The γ-aminobutyric acid type A receptor (GABA\(\alpha\)R) is the target of many depressants, including benzodiazepines, anesthetics, and alcohol. Although the highly prevalent \(\alpha\beta\) \(\gamma\)-aminobutyric acid type A \(\alpha\) subunit mediates the majority of fast synaptic inhibition in the brain, receptors containing \(\delta\) subunits also play a key role, mediating tonic inhibition and the actions of endogenous neurosteroids and alcohol. However, the fundamental properties of \(\delta\)-containing GABA\(\alpha\)Rs, such as subunit stoichiometry, are not well established. To determine subunit stoichiometry of expressed \(\delta\)-containing GABA\(\alpha\)Rs, we inserted the \(\alpha\)-bungarotoxin binding site tag in the \(\alpha\), \(\beta\), and \(\delta\) subunit N termini. An enhanced green fluorescent protein tag was also inserted into the \(\beta\) subunit to shift its molecular weight, allowing us to separate subunits using SDS-PAGE. Tagged \(\alpha\beta\delta\) GABA\(\alpha\)Rs were expressed in HEK293T cells using various ratios of subunit cDNA, and receptor subunit stoichiometry was determined by quantitating fluorescent receptor \(\alpha\)-bungarotoxin bound to each subunit on Western blots of surface immunopurified tagged GABA\(\alpha\)Rs. The results demonstrate that the subunit stoichiometry of \(\alpha\beta\beta\delta\) GABA\(\alpha\)Rs is regulated by the ratio of subunit cDNAs transfected. Increasing the ratio of \(\delta\) subunit cDNA transfected increased \(\delta\) subunit incorporation into surface receptors with a concomitant decrease in \(\beta\) subunit incorporation. Because receptor subunit stoichiometry can directly influence GABA\(\alpha\)R pharmacological and functional properties, considering how the transfection protocols used affect subunit stoichiometry is essential when studying heterologously expressed \(\alpha\beta\beta\delta\) GABA\(\alpha\)Rs. Successful bungarotoxin binding site tagging of GABA\(\alpha\)R subunits is a novel tool with which to accurately quantitate subunit stoichiometry and will be useful for monitoring GABA\(\alpha\)R trafficking in live cells.

The \(\gamma\)-aminobutyric acid type A receptor (GABA\(\alpha\)R) is the main inhibitory ligand-gated ion channel in the brain and is the target for a wide range of drugs, including benzodiazepines, anesthetics, neurosteroids, and barbiturates. The actions of these drugs are dependent on the GABA\(\alpha\)R subunit isoforms present, 16 of which have been identified, including \(\alpha\)-6, \(\beta\)-3, \(\gamma\)-3, \(\delta\), \(\epsilon\), \(\theta\), and \(\pi\). Although the highly prevalent GABA\(\alpha\)R subtype comprised of \(\alpha\), \(\beta\), and \(\gamma\) subunits mediates the majority of fast synaptic inhibition in the brain, \(\alpha\beta\delta\) GABA\(\alpha\)Rs also play a key role, mediating tonic inhibition and the molecular actions of endogenous neuroactive steroids, alcohol, and several anesthetic agents (1).

Heterologous expression of GABA\(\alpha\)R subtypes in cell lines (e.g. HEK293 and Chinese hamster ovary cells) and Xenopus laevis oocytes has been used extensively to study their structural, electrophysiological, and pharmacological properties. Although the subunit stoichiometry of \(\alpha\beta\) GABA\(\alpha\)Rs has been established as 2\(\alpha\):1\(\beta\) using a variety of methods, including using a reporter mutation (2), quantifying antibody bound per subunit (3), fluorescence resonance energy transfer (4), and using concatenated subunits (5, 6), the stoichiometry of \(\delta\)-containing receptors is not as well established. Using atomic force microscopy, the stoichiometry of \(\alpha\beta\delta\) GABA\(\alpha\)Rs expressed in tsA201 cells, was determined to be 2\(\alpha\):2\(\beta\):1\(\delta\) (7), suggesting that the \(\delta\) subunit simply replaces the \(\gamma\) subunit in a pentameric receptor. However, in recent studies using concatenated subunits to express \(\alpha\beta\beta\delta\) GABA\(\alpha\)Rs in X. laevis oocytes, functional receptors with more than one \(\delta\) subunit were observed, and when the subunit stoichiometry was constrained to 2\(\alpha\):2\(\beta\):1\(\delta\), multiple subunit arrangements were capable of forming functional receptors (8, 9).

Generally, cDNA or cRNA ratios of 1:1:5 to 1:1:10 are used to heterologously express \(\alpha\beta\delta\) receptors (8, 10, 11). Interestingly, when the ratio of \(\delta\) cRNA was increased from 1\(\alpha\):1\(\beta\):1\(\delta\) to 1\(\alpha\):1\(\beta\):5\(\delta\) and 1\(\alpha\):1\(\beta\):10\(\delta\), the GABA EC\(_{50}\) values increased, and the Hill slope decreased, suggesting changes in receptor subunit stoichiometry (12). If different ratios of cDNA/cRNA alter subunit stoichiometry of surface-expressed \(\alpha\beta\delta\) GABA\(\alpha\)Rs, this may contribute to the discrepancies in the functional properties (e.g. alcohol sensitivity, GABA EC\(_{50}\) values, maximal GABA currents) of expressed \(\alpha\beta\) GABA\(\alpha\)Rs reported in the literature (13–16). In heteromeric neuronal nicotinic acetylcholine receptors, varying the transfection ratios of \(\alpha\) and \(\beta\) subunits results in receptors with alternate stoichiometries of 3\(\alpha\):2\(\beta\) or 2\(\alpha\):3\(\beta\) (17). Similarly, the stoichiometry of recombinant heteromeric P2X receptors is regulated by subunit transfection ratios (18).

Here, we examined whether the ratio of subunit cDNA in a transfection influences the subunit stoichiometry of cell surface \(\alpha\beta\delta\) GABA\(\alpha\)Rs expressed in HEK293T cells. We inserted a 13-amino acid sequence that encodes for a high affinity \(\alpha\)-bunga-
Subunit Stoichiometry of $\alpha_4\beta_2\delta$ GABA$_A$Rs

garotoxin binding site (BBS) into the $\alpha_4$, $\beta_2$, and $\delta$ subunits (see Fig. 1). This sequence comes from the nicotinic acetylcholine receptor, where $\alpha$-bungarotoxin (\(\alpha\)-BTX) is a native antagonist. The BBS tag has been successfully engineered into GABA$_A$ receptors (19) and other proteins, including GABA$_B$ receptors (20) and ionotropic glutamate receptor, (21) and used to monitor receptor trafficking. BBS-tagged $\alpha_4\beta_2\delta$ GABA$_A$Rs were heterologously expressed in HEK293T cells using a variety of subunit cDNA ratios. Receptor subunit stoichiometry was determined by immunopurifying surface tagged $\alpha_4\beta_2\delta$ GABA$_A$Rs and quantitating fluorophore-conjugated $\alpha$-BTX binding to each subunit. Our data demonstrate that the subunit stoichiometry of cell surface $\alpha_4\beta_2\delta$ receptors is highly influenced by the ratio of subunit cDNA used in a transfection.

EXPERIMENTAL PROCEDURES

**GABA$_A$ Receptor cDNA Constructs**—cDNAs encoding rat $\alpha_2$, $\alpha_4$, $\beta_2$, and $\delta$ subunit polypeptides including their signal peptides were inserted into the pUNIV vector as described previously (22). The cDNA encoding the BBS tag, WRYYES-SLEEPYPD, was introduced between the third and fourth amino acids similarly in the mature $\beta_2$ subunit. All of the cDNA constructs were verified by double-stranded DNA sequencing.

**Cell Culture and Transfection**—Human embryonic kidney cells (HEK293T), a gift from Vsevolod V. Gurevich (Vanderbilt University), were incubated at 37°C in humidified 5% CO$_2$, 95% air and grown in minimum essential medium with Earle’s salts and l-glutamine (Mediatech, Inc., Manassas, VA) supplemented with 10% fetal bovine serum (HyClone Laboratories, New Brunswick, NJ) and 50 $\mu$g/ml gentamicin (Invitrogen). In general, the cells were transfected with a total of 21 $\mu$g of cDNA/100-mm dish with a variety of different subunit cDNA ratios (as noted in text) using a standard CaHPO$_4$ precipitation method (23) to heterologously express wild type $\alpha_4\beta_2\delta$ receptors, tagged $\alpha_4$BBS$\beta_2$HBS-EGFP$\delta$HBS receptors, tagged $\alpha_4$BBS$\beta_2$HBS-EGFP receptors, or tagged $\alpha_4$BBS$\beta_2$HBS-EGFP receptors. For nontransfected controls, the cells were treated similarly to transfected cells (e.g. medium changes, washes, and incubations), but no CaHPO$_4$ solution or cDNA was added to the cells. For mock transfected controls, the cells were treated similarly to transfected cells, and CaHPO$_4$ solution was added, but the solution contained no cDNA.

**Radioligand Binding Assays**—Approximately 60 h post-transfection, HEK293T cells transfected with either wild type or tagged subunit cDNAs at a 2:1:4 ratio ($\alpha_4:\beta_2:\delta$) were harvested, and membrane homogenates were prepared as described previously (24). 100 $\mu$g of membrane protein was incubated at room temperature for 40 min with a sub-$K_d$ concentration of $[^3]$H]muscimol (30 Ci/mmol; PerkinElmer Life Sciences) in the absence or presence of seven different concentrations of non-radioactive muscimol in a final volume of 250 $\mu$l. The data were fit using a nonlinear least squares method to a one-site competition curve defined by the equation $y = B_{max}/[1 + (x/IC_{50})]$, where $y$ is the total bound $[^3]$H]muscimol in disintegrations/ min, $B_{max}$ is maximal binding, and $x$ is the concentration of displacing drug (Prism v5.02; GraphPad Software, San Diego, CA). Equilibrium dissociation constant values for unlabeled muscimol ($K_d$) were calculated according to the Cheng-Prusoff-Chou equation: $K_d = IC_{50}/[1 + L/K_d]$, where $K_d$ is the equilibrium dissociation constant of the radioligand, and $L$ is the concentration of radioligand (25, 26).

**Determining GABA EC$_{50}$ and Expression of Tagged Receptors in X. laevis Oocytes**—Capped cRNA encoding wild type or tagged $\alpha_4$, $\beta_2$, and $\delta$ subunits in the pUNIV vector was prepared as described previously (27). The oocytes were harvested from X. laevis and prepared as described in Ref. 28 before injection with 27 nl of cRNA mixture (370 pg/ml) in the ratio 1:1:10 ($\alpha_4:\beta_2:\delta$). The oocytes were incubated 5–7 days after injection with cRNA before being used for two-electrode voltage clamp electrophysiological recordings to determine GABA EC$_{50}$ values for either wild type or tagged receptors. For detailed methods regarding oocyte storage, two-electrode voltage clamp and concentration response analysis, see Ref. 27. GABA concentration response data were fit by the following equation: $I = I_{max}/[1 + (EC_{50}/[A^n])]$, where $I$ is the peak response to a given drug concentration, $I_{max}$ is the maximum current amplitude, EC$_{50}$ is the drug concentration that produces a half-maximal response, $[A]$ is drug concentration, and $n$ is the Hill coefficient using Prism v5.02 (GraphPad Software).

**Immunopurification of Surface Receptors**—30–60 h post-transfection, intact cells expressing GABA$_A$Rs were washed with ice-cold PBS (2.7 mM KCl, 1.5 mM KH$_2$PO$_4$, 0.5 mM MgCl$_2$, 137 mM NaCl, and 14 mM Na$_2$HPO$_4$, pH 7.1) and incubated with 1 $\mu$l/ml rabbit polyclonal anti-$\delta$ subunit antibody (Millipore, Billerica, MA) or 0.75 $\mu$g/ml mouse monoclonal anti-$\delta$-GFP antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in PBS for 1 h at 4°C. The cells were washed twice in ice-cold PBS and incubated in 10 mM N-ethylmaleimide in PBS for 15 min at 4°C. The cells were washed three more times in ice-cold PBS and were then solubilized with 1 ml of lysis buffer (1% Triton X-100, 50 mM Tris-Cl, 150 mM NaCl, 140 mM Na$_2$HPO$_4$, pH 7.1) and incubated with 1 $\mu$l/ml rabbit polyclonal anti-$\delta$ subunit antibody (Millipore, Billerica, MA) or 0.75 $\mu$g/ml mouse monoclonal anti-$\delta$-GFP antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in PBS for 1 h at 4°C. The cells were washed twice in ice-cold PBS and incubated in 10 mM N-ethylmaleimide in PBS for 15 min at 4°C. The cells were washed three more times in ice-cold PBS and were then solubilized with 1 ml of lysis buffer (1% Triton X-100, 50 mM Tris-Cl, 150 mM NaCl, 5 mM EDTA, pH 7.5, 10 mM N-ethylmaleimide) supplemented with Complete Protease Inhibitor tablets (Roche Applied Science), scraped into a fresh tube, and passed through a 25-gauge needle three times. The solubilized cells were incubated at 4°C for 3–6 h and were then centrifuged at 10,000 $\times$ g for 10 min at 4°C. A 25-$\mu$l aliquot of lysate supernatant was removed for Western blotting, and the remaining cell lysate labeled with anti-$\delta$ or anti-GFP antibodies was incubated with 120 $\mu$l of 20% slurry of protein A-Sepharose or protein G-Sepharose (Sigma-Aldrich), respectively. The samples were incubated at 4°C while rotating overnight and then centrifuged at 16,000 $\times$ g for 5 min at 4°C. The supernatant was discarded, and the beads were washed six times with 1 ml of buffer (0.5% Triton X-100, 50 mM Tris-Cl, 150 mM NaCl, and 5 mM EDTA, pH 7.5). The wash buffer was completely removed, and surface GABA$_A$R protein was eluted with 30 $\mu$l of 2 $\times$ Laemmli sample buffer (6% SDS, 20% glycerol, 125 mM Tris-Cl, pH 6.8) at room temperature for 1 h with rotation. Dithiothreitol was added to a final concentration of 55 mM as well as 1 $\mu$l of 50× bromphenol blue prior to SDS-PAGE.

**Western Blotting**—The proteins were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (0.45 $\mu$m NitroPure; GE Water and Process Technologies)
for 1 h at 100 V. The blots were incubated overnight at 4 °C in blocking buffer containing 2% nonfat milk in PBST (PBS + 0.1% Tween 20, pH 7.1). The blots were washed in PBST once for 5 min at room temperature and were then incubated in 1 μg/ml α-BTX-Alexa680 (molecular weight = 8,800; Molecular Probes) in PBST, unless noted differently, for 1 h at room temperature in the dark. The blots were kept in the dark for all of the subsequent steps until scanned. The blots were washed with PBST three times for 5 min and then dried on gel blot paper (Whatman, Sanford, ME) and stored at room temperature.

Determining α-BTX-Alexa680 Affinity for BBS-tagged Subunits—The cells were transfected to express GABA<sub>α</sub> receptors that were tagged with BBS on a single subunit (α<sub>BBS</sub>β<sub>2</sub>δ, α<sub>BBS</sub>β<sub>2</sub>β<sub>RS</sub>:EGFP<sub>βRS</sub>δ or α<sub>BBS</sub>β<sub>2</sub>δ<sub>BBS</sub>). Approximately 45 h after transfection, the cells were lysed with buffer (1% Triton X-100, 150 mm NaCl, 5 mm glucose, 20 μM NaEDTA, 10 μM NaEGTA, and 25 mm Tris-HCl, pH 7.4) supplemented with Complete Protease Inhibitor (Roche Applied Science). The cell lysate was passed through a 25-gauge needle three times and then incubated overnight at 4 °C with rotation. The cell lysates were spun down at 1,000 × g for 5 min at 4 °C, and the supernatant was collected. 150 μl of cell lysate supernatant was diluted with 150 μl of 2X Laemmli sample buffer and bromphenol blue and dithiothreitol were added, as described above. Eight 30-μl aliquots of each lysate were run on two 10% SDS-PAGE gels and transferred to nitrocellulose as described above. Each lane on the blot was cut out, washed, and blocked (as described above) and then incubated in varying concentrations of α-BTX-Alexa680 in PBST (ranging from 0.1 to 25 μg/ml) for 1 h at room temperature, rotating. All of the blots were kept in the dark during fluorophore labeling and for the remainder of the experiment. For a loading control, each blot below 30 kDa was determined the apparent affinity (K<sub>d</sub>) of the fluorescence intensity of each tagged subunit band was measured as described above. The fluorescence intensities of the α<sub>BBS</sub>β<sub>2</sub>β<sub>RS</sub>:EGFP<sub>βRS</sub>δ subunit bands in each lane were summed to determine total fluorescence signal associated with the purified receptors in that lane. Because GABA<sub>α</sub>Rs are pentamers, the total fluorescence signal in a lane was divided by 5 to calculate the amount of fluorescence/subunit. The experimentally determined fluorescence intensity for each subunit was divided by the calculated fluorescence/subunit to determine the average subunit stoichiometry of surface GABA<sub>α</sub>Rs.

RESULTS

Characterization of BBS-tagged and EGFP-tagged GABA<sub>α</sub>R Subunits—We inserted a BBS tag in the extracellular N terminus of the α<sub>2</sub>, β<sub>2</sub>, and δ subunits (Fig. 1A) to allow us to measure fluorophore-conjugated α-BTX binding to each of the subunits on a Western blot. We also inserted EGFP (27 kDa) into the N terminus of the β<sub>2</sub> subunit (Fig. 1A) to distinguish it from the δ subunit, because β<sub>2</sub> and δ subunits have approximately the same molecular mass (~50 kDa). Initially, we measured the binding of [3H]muscimol, a GABA binding site agonist, to WT α<sub>2</sub>β<sub>2</sub>δ and tagged α<sub>BBS</sub>β<sub>2</sub>β<sub>RS</sub>:EGFP<sub>βRS</sub>δ GABA<sub>α</sub>Rs to examine whether inserting the BBS tag and EGFP altered α<sub>BBS</sub>β<sub>2</sub>δ receptor expression or function. Muscimol bound with similar affinity to WT α<sub>2</sub>β<sub>2</sub>δ receptors (K<sub>d</sub> = 32 ± 2 nM) and tagged α<sub>BBS</sub>β<sub>2</sub>β<sub>RS</sub>:EGFP<sub>βRS</sub>δ receptors (K<sub>d</sub> = 21 ± 3 nM) (Fig. 1B). In addition, the maximal number of [3H]muscimol binding sites (B<sub>max</sub>) was not significantly different for wild type α<sub>2</sub>β<sub>2</sub>δ receptors and tagged α<sub>BBS</sub>β<sub>2</sub>β<sub>RS</sub>:EGFP<sub>βRS</sub>δ receptors (WT, B<sub>max</sub> = 90.0 ± 0.6 pmol/mg; tagged, B<sub>max</sub> = 0.41 ± 0.3 pmol/mg). The tags also had no effect on the GABA EC<sub>50</sub> values or the maximum GABA-elicited currents (I<sub>max</sub>) as determined using two-electrode voltage clamping of oocytes expressing wild type α<sub>2</sub>β<sub>2</sub>δ or tagged α<sub>BBS</sub>β<sub>2</sub>β<sub>RS</sub>:EGFP<sub>βRS</sub>δ GABA<sub>α</sub>Rs (supplemental Fig. S1; WT, GABA EC<sub>50</sub> = 1.3 ± 0.1 μM (n = 3); tagged, GABA EC<sub>50</sub> = 1.1 ± 0.23 μM (n = 7); means ± S.E.). At 7 days post-injection, I<sub>max</sub> ranged from 2.6 to 3.7 μA for wild type receptors and from 2.0 to 3.6 μA for tagged receptors. The above data indicate that insertion of the BBS tags and EGFP is tolerated and has little effect on α<sub>BBS</sub>β<sub>2</sub>δ GABA<sub>α</sub>R expression, muscimol binding, and GABA-activated functional responses.

α-BTX-Alexa680 Binding to BBS-tagged GABA<sub>α</sub>R Subunits—Western blots of lysates prepared from nontransfected cells are fits of normalized data compiled from all of the experiments for each tagged subunit.

Calculating Surface GABA<sub>α</sub>R Receptor Subunit Stoichiometry—Western blots of surface immunopurified α<sub>BBS</sub>β<sub>2</sub>β<sub>RS</sub>:EGFP<sub>βRS</sub>δ receptors were blocked (as described above) and then incubated in PBST with 1 μg/ml α-BTX-Alexa680 for 1 h at room temperature in the dark. In a few experiments, 5 μg/ml α-BTX-Alexa680 was used to label blots, which had no effect on the receptor subunit stoichiometry calculated. The blots were washed three times for 5 min in PBST and dried between filter papers before scanning with the Odyssey infrared imaging system. The fluorescence intensity of each tagged subunit band was measured as described above. The fluorescence intensities of the α<sub>BBS</sub>β<sub>2</sub>β<sub>RS</sub>:EGFP<sub>βRS</sub>δ subunit bands in each lane were summed to determine total fluorescence signal associated with the purified receptors in that lane. Because GABA<sub>α</sub>Rs are pentamers, the total fluorescence signal in a lane was divided by 5 to calculate the amount of fluorescence/subunit. The experimentally determined fluorescence intensity for each subunit was divided by the calculated fluorescence/subunit to determine the average subunit stoichiometry of surface GABA<sub>α</sub>Rs.

For example, if the measured fluorescence intensity for α<sub>2</sub> = 200, β<sub>2</sub> = 200, and δ = 600, then the total fluorescence signal = 1000, and the calculated fluorescence signal per subunit is 200. Thus, the average subunit stoichiometry = 1α<sub>2</sub>β<sub>2</sub>δ.

Fig. 3 are fits of normalized data compiled from all of the experiments for each tagged subunit.

Subunit Stoichiometry of α<sub>4</sub>β<sub>2</sub>δ GABA<sub>α</sub>Rs
Subunit Stoichiometry of $\alpha_4\beta_2\delta$ GABA$_A$Rs

and cells expressing $\alpha_4\beta_2\delta$ GABA$_A$Rs, in which only one subunit was BSS-tagged, were incubated with $\alpha$-BTX-Alexa680 to test the specificity of its binding. As shown in Fig. 2, $\alpha$-BTX-Alexa680 only labeled the subunits containing the BBS tag, demonstrating that $\alpha$-BTX-Alexa680 binding was specific. The tagged subunits ran at their predicted molecular masses ($\alpha_4$ $^{\text{BBS}}$ $\approx$ 64 kDa, $\beta_2$ $^{\text{BSS:EGFP}}$ $\approx$ 77 kDa, $\delta$ $^{\text{BBS}}$ $\approx$ 51 kDa) and were easily distinguished from one another. The $\delta$ subunit signal contained multiple bands, which reflect varying amounts of glycosylation (data not shown).

Our method for calculating subunit stoichiometry is dependent on $\alpha$-BTX-Alexa680 binding to each subunit with a similar affinity. Western blots of lysates prepared from cells expressing $\alpha_\beta_\delta$ receptors in which only one subunit was tagged (e.g. $\alpha_4$ $^{\text{BBS}}$, $\beta_2$ $^{\text{BSS:EGFP}}$) were incubated in increasing concentrations of $\alpha$-BTX-Alexa680 (Fig. 3A). The fluorescence signals associated with the BBS-tagged subunit were quantitated and fit by nonlinear regression to determine the $\alpha$-BTX-Alexa680 binding affinity ($K_d$) to each tagged subunit (Fig. 3B). The $\alpha$-BTX-Alexa680 binding affinities were not significantly different, demonstrating that $\alpha$-BTX-Alexa680 bound to each BSS-tagged subunit with a similar affinity ($\alpha_4$ $^{\text{BBS}}, \delta$ receptors, $K_d$ = 250 ± 130 nM (n = 3); $\alpha_4$ $^{\text{BBS:EGFP}}, \delta$ receptors, $K_d$ = 190 ± 100 nM (n = 4); $\alpha_4$ $^{\text{BRS}}, \delta$ receptors, $K_d$ = 390 ± 110 nM (n = 3); means ± S.E.).

Analysis of $\alpha_\beta_\delta$ GABA$_A$R Subunit Stoichiometry—When calculating receptor stoichiometry, we needed to avoid including unassembled receptor subunits and/or partially assembled receptors. Thus, we determined the subunit stoichiometry of cell surface GABA$_A$Rs. Moreover, we purified cell surface $\alpha_\beta_\delta$ GABA$_A$Rs using two different antibodies, either a GFP antibody or a $\delta$ subunit antibody. If high levels of $\alpha_4\beta_2$ receptors were contaminating our $\alpha_4\beta_2\delta$ receptor population or if significant amounts of the $\delta$ subunit trafficked to the cell surface alone or as a homo-oligomeric receptor, we would expect to see different subunit stoichiometries for receptors purified using the different antibodies. Initially, we assessed the subunit stoichiometry of surface $\alpha_4$ $^{\text{BBS}}, \beta_2$ $^{\text{BSS:EGFP}}, \delta$ $^{\text{BBS}}$ GABA$_A$Rs using a transfection ratio of 1:1:5 ($\alpha_4\beta_2\delta$), because this is a commonly reported ratio used in the field (8, 10, 11). For $\alpha_4$ $^{\text{BRS}}, \beta_2$ $^{\text{BSS:EGFP}}, \delta$ $^{\text{BBS}}$ GABA$_A$Rs immunoprecipitated using a $\delta$ subunit antibody, the average subunit stoichiometry was calculated to be $0.4_{\alpha_4}, 0.5_{\beta_2}, 4.1_{\delta}$ (Fig. 4A and Table 1). The stoichiometry of anti-GFP-purified receptors was similar: $0.5_{\alpha_4}, 0.5_{\beta_2}, 3.9_{\delta}$ (Fig. 4A and Table 1). The calculated subunit stoichiometries are the average values for the entire pool of surface GABA$_A$Rs, which were expressed and purified. Non-integer values likely reflect surface receptors with mixed stoichiometries, because a single receptor cannot contain half of a subunit.

Regardless of the antibody used to immunoprecipitate the surface receptors, the calculated subunit stoichiometries were not significantly different, indicating that there were few to no $\alpha_4\beta_2$ receptors in our receptor preparations. In addition,
Subunit Stoichiometry of α₄β₂δ GABAₐRs

A

| kDa | 0.1 | 0.5 | 1.0 | 3.0 | 7.0 | 10 | 15 | 25 |
|-----|-----|-----|-----|-----|-----|----|----|----|
| α₄²⁺BBS δ | [α-BTX-Alexa680] (µg/ml) |
| cyclophilin B |

B

- FIGURE 3. α-BTX-Alexa680 binds with similar affinity to the α-BTX binding site tag on α₄β₂ and δ subunits. A, representative Western blot of whole cell lysates from cells expressing α₄²⁺BBS δ receptors. Individual lanes containing equal amounts of cell lysate were incubated in different concentrations of α-BTX-Alexa680, ranging from 0.1 to 25 µg/ml. The endogenous protein cyclophilin B was used as a loading control. δ, normalized α-BTX-Alexa680 fluorescence was fit by nonlinear regression as described under "Experimental Procedures." KD values for α-BTX-Alexa680 binding to each BBS-tagged subunit were calculated for receptors purified with either [α-BTX-Alexa680] ratio = 1:1:5 transfection ratio does not result in detectable amounts of the δ subunit being trafficked to the cell surface alone or as a homo-oligomeric protein because the same average stoichiometry was measured to be 3.2 ± 1.9 δ (Table 1), indicating that the stoichiometry of αβ GABAₐRs is influenced by the subunit isoforms present.

- FIGURE 4. Varying the subunit cDNA ratios used to transfect cells changes the subunit stoichiometry of surface-expressed α₄β₂δ GABAₐRs. Representative Western blots of anti-GFP or anti-δ surface immunoprecipitations (IP) from mock transfected cells (mock) and from cells transfected with α₄²⁺BBS δ or δBBS at a 1:1:5 subunit ratio (A) and 2:1:5 subunit ratio (B). The subunits were visualized using α-BTX-Alexa680. Subunit stoichiometry of surface GABAₐRs was determined by quantitating the amount of α-BTX-Alexa680 bound to each tagged subunit normalized to the total fluorescence signal in each lane as described under "Experimental Procedures." The average receptor subunit stoichiometries for each condition are shown and reported in Table 1. Increasing the ratio of δ subunit cDNA transfected increased δ subunit incorporation into surface receptors. Similar results were obtained regardless of how surface GABAₐRs were immunopurified.

the data suggest that overexpression of the δ subunit using a 1:1:5 transfection ratio does not result in detectable amounts of the δ subunit being trafficked to the cell surface alone or as a homo-oligomeric protein because the same average stoichiometry was calculated for receptors purified with either an antibody to the δ subunit or to the δ subunit. When we altered the cDNA transfection ratio to 2:1:0.5 (αγβδ), the subunit stoichiometry changed to 1.5αγ2.3β1.1δ for anti-GFP-purified receptors and 1.4αγ2.2β1.4δ for anti-δ-purified receptors (Fig. 4B and Table 1), indicating that changing the cDNA transfection ratio alters the subunit stoichiometry of surface-expressed GABAₐRs.

Dependence of Subunit Stoichiometry on the Ratio of Transfected cDNA—To extend our analysis further, we determined the subunit stoichiometry of GABAₐRs that were expressed using nine different cDNA transfection ratios ranging from 2:1:0 to 2:1:5 (αγβδ). As seen in Fig. 5, increasing the ratio of δ cDNA increased the incorporation of the δ subunit into surface α₄β₂δ GABAₐRs almost exclusively at the expense of the β₂ subunit. Again, regardless of the antibody used to immunoprecipitate the surface receptors, the calculated subunit stoichiometries were not significantly different (Fig. 5 and Table 1). Moreover, the total amount of cDNA used in a transfection did not influence subunit stoichiometry. HEK293T cells were transfected with either 21 or 10.5 µg of total cDNA using ratios of 2:1:0.1 and 2:1:0.5 (αγβδ). Regardless of the amount of cDNA transfected, the calculated subunit stoichiometries of surface α₄β₂δ GABAₐRs were not significantly different (supplemental Fig. S2).

Subunit Stoichiometry of α₄β₂ and α₁β₂ Receptors—We also determined the subunit stoichiometry of α₄β₂ and α₁β₂ GABAₐRs. The stoichiometry of α₁β₂ receptors was 2α₁γ3β₂, using cells transfected with a 2:1 (αγβδ) cDNA ratio (Fig. 5 and Table 1). Surprisingly, the subunit stoichiometry is reversed in αβ receptors containing the α₁ subunit. In cells transfected with a 1:1 (αγβδ) cDNA ratio, the subunit stoichiometry was determined to be 3.2α₁γ1.9β₂ (Table 1), indicating that the stoichiometry of αβ GABAₐRs is influenced by the subunit isoforms present.

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JOURNAL OF BIOLOGICAL CHEMISTRY 14191
**DISCUSSION**

Here, we demonstrate that the subunit stoichiometry of heterologously expressed $\alpha_4\beta_2\delta$ GABA$_A$Rs is highly influenced by the ratio of subunit cDNAs used in a transfection. A transfection cDNA ratio of 2:1:0.25 ($\alpha\beta\delta$) yielded GABA$_A$Rs with an average subunit stoichiometry of $2\alpha:2\beta:1\delta$, whereas a 2:1:5 cDNA ratio yielded receptors with an average stoichiometry of $1\alpha:1\beta:3\delta$ (Table 1). As the amount of $\delta$ cDNA transfected increased, $\delta$ subunit incorporation into surface GABA$_A$Rs increased with a concomitant decrease in $\beta_2$ subunit incorporation (Fig. 5C). In addition, we determined that the subunit stoichiometry of recombinant $\alpha\beta$ GABA$_A$Rs was dependent on the $\alpha$ subunit isoform, such that $\alpha_1\beta_2$ GABA$_A$Rs had a stoichiometry of $2\alpha:3\beta$, whereas the stoichiometry $\alpha_1\beta_2$ GABA$_A$Rs was $3\alpha:2\beta$ (Table 1). The ability to alter receptor stoichiometry by subunit transfection ratios has been observed in other related pentameric ligand-gated ion channels including neuronal nicotinic acetylcholine receptors and P2X receptors (17, 18).

Varying the amount and availability of the $\delta$ subunit during GABA$_A$Rs assembly resulted in surface $\alpha_4\beta_2\delta$ GABA$_A$Rs with different amounts of $\delta$ subunit incorporated. One possible reason for this is that the $\delta$ subunit possesses promiscuous assembly properties, similar to the $\epsilon$ subunit (29). Recently, it has been shown for concatenated $\alpha_2\beta_3\delta$ receptors that the $\delta$ subunit can assemble in either of the two $\beta$ subunit positions, in the $\gamma$ subunit position, or in the position of the $\alpha$ subunit between the $\beta$ subunits (9, 30). Our data suggest that for recombinant $\alpha_4\beta_2\delta$ GABA$_A$Rs, the $\delta$ subunit preferentially replaces the $\beta_2$ subunit (Fig. 5C). Phylogenetic analysis of GABA$_A$R subunits suggests that the $\delta$ subunit is more related to $\beta$ subunits than $\alpha$ subunits (31), consistent with its substitution for the $\beta_2$ subunit in our studies.

Our data have important implications when interpreting data from recombinant $\alpha\beta\delta$ GABA$_A$Rs because the transfection protocols used to express these receptors vary between lab groups. For example, alcohol potentiation of GABA-mediated tonic current from $\alpha\beta\delta$ GABA$_A$Rs is routinely observed in whole cell recordings of hippocampal granule cells and cerebellar granule cells (32–34), but it is not reproducibly observed in recombinant systems. Although some studies have reported that GABA-activated currents from recombinant $\alpha\beta\delta$ GABA$_A$Rs are potentiated by low concentrations of...
alcohol (1–30 mm) (13, 14), others have reported that recombinant αβδ receptors are not responsive to alcohol (15, 16).

Numerous groups expressing recombinant αβδ GABA<sub>A</sub>Rs use a greater ratio of δ cDNA or cRNA to ensure adequate δ subunit expression and assembly (8, 10, 11, 35). However, we show that increasing the δ cDNA transformation ratio can dramatically change receptor stoichiometry. Thus, differences in subunit cDNA/cRNA ratios used for transient expression, coupled with the effects of using cDNA/cRNA from different species, vectors, and cell types, likely result in expression of GABA<sub>A</sub>Rs with varying subunit stoichiometries and arrangements, each of which can have different pharmacological and functional properties. This could be one reason for the variability in alcohol-sensitive GABA<sub>A</sub> data between independent groups, particularly if only αβδ GABA<sub>A</sub>Rs with a specific subunit stoichiometry and arrangement are responsive to low concentrations of alcohol.

Functional studies of α<sub>4</sub>β<sub>2</sub>δ GABA<sub>A</sub>Rs expressed in cells using a variety of cDNA ratios are needed to determine which subunit stoichiometries form functional GABA-activated channels and to tease apart the pharmacological and kinetic properties of α<sub>4</sub>β<sub>2</sub>δ GABA<sub>A</sub>Rs with different subunit stoichiometries. Two studies in oocytes have examined the effects of varying the subunit cRNA ratio used to express α<sub>4</sub>β<sub>2</sub>δ receptors. In one study, when the ratio of δ cRNA was increased from 1α1β2:16 to 1α1β2:56 and 1α1β2:108, the GABA EC<sub>50</sub> values increased, and the Hill slope decreased, suggesting changes in receptor subunit stoichiometry (12). However, in another study, no changes in GABA EC<sub>50</sub> values or the sensitivity to zinc block were seen for α<sub>4</sub>β<sub>2</sub>δ GABA<sub>A</sub>Rs expressed using cRNA ratios of 1α1β2:016, 1α1β2:16, 1α1β2:36, or 1α1β2:106 (36). A recent functional study examining α<sub>4</sub>β<sub>2</sub>δ GABA<sub>A</sub>Rs in HEK293 cells reported that maximal GABA-activated peak currents from α<sub>4</sub>β<sub>2</sub>δ GABA<sub>A</sub>Rs were obtained by decreasing the ratio of δ subunit cDNA transfected to 1:1:0.1 (α<sub>4</sub>β<sub>2</sub>δ), suggesting that this transfection ratio was optimum for maximal expression of functional α<sub>4</sub>β<sub>2</sub>δ receptors (37).

The results of our study provide guidelines for heterologously expressing α<sub>4</sub>β<sub>2</sub>δ GABA<sub>A</sub>Rs with a particular average stoichiometry. It is important to note that the use of different vectors, cell types, or especially subunit isoforms, may change the receptor subunit composition. Here, we show that the stoichiometry of αβ GABA<sub>A</sub>Rs is affected by the subunit isoforms present. The stoichiometry of recombinant α<sub>4</sub>β<sub>2</sub>δ GABA<sub>A</sub>Rs was 2α<sub>4</sub>δ:3β<sub>2</sub>, whereas the α<sub>4</sub>β<sub>2</sub>δ GABA<sub>A</sub>Rs stoichiometry was 3α<sub>4</sub>:1β<sub>2</sub> (Table 1). Our previous work, using tandem subunit constructs, also indicated that α<sub>4</sub>β<sub>2</sub> GABA<sub>A</sub>Rs stoichiometry was 3α<sub>4</sub>:2β<sub>2</sub> (6), but others have reported a 2α<sub>4</sub>:3β<sub>2</sub> stoichiometry (38, 39), suggesting that both stoichiometries are possible. Studies examining α<sub>4</sub>β<sub>2</sub>δ GABA<sub>A</sub>Rs subtypes report that the subunit stoichiometry is 3α<sub>4</sub>:2β<sub>2</sub> (40), whereas for α<sub>4</sub>β<sub>2</sub>δ GABA<sub>A</sub>Rs the stoichiometry is 2α<sub>4</sub>:3β<sub>2</sub> (3). Taken together, the data indicate that the subunit composition of recombinant GABA<sub>A</sub>Rs is influenced by the subunit isoforms present.

An important question that arises from our results is which of the different α<sub>4</sub>β<sub>2</sub>δ GABA<sub>A</sub>Rs stoichiometries that are obtained in recombinant systems correspond to those found in native neuronal α<sub>4</sub>β<sub>2</sub>δ GABA<sub>A</sub>Rs. The subunit stoichiometry and subunit arrangement of native α<sub>4</sub>β<sub>2</sub>δ GABA<sub>A</sub>Rs are currently unknown. It is possible that a native alcohol-sensitive α<sub>4</sub>β<sub>2</sub>δ GABA<sub>A</sub>R subtype may have more than one δ subunit incorporated in a receptor pentamer. The subunit composition, subunit stoichiometry and arrangement of native GABA<sub>A</sub>Rs likely depend on subunit availability as well as a variety of factors, including subunit coassembly affinities and the accessory proteins present. Thus, it can be difficult to replicate expression of native type receptors using recombinant expression systems. Nonetheless, recombinant expression remains an invaluable tool for studying GABA<sub>A</sub>Rs and other ligand-gated ion channels. The technique has distinct advantages, including the ability to express receptors in isolation and with mutations that can be probed with a variety of compounds and labels to study the structure and function of a particular residue or region of the receptor. These unique capabilities make recombinant expression a powerful and essential tool. Thus, despite its limitations, improving and understanding the recombinant expression of α<sub>4</sub>β<sub>2</sub>δ GABA<sub>A</sub>Rs remains critical.

We studied α<sub>4</sub>β<sub>2</sub>δ GABA<sub>A</sub>Rs in our experiments, which are expressed predominately in thalamic relay neurons and dentate gyrus granule cells (41–44). Although it is hypothesized and often assumed that the stoichiometry of these receptors is 2α2βδ1δ with a similar arrangement to αβγδ GABA<sub>A</sub>Rs, additional studies are needed to confirm this idea. Given the extrasynaptic localization of δ-containing GABA<sub>A</sub>Rs and their role in mediating tonic inhibition and the effects of volatile anesthetics, neurosteroids, and ethanol, characterizing their structural, physiological, and pharmacological properties remains an important task. Moreover, recent studies have shown that the actions of neurosteroids likely alter the trafficking and expression of δ-containing receptors (45–47). Successful BBS tagging of GABA<sub>A</sub>R subunits is a novel tool with which to accurately quantify subunit/receptor expression and receptor subunit stoichiometry that is suitable for any multisubunit membrane protein. The method will also be useful for monitoring the trafficking of GABA<sub>A</sub>Rs in live cells.

REFERENCES

1. Belelli, D., Harrison, N. L., Maguire, J., MacDonald, R. L., Walker, M. C., and Cope, D. W. (2009) J. Neurosci. 29, 12757–12763
2. Chang, Y., Wang, R., Barot, S., and Weiss, D. S. (1996) J. Neurosci. 16, 5415–5424
3. Trettter, V., Ehya, N., Fuchs, K., and Sieghart, W. (1997) J. Neurosci. 17, 2728–2737
4. Farrar, S. J., Whiting, P. J., Bonnert, T. P., and McKernan, R. M. (1999) J. Biol. Chem. 274, 10100–10104
5. Baumann, S. W., Baur, R., and Sigel, E. (2002) J. Biol. Chem. 277, 46020–46025
6. Boileau, A. J., Pearce, R. A., and Czajkowski, C. (2005) J. Neurosci. 25, 11219–11230
7. Barrera, N. P., Betts, J., You, H., Henderson, R. M., Martin, I. L., Dunn, S. M., and Edwardson, J. M. (2008) Mol. Pharmacol. 73, 960–967
8. Kaur, K. H., Baur, R., and Sigel, E. (2009) J. Biol. Chem. 284, 7889–7896
9. Baur, R., Kaur, K. H., and Sigel, E. (2009) J. Neurochem. 111, 1172–1181
10. Meera, P., Olsen, R. W., Otis, T. S., and Wallner, M. (2009) Neuropharmacology 56, 155–160
11. Keramidas, A., and Harrison, N. L. (2008) J. Gen. Physiol. 131, 163–181
12. You, H., and Dunn, S. M. (2007) J. Neurochem. 103, 1092–1101
13. Sundstrom-Poromaa, I., Smith, D. H., Gong, Q. H., Sabado, T. N., Li, X., Light, A., Wiedmann, M., Williams, K., and Smith, S. S. (2002) Nat. Neu-
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14. Wallner, M., Hanchar, H. J., and Olsen, R. W. (2003) Proc. Natl. Acad. Sci. U.S.A. 100, 15218–15223
15. Borghese, C. M., Störkstovu, S., Ebert, B., Herd, M. B., Belelli, D., Lambert, J. J., Marshall, G., Wafford, K. A., and Harris, R. A. (2006) J. Pharmacol. Exp. Ther. 316, 1360–1368
16. Yamashita, M., Marszalec, W., Yeh, J. Z., and Narahashi, T. (2006) J. Pharmacol. Exp. Ther. 319, 431–438
17. Nelson, M. E., Kuryatov, A., Choi, C. H., Zhou, Y., and Lindstrom, J. (2003) Mol. Pharmacol. 63, 332–341
18. Barrera, N. P., Henderson, R. M., Murrell-Lagnado, R. D., and Edwardson, J. M. (2007) Biophys. J. 93, 505–512
19. Bogdanov, Y., Michels, G., Armstrong-Gold, C., Haydon, P. G., Lindstrom, J., Pangalos, M., and Moss, S. J. (2006) EMBO J. 25, 4381–4389
20. Wilkins, M. E., Li, X., and Smart, T. G. (2008) J. Biol. Chem. 283, 34745–34752
21. Sekine-Aizawa, Y., and Huganir, R. L. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 17114–17119
22. Venkatachalan, S. P., Bushman, J. D., Mercado, J. L., Sancar, F., Christopherson, K. R., and Boileau, A. J. (2007) Pflugers Arch. 454, 155–163
23. Graham, F. L., and van der Eb, A. J. (1973) Virology 54, 536–539
24. Boileau, A. J., Kucken, A. M., Evers, A. R., and Czajkowski, C. (1998) Mol. Pharmacol. 53, 295–303
25. Cheng, Y., and Prusoff, W. H. (1973) Biochem. Pharmacol. 22, 3099–3108
26. Chou, T. (1974) Mol. Pharmacol. 10, 235–247
27. Hanson, S. M., and Czajkowski, C. (2008) J. Neurosci. 28, 3490–3499
28. Boileau, A. J., Evers, A. R., Davis, A. F., and Czajkowski, C. (1999) J. Neurosci. 19, 4847–4854
29. Bollan, K. A., Baur, R., Hales, T. G., Sigel, E., and Connolly, C. N. (2008) Mol. Cell Neurosci. 37, 610–621
30. Sigel, E., Kaur, K. H., Lüscher, B. P., and Baur, R. (2009) Biochem. Soc. Trans. 37, 1338–1342
31. Simon, J., Wakimoto, H., Fujita, N., Lalande, M., and Barnard, E. A. (2004) J. Biol. Chem. 279, 41422–41435
32. Wei, W., Faria, L. C., and Mody, I. (2004) J. Neurosci. 24, 8379–8382
33. Hanchar, H. J., Dodson, P. D., Olsen, R. W., Otis, T. S., and Wallner, M. (2005) Nat. Neurosci. 8, 339–345
34. Fleming, R. L., Wilson, W. A., and Swartzwelder, H. S. (2007) J. Neurophysiol. 97, 3806–3811
35. Smith, S. S., and Gong, Q. H. (2005) J. Physiol. 564, 421–436
36. Borghese, C. M., and Harris, R. A. (2007) Alcohol 41, 155–162
37. E. J. Botzolakis, A. K., Feng, H. J., Gurba, K. N., Tian, M., and Macdonald, R. L. (2007) Society for Neuroscience Annual Meeting Program 441.3, San Diego, CA, November 3–7, 2007
38. Baumann, S. W., Baur, R., and Sigel, E. (2001) J. Biol. Chem. 276, 36275–36280
39. Gondoles, E. B., Bell-Horner, C. L., Dibas, M. I., Huang, R. Q., and Dillon, G. H. (2008) Neurosci. Lett. 431, 184–189
40. Im, W. B., Pregenzer, J. F., Binder, J. A., Dillon, G. H., and Alberts, G. L. (1995) J. Biol. Chem. 270, 26063–26066
41. Wisden, W., Laurie, D. J., Monyer, H., and Seeburg, P. H. (1992) J. Neurosci. 12, 1040–1062
42. Pirker, S., Schwarzer, C., Wiesethaler, A., Sieghart, W., and Sperk, G. (2000) Neuroscience 101, 815–850
43. Chandra, D., Jia, F., Liang, J., Peng, Z., Suryanarayanan, A., Werner, D. F., Spigelman, I., Houser, C. R., Olsen, R. W., Harrison, N. L., and Homanics, G. E. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 15230–15235
44. Herd, M. B., Haythornthwaite, A. R., Rosahl, T. W., Wafford, K. A., Homanics, G. E., Lambert, J. J., and Belelli, D. (2008) J. Physiol. 586, 989–1004
45. Maguire, J., and Mody, I. (2007) J. Neurosci. 27, 2155–2162
46. Shen, H., Gong, Q. H., Yuan, M., and Smith, S. S. (2005) Neuropeharmacology 49, 573–586
47. Shen, H., and Smith, S. S. (2009) Biochem. Soc. Trans. 37, 1378–1384