known to be present mainly at extrasynaptic sites. We first demonstrated that both α6β3δ and α2β3γ2-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>A</sub>-Rs in HEK cells. Bath application of GABA (100 μM) evoked a significant current in HEK cells expressing α6β3δ receptors (Fig. 1B). Pre-application of the neurosteroid THDOC (100 nM) for 30 s 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>A</sub>-Rs (45, 46). In contrast, THDOC (100 nM) negatively regulated the GABA current mediated by α2β3γ2 receptors (Fig. 1, E 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 α2β3γ2 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 α6β3δ and α2β3γ2 receptors.

Distinct Kinetic Properties of GABAergic Events Mediated by Different Subtypes of GABA<sub>A</sub>-Rs—We previously demonstrated that NL2-transfected HEK cells receive GABAergic innervation from surrounding neurons in the HEK cell neurone co-culture system (34). Orthogonal views of Z-stack confocal images showed GABAergic terminals labeled by GAD staining (green) wrapping 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>A</sub>-Rs. We found that GABA (100 μM) induced large whole-cell currents in HEK cells expressing α2β3, α6β3, α2β3γ2, α6β3γ2, or α6β3δ receptors (Fig. 2B; α2β3, 1158 ± 277 pA, n = 16; α6β3, 1192 ± 295 pA, n = 33; α2β3γ2, 1348 ± 195 pA, n = 13; α6β3γ2, 793 ± 172 pA, n = 9; and α6β3δ, 417 ± 89 pA, n = 16). The large GABA currents coincided with the observation of synaptic like events recorded from the co-cultured HEK cells expressing these receptor subtypes (Fig. 2, C–G). All synaptic events in HEK cells were abolished by bicuculline (20 μM, data not shown), indicating that they were GABAergic IPSCs (34). In contrast, HEK cells expressing α2β3δ subunits showed small whole-cell currents after bath application of 100 μM GABA (Fig. 2B, α2β3δ, 45 ± 23 pA, n = 14), and no IPSCs were detected in these cells (data not shown).

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 with the rapid rising time of IPSCs mediated by the α2β3γ2 receptors (Fig. 2E), the 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, but even in the presence of tetrodotoxin (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 GABA_A receptors in the presence of tetrodotoxin (TTX) (0.5 μM). Lower panels show the expanded views of the boxed IPSCs from the top traces. THDOC (100 nM) increases the amplitude of IPSCs in HEK cells co-expressing α6β3δ and NL2. J, application of bicuculline (BIC) (20 μM) reduces the base-line current and the noise level, revealing the tonic current in HEK cells expressing NL2 and α6β3δ receptors.

We next quantitatively compared the kinetics of IPSCs mediated by different GABA_A-Rs in HEK cells with those of IPSCs recorded from neurons (Fig. 3, A–D). The IPSCs recorded from α2β3γ2-expressing HEK cells showed a slightly yet significantly slower rise phase compared with neuronal IPSCs (Fig. 3, A, B, E, and F; α2β3γ2 τ_{20–80%Rise} = 1.7 ± 0.2 ms, n = 16; neuron τ_{20–80%Rise} = 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_{weighted}) significantly faster than that of neuronal IPSCs (Fig. 3G; neuron τ_{weighted} = 52.2 ± 5.4 ms, n = 9; α2β3γ2 τ_{weighted} = 29.2 ± 2.9 ms, n = 16; p < 0.001). Co-expression of gephyrin with α2β3γ2-Rs in HEK cells did not change the kinetics of IPSCs (T_{20–80%Rise} = 1.9 ± 0.3 ms, n = 9).

FIGURE 2. GABA_A receptors with distinct subunit combinations mediate IPSCs in HEK cells. A, three-dimensional reconstitution of Z-stack confocal images showing the GABAAergic nerve terminals (green) on the surface of an NL2-transfected HEK cell (blue). Scale bar, 20 μm. B, whole-cell currents (means ± S.E.) induced by 100 μM GABA in HEK cells expressing α2β3, α6β3, α2β3γ2, α6β3γ2, α2β3δ, and α6β3δ receptors. C–G, representative traces showing the IPSCs recorded in HEK cells co-expressing NL2 with α2β3 (C), α6β3 (D), α2β3γ2 (E), α6β3γ2 (F), or α6β3δ (G) GABA_A receptors. H, miniature IPSCs recorded from a HEK cell expressing NL2 and α6β3δ receptors in the presence of tetrodotoxin (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 co-expressing α6β3δ and NL2. J, application of bicuculline (BIC) (20 μM) reduces the base-line current and the noise level, revealing the tonic current in HEK cells expressing NL2 and α6β3δ receptors.

FIGURE 3. Quantitative analysis of the kinetics of IPSCs recorded from HEK 293T cells transfected with NL2 and different sets of GABA_A-R subunits. A, average trace of IPSCs in a neuron. B–D, average traces of IPSCs mediated by α2β3γ2 (B), α6β3γ2 (C), or α6β3δ (D) receptors. E, scaled overlay of IPSCs from A–D, showing the difference in rising and decay phases. F and G, pooled kinetics data of the IPSCs recorded from neurons and HEK cells expressing α2β3γ2, α6β3γ2, and α6β3δ receptors. F, 20–80% rising time, and G, weighted time constant (τ_{weighted}) of sIPSCs. *, p < 0.05; ***, p < 0.001 (one-way ANOVA followed by Bonferroni’s pairwise comparison).
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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 supplemental 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–G; T_{20–80% rise} = 21.9 ± 2.6 ms; \( \tau_{\text{weighted}} = 140.6 \pm 12.8 \text{ ms} ; n = 13; p < 0.001 \)). The very slow rise phase was consistent with slow IPSCs previously observed in cerebellar granule cells, which are mediated by α6 subunit-containing GABAA-Rs localized far from GABA release sites (47). Our data suggest that α6β3δ receptors assume an extrasynaptic localization, whereas α2β3γ2 receptors cluster at synaptic sites in the hetero-synapse model.

Interestingly, the rise phase of α6β3δγ2 IPSCs was significantly faster than that of α6β3δ IPSCs, yet significantly slower than that of α2β3γ2 IPSCs (Fig. 3 C, E and F; T_{20–80% rise} = 3.7 ± 0.4 ms, n = 9; α6β3δγ2 versus α6δ3, p < 0.001; α6β3δ versus 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_{20–80% rise} = 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_α-Rs Composed of Different Subunits—We wondered whether the onset kinetics of different receptors might explain such a 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. 4, B and G, T_{20–80% rise} = 1.0 ± 0.2 ms, n = 8), faster than the rise phase of α2β3γ2-mediated IPSCs in HEK cells but comparable with neuronal IPSCs. This result suggests that GABA_α receptors in HEK cells are not clustered as tightly in neuronal cells. The rise phase of α6β3γ2-Rs was indistinguishable from that of α2β3γ2-Rs (Fig. 4, B and G, T_{20–80% rise} = α6β3δ = 1.0 ± 0.2 ms, n = 8; p > 0.9). However, the rise phase of α6β3δ-mediated GABA currents was significantly slower than that of α2β3γ2 or α6β3γ2 receptors (Fig. 4, B and G, T_{20–80% rise} = α6β3δ = 2.3 ± 0.3 ms, n = 10; p < 0.01 for both comparisons, one-way ANOVA followed by Bonferroni’s pairwise comparison), yet it was still an order of magnitude faster than that of α6β3δ-IPSCs in HEK cells. Because the difference in receptor kinetics is too small to explain the drastic 10-fold difference between the rise phase of α2β3γ2 and α6β3δ IPSCs, the slow α6β3δ-IPSCs is likely a result of the extrasynaptic localization of α6β3δ receptors.

Ultrastructural Localization of GABA_α-Rs—We further carried out immunoelectron microscopic studies to reveal the ultrastructural localization of α6β3δ and α2β3γ2 receptors in neuron-HEK cell co-cultures. HEK cells expressing α6β3δ or α2β3γ2 receptors were identified by silver-enhanced gold particles immunolabeling the δ or mGlu2 subunit. Nerve terminals containing synaptic vesicles were found in close contact with HEK cells. Importantly, gold particles immunopositive for δ receptors were localized mostly at extrasynaptic membranes, whereas γ2-positive particles were mainly at synaptic cleft (Fig. 5, A 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, whereas only 12.7 and 7.3% were localized perisynaptically or synaptically (Fig. 5C). For comparison, 6 sections containing 18 synapses from α2β3γ2-expressing HEK cells were assessed. Among 81 γ2-immunoreactive particles analyzed, 63% were synaptic and 16% perisynaptic, with the remaining 21% being extrasynaptic (Fig. 5C). The immuno-EM results confirmed...
that the α6β3δ GABA<sub>A</sub>-Rs are preferentially localized at extrasynaptic membranes in the hetero-synapse model. Together with the kinetics analysis (Figs. 2–4), the IPSC rise phase seems to be a faithful indicator of receptor localization in our hetero-synapse model; fast rise phase indicates synaptic localization, and slow rise phase indicates extrasynaptic or perisynaptic localization.

α2 and α6 Subunits Directly Target Chimeric Receptors to Synaptic and Extrasynaptic Sites—Given the significant difference in the rise phase of α2β3 versus α6β3 IPSCs and α2β3γ2 versus α6β3γ2 IPSCs (Figs. 2 and 3), we hypothesized that α2 and α6 subunits play a distinctive role in receptor targeting. To test this hypothesis, a series of δ/γ2 chimeras were co-expressed with either α2 or α6 subunits, together with β3 subunit and NL2 in HEK cells. Fig. 6A illustrates the domain compositions of the δ/γ2 chimeras. Interestingly, when different δ/γ2 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, gray traces). Importantly, for each individual δ/γ2 chimera, the IPSC rise phase was always slower when it was co-assembled with the α6 compared with the α2 subunit (Fig. 6C and supplemental Table 1). Similarly, the decay phase of the IPSCs mediated by each δ/γ2 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 δ/γ2ILTM4 and δ/γ2 M1i were analyzed as well (Fig. 4, C and G). Interestingly, different α subunits (α2 versus α6) did not change the receptor onset kinetics (α2β3δ/γ2ILTM4, T<sub>20–80%Rise</sub> = 1.8 ± 0.3 ms, n = 8; α6β3δ/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; α6β3δ/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 recep-
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![Figure 7](image)

**FIGURE 7. α2 subunit intracellular domain was not sufficient to determine synaptic receptor targeting.** A, schematic diagram indicating the structure of α2<sub>α6IL</sub> and α6<sub>α2IL</sub> chimeras. B, whole-cell current induced by 100 µM GABA in HEK cells expressing α2<sub>α6IL</sub>β3γ2, α6<sub>α2IL</sub>β3γ2, α6<sub>α2IL</sub>β3δ, and α6<sub>α2IL</sub>β3δ receptors. C and D, averaged sIPSC traces recorded from HEK cells expressing α6β3γ2 or α6β3δ γ2 receptors. E, scaled overlay of α6β3γ2 or α6β3δ γ2 receptor-mediated IPSCS. F and G, pooled data (mean ± S.E.) showing the comparison of the 20–80% rising and τ<sub>weighted</sub> of α6β3γ2 and α6β3δ γ2 receptor-mediated IPSCS. H and I, representative traces showing the averaged sIPSC events from HEK cells expressing α6β3δ (H) and α6<sub>α2IL</sub>β3δ (I) receptors. J, scaled overlay of α6β3δ or α6<sub>α2IL</sub>β3δ receptor-mediated IPSCS. K and L, pooled data comparing the 20–80% rising and τ<sub>weighted</sub> of α6β3δ and α6<sub>α2IL</sub>β3δ receptor-mediated IPSCS. *, p < 0.05; **, p < 0.01.

The IPSCs mediated by α6<sub>α2IL</sub>β3γ2 and α6<sub>α2IL</sub>β3δ receptors were compared with those mediated by receptors containing the wild type α6 subunit (α6β3γ2 and α6β3δ). Unexpectedly, the α6<sub>α2IL</sub>β3γ2 IPSCs showed slower rise and decay phases than wild type α6β3γ2 IPSCs (Fig. 7, F and G, α6<sub>α2IL</sub>β3γ2, T<sub>20–80% Rise</sub> = 0.66 ± 0.7 ms, τ<sub>weighted</sub> = 80.7 ± 9.6 ms, n = 10; α6β3γ2, T<sub>20–80% Rise</sub> = 3.7 ± 0.4 ms, p < 0.01, τ<sub>weighted</sub> = 50.6 ± 5.3 ms, p < 0.05). As for the α6<sub>α2IL</sub>β3δ IPSCS, the rise phase was not different from that of α6β3δ IPSCS (Fig. 7K; α6<sub>α2IL</sub>β3δ, 16.8 ± 2.0 ms, n = 13; α6β3δ, 21.9 ± 2.6 ms, n = 13, p > 0.1), but the decay time constant of α6<sub>α2IL</sub>β3δ IPSCS was increased by 45% compared with that of α6β3δ IPSCS (Fig. 7L; α6<sub>α2IL</sub>β3δ, τ<sub>weighted</sub> = 203.7 ± 22.7 ms, n = 13; α6β3δ, τ<sub>weighted</sub> = 140.6 ± 12.8 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 cannot direct GABA<sub>4</sub>-Rs to synaptic sites.

The onset kinetics of α6<sub>α2IL</sub>-containing receptors was similar to those of receptors containing the wild type α6 subunit (Fig. 4, D, E, and G). The 20–80% rise time of GABA-induced currents was 1.5 ± 0.2 ms for the α6<sub>α2IL</sub>β3γ2-Rs (n = 9, p > 0.1 compared with α6β3γ2-Rs) and 2.2 ± 0.2 ms for the α6<sub>α2IL</sub>β3δ-Rs (n = 9, p > 0.8 compared with α6β3δ-Rs).

**Recruiting α6β3δ GABA<sub>4</sub>-Rs to Synaptic Sites through Forced Interaction with Gephyrin**—Gephyrin is known to cluster at inhibitory synapses, where it stabilizes synaptic GABA<sub>4</sub>-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 α6<sub>GBS</sub> and δ<sub>GBS</sub> chimeras (Fig. 8A). The interaction between δ<sub>GBS</sub> and gephyrin was demonstrated by co-immunoprecipitation assay (Fig. 8B). The δ<sub>GBS</sub> and α6<sub>GBS</sub> 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 α6GBSβ3δ receptors co-localized with gephyrin aggregates, whereas the wild type 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, whereas α6GBSβ3δ and α6GBSβ3δGBS receptors showed a very small GABA-induced current (Fig. 8D; I_{GABA}: δGBS: α6β3δ, 417 ± 89 pA, n = 16; α6β3δGBS, 504 ± 113 pA, n = 20; α6GBSβ3δ, 100 ± 32 pA, n = 6, p < 0.05; α6GBSβ3δGBS, 47 ± 14 pA, n = 6, p < 0.001). Thus, we focused on analyzing the IPSCs mediated by α6β3δ or α6β3δGBS receptors. The IPSCs had a significantly faster rise phase compared with those mediated by α6β3δ receptors (α6β3δGBS, T_{20–80% Rise} = 13.9 ± 1.4 ms, n = 8; α6β3δ, T_{20–80% Rise} = 21.9 ± 2.6 ms, n = 13; p < 0.05). Interestingly, co-expression of gephyrin with α6β3δGBS receptors did not shorten the IPSC rise time (Fig. 8F; α6β3δGBS + gephyrin, T_{20–80% Rise} = 12.1 ± 1.5 ms, n = 10, p > 0.4 compared with α6β3δGBS). Similarly, further addition of collybistin (CB3_{SH3} or CB3_{SH3+} (41)) resulted in sIPSCs with rising time similar to that of α6β3δGBS alone (Fig. 8F, α6β3δGBS + gephyrin + CB3_{SH3} versus α6β3δGBS, p > 0.3; α6β3δGBS + gephyrin + CB3_{SH3+} versus 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 (supplemental Fig. 1). Our kinetics analysis revealed that the α6β3δGBS-Rs (co-expressed with gephyrin and CB3_{SH3+}) responded to GABA application at a rate similar to α6β3δ-Rs (Fig. 4, F and G, δGBS, T_{20–80% Rise} = 2.0 ± 0.3 ms, n = 7, p < 0.04), indicating that the insertion of GBS did not change the receptor kinetics.

The targeting of α6GBS and δGBS subunits was further analyzed in neurons co-transfected with α6, β3, and δGBS subunits. Transfected neurons were double immunolabeled to visualize the co-localization 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 apposed 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 overexpressing δGBS-containing receptors showed punctate staining, which was also co-localized with punctate gephyrin staining (Fig. 9C). More importantly, some of the δGBS-containing puncta were found jux-
tapped to GAD puncta (Fig. 9D), suggesting that these GBS-containing chimeric receptors were recruited to postsynaptic sites in neurons, likely through interaction with gephyrin.

**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. Fig. 10 is a schematic diagram illustrating the relative subcellular localiza-
tions of different subtypes of GABA<sub>α</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 perisynaptic sites, different from the synaptic α2β3γ2 or extrasynaptic α6β3ε 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 hetero-co-culture 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 co-expressing NL2 and αβ3γ2 GABA<sub>α</sub>-Rs (34). Here, we further developed the hetero-synapses as a model system to study GABA<sub>α</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>α</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 a model system is useful for the screening of human disease-related gene mutations by co-expressing GABA<sub>α</sub>-Rs with wild type or mutant NL2 identified from patients with schizophrenia (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>α</sub>-Rs**—Previous studies suggest that γ2 subunit-containing GABA<sub>α</sub>-Rs are mainly concentrated at postsynaptic sites (28–30), whereas δ subunit-containing GABA<sub>α</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). Conversely, deletion of α4, α5, or α6 subunit greatly reduced tonic currents, suggesting an extrasynaptic localization (64–66). These knock-out experiments suggest that the α subunit is required for functional assembly of synaptic (α1–3) and extrasynaptic (α4–6) GABA<sub>α</sub>-Rs, but they did not address whether the α subunit is involved in receptor targeting.

In this work, we directly investigated the role of α2 and α6 subunits in GABA<sub>α</sub>-R targeting. We first observed a 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 the 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<sub>α</sub>-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 the α subunit with which it is co-assembled. These experiments suggest that different α subunits directly play a targeting role in guiding GABA<sub>α</sub>-Rs to synaptic versus extrasynaptic sites.
Gephyrin and GABA<sub>A</sub>-R Targeting—Synaptic GABA<sub>A</sub>-Rs are thought to be first inserted to extrasynaptic membranes and then laterally diffused into postsynaptic sites, where they are stabilized by the scaffolding protein complex (40, 50, 67, 68). Both knock-out and knockdown of gephyrin expression disrupted the clustering of a major subset of synaptic GABA<sub>A</sub>-Rs and resulted in decreased GABAergic neurotransmission (28, 40, 50, 69, 70). However, not all GABA<sub>A</sub>-R clusters are dependent on gephyrin (70, 71). For example, α-1-containing receptors in pyramidal neurons are likely stabilized by the dystrophin-glycoprotein complex (27).

The α1–3 subunits, but not the α6 subunit, have been shown to directly bind with gephyrin through their large IL (48, 52, 53). By swapping the IL domain between α2 and α6 subunits, we generated α2<sub>α6IL</sub> and α6<sub>α2IL</sub> chimeras to test their targeting role. However, the α6<sub>α2IL</sub>β3γ2-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<sub>A</sub>-Rs. In agreement with our finding, a recent study showed that the interaction between GABA<sub>A</sub>-R α2 subunit and gephyrin is much weaker than that between GlyR β subunit and gephyrin (54).

We hypothesized that the extrasynaptic localization of α6β3δ 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 α6β3δ<sub>GRS</sub> IPSCs in HEK cells (with or without gephyrin overexpression) have faster kinetic properties than α6β3δ IPSCs, suggesting that the δ<sub>GRS</sub> subunit-containing receptors are localized closer to synaptic sites than native δ subunit-containing receptors (Fig. 8). Furthermore, immunostaining in neurons demonstrated that α6<sub>GRS</sub>β3<sub>GRS</sub> receptors form clusters that co-localized with gephyrin and GAD at synapses (Fig. 9). These results suggest that forced interaction with gephyrin is capable of bringing extrasynaptic α6β3δ receptors close to synaptic sites.

To our surprise, gephyrin or collybistin co-expression was not required for the δ<sub>GRS</sub>-Rs to mediate faster IPSC events (Fig. 8F). We hypothesize that endogenous gephyrin in HEK cells is sufficient to interact with δ<sub>GRS</sub> and target the receptors closer to synaptic membranes. Indeed, we found that a subpopulation of HEK cells expressed a high level of gephyrin, although 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 (supplemental Fig. 1). Because gephyrin is a microtubule-binding protein, we suspect that such a high level of expression might indicate a potential role of gephyrin during cell division, which is worthy of future study but is beyond the scope of this work.

Besides GABA<sub>A</sub>-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<sub>A</sub>-Rs to perisomatic membranes (73). NL2 overexpression may also change GABA<sub>A</sub>-R subunit composition as shown in cerebellar granule cells (74). Collybistin can facilitate gephyrin localization to submembrane sites (75) and increase synaptic GABA<sub>A</sub>-R accumulation (76). Collybistin deficiency results in region-specific loss of gephyrin and a subset of GABA<sub>A</sub>-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 α2 subunit (79). In this study, we have co-expressed collybistin (CB3<sub>H9251</sub> or CB3<sub>H9254</sub>) with α6β3δ<sub>GRS</sub> 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<sub>A</sub>-R trafficking to the membrane surface but does not affect receptor localization.

Conclusion—Our studies suggest that different GABA<sub>A</sub>-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<sub>A</sub> receptors with a variety of subunit partnership.

REFERENCES

1. Luscher, B., Fuchs, T., and Kilpatrick, C. L. (2011) GABA<sub>A</sub>-R trafficking-mediated plasticity of inhibitory synapses. Neuron 70, 385–409.
2. Jacob, T. C., Moss, S. J., and Jurd, R. (2008) GABA<sub>A</sub>-R trafficking and its role in the dynamic modulation of neuronal inhibition. Nat. Rev. Neurosci. 9, 331–343.
3. Olsen, R. W., and Sieghart, W. (2009) GABA<sub>A</sub>-R subtypes. Subtypes provide diversity of function and pharmacology. Neuropharmacology 56, 141–148.
4. Sigel, E., Baur, R., Bouliveau, N., and Minier, F. (2006) Impact of subunit positioning on GABAA receptor function. Biochem. Soc. Trans. 34, 868–871.
5. Farrant, M., and Nusser, Z. (2005) Variations on an inhibitory theme. Phasic and tonic activation of GABA<sub>A</sub> receptors. Nat. Rev. Neurosci. 6, 215–229.
6. Brickley, S. G., and Mody, I. (2012) Extrasynaptic GABA<sub>A</sub>-R receptors. Their function in the CNS and implications for disease. Neuron 73, 23–34.
7. Nusser, Z., Sieghart, W., and Somogyi, P. (1998) Segregation of different GABA<sub>A</sub> receptors to synaptic and extrasynaptic membranes of cerebellar granule cells. J. Neurosci. 18, 1693–1703.
8. Wei, W., Zhang, N., Peng, Z., Houser, C. R., and Mody, I. (2003) Perisynaptic localization of δ subunit-containing GABA<sub>A</sub>-R receptors and their activation by GABA spillover in the mouse dentate gyrus. J. Neurosci. 23, 10650–10661.
9. Glykys, J., Peng, Z., Chandra, D., Homanics, G. E., Houser, C. R., and Mody, I. (2007) A new naturally occurring GABA<sub>A</sub>-R subunit partner with high sensitivity to ethanol. Nat. Neurosci. 10, 40–48.
10. Serwanski, D. R., Miralles, C. P., Christie, S. B., Mehta, A. K., Li, X., and De Blas, A. L. (2006) Extrasynaptic and non-synaptic localization of GABA<sub>A</sub>-R receptors containing the δ5 subunit in the rat brain. J. Comp. Neurol. 499, 458–470.
11. Swanwick, C. C., Murthy, N. R., Mchelidishvili, Z., Sieghart, W., and Kapur, I. (2006) Development of γ-aminobutyric acid agonist and antagonist receptors in cultured hippocampal neurons. J. Comp. Neurol. 495, 497–510.
12. Brüning, I., Scotti, E., Sidler, C., and Fritschy, J. M. (2002) Intact sorting, targeting, and clustering of γ-aminobutyric acid A receptor subtypes in hippocampal neurons in vitro. J. Comp. Neurol. 443, 43–55.
13. Hamann, M., Rossi, D. J., and Attwell, D. (2002) Tonic and spillover inhibition of granule cells control information flow through cerebellar cortex. Neuron 33, 625–633.
14. Semyanov, A., Walker, M. C., and Kullmann, D. M. (2003) GABA uptake regulates cortical excitability via cell type-specific tonic inhibition. Nat. Neurosci. 6, 484–490.
Synaptic and Extrasynaptic Targeting of GABA_A-Rs

specify distinct desensitization, deactivation, and neurosteroid modulation of GABA_A receptors containing the δ subunit. Neurropharmacology 43, 492–502

37. Nagaya, N., and Macdonald, R. L. (2001) Two γ2L subunit domains confer low Zn^{2+} sensitivity to ternary GABA_A receptors. J. Physiol. 532, 17–30

38. Meyer, G., Kirsch, J., Betz, H., and Langosch, D. (1995) Identification of a gephyrin-binding motif on the glycine receptor β subunit. Neuron 15, 563–572

39. Chih, B., Engelman, H., and Scheiffele, P. (2005) Control of excitatory and inhibitory synapse formation by neuregulins. Science 307, 1324–1328

40. Yu, W., Jiang, M., Miralles, C. P., Li, R. W., Chen, G., and de Blas, A. L. (2007) Gephyrin clustering is required for the stability of GABAergic synapses. Mol. Cell. Neurosci. 36, 484–500

41. Kalscheuer, V. M., Musante, L., Fang, C., Hoffmann, K., Fuchs, C., Carta, E., Deas, E., Venekateswaru, K., Menzel, C., Ullmann, R., Tommerup, N., Dalprà, L., Tschach, A., Selicorni, A., Lüscher, B., Ropers, H. H., Harvey, K., and Harvey, R. J. (2009) A balanced chromosomal translocation disrupting ARHGEF9 is associated with epilepsy, anxiety, aggression, and mental retardation. Hum. Mutat. 30, 61–68

42. Dong, L., Yao, J., Fang, C., Dong, N., Lüscher, B., and Chen, G. (2007) Sequential postsynaptic maturation governs the temporal order of GABAergic and glutamatergic synaptogenesis in rat embryonic cultures. J. Neurosci. 27, 10860–10869

43. Liu, Y., and Dilger, J. P. (1991) Opening rate of acetylcholine receptor channels. Biophys. J. 60, 424–432

44. Andersen, N., Corradi, J., Bartos, M., Sine, S. M., and Bouzat, C. (2011) Functional relationships between agonist-binding sites and coupling regions of homomeric Cys-loop receptors. J. Neurosci. 31, 3662–3669

45. Wohlfarth, K. M., Bianchi, M. T., and Macdonald, R. L. (2002) Enhanced neurosteroid potentiation of ternary GABA_A receptors containing the δ subunit. J. Neurosci. 22, 1541–1549

46. Krosggaard-Larsen, P., Frølund, B., Liljefors, T., and Ebert, B. (2004) GABA_A agonists and partial agonists. THIP (Gaboxadol) as a nonopioid analgesic and a novel type of hypnotic. Biochem. Pharmacol. 68, 1573–1580

47. Rossi, D. I., and Hamann, M. (1998) Spillover-mediated transmission at inhibitory synapses promoted by high affinity α6 subunit GABA_A receptors and glomerular geometry. Neuron 20, 783–795

48. Tretter, V., Jacob, T. C., Mukherjee, J., Fritschi, J. M., Pangalos, M. N., and Moss, S. J. (2008) The clustering of GABA_A receptor subtypes at inhibitory synapses is facilitated via the direct binding of receptor α2 subunits to gephyrin. J. Neurosci. 28, 1356–1365

49. Craig, A. M., Banker, G., Chang, W., McGrath, M. E., and Serpinskaya, A. S. (1996) Clustering of gephyrin at GABAergic but not glutamatergic synapses in cultured rat hippocampal neurons. J. Neurosci. 16, 3166–3177

50. Jackson, A. S. (1996) Clustering of gephyrin at GABAergic but not glutamatergic synapses in cultured rat hippocampal neurons. J. Neurosci. 16, 3166–3177

51. Jacob, T. C., Vogt, P. J., Magnus, C., Saliba, R. S., Ktittler, J. T., Haydon, P. G., and Moss, S. J. (2005) Gephyrin regulates the cell surface dynamics of synaptic GABA_A receptors. J. Neurosci. 25, 10469–10478

52. Sassoé-Pognetto, M., and Fritschy, J. M. (2000) Mini-review. Gephyrin, a postsynaptic density protein involved in GABAergic synapses. Eur. J. Neurosci. 12, 2205–2210

53. Tretter, V., Kerschner, B., Milenkovic, I., Ramdass, S. L., Ramerstorfer, I., Saeipour, L., Mari, H. M., Moss, S. J., Schindelin, H., Harvey, R. J., Sieghart, W., and Harvey, K. (2011) Molecular basis of the γ-amino butyric acid A receptor α3 subunit-null mice. Eur. J. Neurosci. 24, 1307–1315

54. Jiang, M., and Chen, G. (2007) Sequential postsynaptic maturation governs the temporal order of GABAergic and glutamatergic synaptogenesis in rat embryonic cultures. J. Neurosci. 27, 10860–10869

55. Kim, H., Weinberg, R. J., and Kim, E. (2006) NGL family PSD-95-interact-
Synaptic and Extrasynaptic Targeting of GABA\(_\text{A}\)-Rs

Scheiffele, P., Fan, J., Choib, J., Fetter, R., and Serafini, T. (2000) Neuroligin expressed in non-neuronal cells triggers presynaptic development in contacting axons. *Cell* **101**, 657–669

Biederer, T., Sara, Y., Mozhaevva, M., Atasoy, D., Liu, X., Kavalali, E. T., and Südhof, T. C. (2002) SynCAM, a synaptic adhesion molecule that drives synapse assembly. *Science* **297**, 1525–1531

Linhoff, M. W., Laurén, J., Cassidy, R. M., Dobie, F. A., Takahashi, H., Nygaard, H. B., Airaksinen, M. S., Strittmatter, S. M., and Craig, A. M. (2009) An unbiased expression screen for synaptogenic proteins identifies the LRRTM protein family as synaptic organizers. *Neuron* **61**, 734–749

Fu, Z., Washbourne, P., Ortinski, P., and Vicini, S. (2003) Functional excitatory synapses in HEK 293 cells expressing neurilolin and glutamate receptors. *J. Neurophysiol.* **90**, 3950–3957

Graf, E. R., Zhang, X., Jin, S. X., Linhoff, M. W., and Craig, A. M. (2004) Neuroligins mediate GABAergic synapse assembly. *J. Neurosci.* **24**, 207–217

Knevessel, M., Brandstätter, J. H., Laube, B., Stahl, S., Müller, U., and Betz, H. (1999) Loss of postsynaptic GABA\(_{\text{A}}\) receptor clustering in gephyrin-deficient mice. *J. Neurosci.* **19**, 9289–9297

Lévi, S., Logan, S. M., Tovar, K. R., and Craig, A. M. (2004) Gephyrin is critical for glycine receptor clustering but not for the formation of functional GABAergic synapses in hippocampal neurons. *J. Neurosci.* **24**, 3662–3667

Kins, S., Kuhse, J., Laube, B., Betz, H., and Kirsch, J. (1999) Incorporation of a gephyrin-binding motif targets NMDA receptors to gephyrin-rich domains in HEK 293 cells. *Eur. J. Neurosci.* **11**, 740–744

Poulopoulos, A., Aramuni, G., Meyer, G., Soykan, T., Hoon, M., Papadopoulos, T., Zhang, M., Paarmann, I., Fuchs, C., Harvey, K., Jedlicka, P., Schwarzacher, S. W., Betz, H., Harvey, R. J., Brose, N., Zhang, W., and Yavorouez, F. (2009) Neuroligin 2 drives postsynaptic assembly at perisomatic inhibitory synapses through gephyrin and collybistin. *Neuron* **63**, 628–642

Su, Z., and Vicini, S. (2009) Neuroligin-2 accelerates GABAergic synapse maturation in cerebellar granule cells. *Mol. Cell. Neurosci.* **42**, 45–55

Harvey, K., Duguid, I. C., Allerd, M. J., Beatty, S. E., Ward, H., Keep, N. H., Lingenfelter, S. E., Pearce, B. R., Lundgren, J., Owen, M. J., Smart, T. G., Lüscher, B., Rees, M. I., and Harvey, R. J. (2004) The GDP-GTP exchange factor collybistin. An essential determinant of neuronal gephyrin clustering. *J. Neurosci.* **24**, 5816–5826

Chiou, T. T., Bonhomme, B., Jin, H., Miralles, C. P., Xiao, H., Fu, Z., Harvey, R. J., Harvey, K., Vicini, S., and De Blas, A. L. (2011) Differential regulation of the postsynaptic clustering of GABA\(_{\text{A}}\) receptors by collybistin isoforms. *J. Biol. Chem.* **286**, 22456–22468

Papadopoulos, T., Korte, M., Eulenburg, V., Kubota, H., Retiounskaia, M., Harvey, R. J., Harvey, K., O’Sullivan, G. A., Laube, B., Hülsmann, S., Geiger, J. R., and Betz, H. (2007) Impaired GABAergic transmission and altered hippocampal synaptic plasticity in collybistin-deficient mice. *EMBO J.* **26**, 3888–3899

Jedlicka, P., Papadopoulos, T., Deller, T., Betz, H., and Schwarzacher, S. W. (2009) Increased network excitability and impaired induction of long-term potentiation in the dentate gyrus of collybistin-deficient mice in vivo. *Mol. Cell. Neurosci.* **41**, 94–100

Saiepour, L., Fuchs, C., Patrizi, A., Sassoè-Pognetto, M., Harvey, R. J., and Harvey, K. (2010) Complex role of collybistin and gephyrin in GABA\(_{\text{A}}\) receptor clustering. *J. Biol. Chem.* **285**, 29623–29631