The subunit architecture of \(\gamma\)-aminobutyric acid, type B (GABA\(_B\)) receptors in situ is largely unknown. The GABA\(_B\) receptor variants, characterized by the constituents GBR1a and GBR1b, were therefore analyzed with regard to their subunit composition as well as their regional and subcellular distribution in situ. The analysis was based on the use of antisera recognizing selectively GBR1a, GBR1b, and GBR2. Following their solubilization, GBR1a and GBR1b were both found by immunoprecipitation to occur as heterodimers associated with GBR2. Furthermore, monomers of GBR1a, GBR1b, or GBR2 were not detectable, suggesting that practically all GABA\(_B\) receptors are heterodimers in situ. Finally, there was no evidence for an association of GBR1a with GBR1b indicating that these two constituents represent two different receptor populations. A size determination of solubilized GABA\(_B\) receptors by sucrose density centrifugation revealed two distinct peaks of which one corresponded to dimeric receptors, and the higher molecular weight peak pointed to the presence of yet unknown receptor-associated proteins. The distribution and relative abundance of GBR2 immunoreactivity corresponded in all brain regions to that of the sum of GBR1a and GBR1b, supporting the view that most if not all GBR1 proteins are associated with GBR2. However, GBR1a was present preferentially at postsynaptic densities, whereas GBR1b may be mainly attributed to presynaptic or extrasynaptic sites. Thus, GBR1a and GBR1b are both associated with GBR2 to form heterodimers at mainly different subcellular locations where they are expected to subserve different functions.

Inhibitory neurotransmission is mainly mediated by \(\gamma\)-aminobutyric acid (GABA)\(^1\) that displays a fast and a slow component. Whereas the fast inhibitory response results from the activation of the postsynaptically localized GABA\(_A\) receptors by triggering the opening of an integral Cl\(^-\) channel, the slow GABA action is mediated by the metabotropic GABA\(_B\) receptors. GABA\(_B\) receptors can be localized pre- and postsynaptically, where they interact via G-proteins with a variety of effector systems (reviewed in Refs. 1 and 2). Postsynaptic GABA\(_B\) receptors activate K\(^+\) channels and regulate adenylyl cyclase. Presynaptic GABA\(_B\) receptors inhibit the release of neurotransmitters by modulation of Ca\(^{2+}\) channels.

Despite the various functions of GABA\(_B\) receptors, molecular cloning initially revealed only two receptor subtypes generated by alternative splicing of a single gene. These variants of the GABA\(_B\) receptor, termed GBR1a and GBR1b, differ solely in their N terminus, where the first 147 residues of GBR1a are replaced by a sequence of 18 different amino acids in GBR1b (3). In situ hybridization histochemistry (3) and immunohistochemistry (4) with probes that did not discriminate between the GBR1a and GBR1b variants suggested that GBR1a and GBR1b represent the vast majority of GABA\(_B\) receptors since their widespread distribution resembled that of GABA\(_B\) receptors detected by autoradiography using radioligands. However, GBR1a and GBR1b expressed in COS-1 cells displayed up to 150-fold lower affinity for agonists compared with native GABA\(_B\) receptors, and coupling of GBR1 to certain effector systems was difficult to demonstrate (3, 5). An explanation for this discrepancy was provided by the observation that GBR1 remained largely trapped in the endoplasmic reticulum when expressed transiently in mammalian cells (6). Recently, a second seven-transmembrane domain component of GABA\(_B\) receptors, GBR2, was identified, which is similar in size to GBR1 and shares about 35% sequence identity (7–11). Upon co-expression in heterologous expression systems GBR1 and GBR2 formed heteromers, which were targeted to the cell surface membrane and displayed robust activation of G-protein-regulated K\(^+\) channels as well as inhibition of forskolin-induced cAMP production with half-maximal effector concentrations similar to those observed for native GABA\(_B\) receptors (7–11). Thus, unlike other G-protein-coupled receptors, functional GABA\(_B\) receptors appear to be heteromers formed from two related seven-transmembrane domain proteins, GBR1 and GBR2. However, there is evidence that also GBR1a and GBR1b alone were able to couple to K\(^+\) channels or adenylyl cyclase, although at low efficiency (3, 12). In addition, GBR2 expressed in HEK 293 cells was found to inhibit forskolin-stimulated cAMP production in the presence of GABA (8). Thus, GABA\(_B\) receptors with monomeric or diverse, yet unknown, heteromorphic structures may occur in vivo. It was therefore tested whether both GBR1a and GBR1b are associated with GBR2 in situ and whether GBR1a and GBR1b may be co-assembled to provide further receptor heterogeneity. In addition, it was analyzed whether the GABA\(_B\) receptor components GBR1a, GBR1b, or GBR2 may also be present as monomers in situ and thereby contribute to further receptor heterogeneity. Finally, the regional and subcellular distributions of GBR1a, GBR1b, and GBR2 were analyzed to detect possible differential localizations, which may point to distinct functions of GABA\(_B\) re-

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‡The abbreviations used are: GABA, \(\gamma\)-aminobutyric acid; GABA\(_A\), \(\gamma\)-aminobutyric acid, type A; GABA\(_B\), \(\gamma\)-aminobutyric acid, type B; PSD, postsynaptic densities; PAGE, polyacrylamide gel electrophoresis; IR, immunoreactivity; SPM, synaptosomal membranes.
Association of GABA<sub>B</sub> Receptor Subunit Proteins in Situ

Receptor subtypes. For this purpose variant-selective antisera were used to immunobiochemically characterize the GBR1α and GBR1β subtypes and their relationship to GBR2. The results indicate that GBR1α and GBR1β represent distinct GABA<sub>B</sub> receptor subtypes, which are targeted to largely different subcellular locations. Both GBR1α and GBR1β exist as heterodimers in combination with GBR2. There was no evidence for the presence of a major population of monomeric receptors. Most remarkably, after solubilization, the two GABA<sub>B</sub> receptor variants were both found to exist in two forms differing in molecular size. Thus, receptor-associated components may contribute further to the heterogeneity of GABA<sub>B</sub> receptors.

**EXPERIMENTAL PROCEDURES**

**Generation of Antibodies**—For production of variant-specific antisera the following peptides were custom-synthesized (ANAWA Trading SA, Switzerland): N-terminal amino acids 83–107 of the GBR1α variant (sequence, C5KSYTLTLENGKVLFTGGLPALDQ), N-terminal amino acids 1–18 of the GBR1β variant (sequence, SHSPLHRPRHPVPPHPHS containing an additional cysteine at the C terminus), and the C-terminal amino acids 922–944 common to both GABA<sub>B</sub> receptor variants (sequence, PDPLLRSDDSGVHLYK, containing an additional cysteine at the N terminus); a partially occurring C terminus (position 13 was exchanged by a serine to allow defined coupling to the carrier protein). The peptides were coupled via the cysteine residue to keyhole limpet hemocyanin and used for immunizing rabbits as described previously (13). All resulting antisera were purified by affinity chromatography using the peptide antigens coupled to diethylaminoethyl-Sepharose 6B as matrix (13).

For detection of GBR2, affinity purified anti-bovine serum albumin (BSA) antisera (22) (GBR2 antisera) was used, which was raised against a glutathione S-transferase fusion protein corresponding to the amino acids 806–907 of GBR2 (for details see Ref. 7).

**Preparation of Brain Membranes and Subcellular Fractionation**—Brain tissue from male Harlan Sprague-Dawley rats (200–250 g) was homogenized in 10 volumes of 5 mM Tris/HCl, pH 7.4, containing 0.32 mM sucrose and centrifuged for 15 min at 100,000 × g. The crude membrane fraction was obtained by centrifugation of the resulting supernatant for 30 min at 17,000 × g. The membranes were washed twice with 50 mM Tris/HCl, pH 7.4, resuspended in the same buffer to give a protein concentration of about 5 mg/ml, and stored at −80 °C.

Subcellular fractionation and centrifugation gradients (2.5–40% sucrose containing 1 mM NaHCO<sub>3</sub>) were done according to Ref. 14. Cortex and hippocampus of adult rats were rapidly dissected and homogenized (10% w/v) in 0.32 M sucrose, 1 mM NaHCO<sub>3</sub>; subjected to osmotic lysis for 60 min on ice followed by centrifugation for 20 min at 48,200 × g resulting in the lysed synaptosomal membrane (LM1) and the cytosolic synaptosomal fraction (LS1).

**Western Blotting**—Aliquots of the crude membranes or subcellular fractions were incubated for 15 min at 60 °C with an equal volume of 125 mM Tris/HCl, pH 6.8, 20% glycerol, 0.002% bromphenol blue, 10% β-mercaptoethanol, 4% SDS and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 7.5% mini-gels (Mini Protean II, Bio-Rad). Proteins were transferred onto polyvinylidene difluoride membranes or nitrocellulose membranes in a semi-dry blotting apparatus (Trans-Blot, Bio-Rad) at 15 V for 60 min using 39 mM glycine, 48 mM Tris, 0.04% SDS as transfer buffer. For immunodetection, the blots were blocked for 1–2 h in TBST (10 mM Tris/HCl, pH 8, 0.15 mM NaCl, 0.05% Tween 20) containing 5% non-fat dry milk (=blocker) at room temperature, followed by incubation with affinity purified primary anti-sera overnight at 4 °C. The blots were washed one time for 10 min with 20 mM Tris, pH 7.5, 60 mM NaCl, 2 mM EDTA, 0.4% SDS, 0.4% Triton X-100, 0.4% deoxycholate and three times with TBST. Incubation with secondary antibodies (horseradish peroxidase-conjugated goat anti-rabbit IgG diluted 1:5000 in TBST, 5% blocker; Promega) was carried out for 1 h at room temperature. Following extensive washing (see above), immunoreactivity was detected by the chemiluminescence method (Western Blot Chemiluminescence Reagent Plus, NEN Life Science Products).

Quantification of immunoreactive bands was performed with a high resolution computer-based image analysis system (MCID M2, Imaging Research, Ontario, Canada). To ensure an analysis in the linear ranges, x-ray films were exposed to Western blots of increasing protein concentrations (2.5–40 μg of protein).

**Solubilization of GABA<sub>B</sub> Receptors**—For solubilization of GABA<sub>B</sub> receptors, crude membranes were thawed and washed once with 10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 200 mg/ml bacitracin, 0.1 mM phenylmethylsulfonyl fluoride, 2.3 mg/liter aprotinin, 1 mM benzamidine, 0.05% phosphatidylcholine and resuspended in the same buffer to give a protein concentration of 5 mg/ml. Following addition of the detergent (either 1% Triton X-100, 0.5% sodium deoxycholate, 2% CHAPS, 2% BigChIP, 2% octyl-β-D-glucopyranoside or RIPA (0.5% sodium deoxycholate), 1% Nonidet P-40, 0.1% SDS; final concentrations), the mixture was incubated for 30 min on ice, and insoluble material was removed by centrifugation for 60 min at 100,000 × g. The supernatant was carefully removed, and the remaining pellet was re-suspended in buffer to the original volume. To test the solubilization efficiency, equal aliquots of the supernatant and pellet were subjected to Western blotting.

**Sucrose Density Gradient Centrifugation**—Linear gradients were prepared from 5 and 20% sucrose in 50 mM Tris/HCl, pH 8.0, 0.02% NaN<sub>3</sub> containing either 0.1% deoxycholate or 0.5% Triton X-100. Deoxycholate extracts of brain membranes (200 μl) were layered on the preformulated gradients (2.5–40% sucrose containing 1 mM NaHCO<sub>3</sub>, 1% sodium deoxycholate) and applied to the gradients run in parallel. The presence of marker proteins in each fraction was determined by SDS-PAGE and by measuring the optical density of the fractions at a wavelength of 280 nm. In addition, the position of GABA<sub>B</sub> receptors was determined by Western blotting using anti-sera against the α1- and α3-subunits (15).

**Histoblotting**—The regional distribution of the GBR1α, GBR1β, and GBR2 proteins in the adult rat brain was analyzed using the histoblot method (17–19). Five-μm thick sections (16 μm) of rat brains were collected onto nitrocellulose membranes (0.45 μm, Bio-Rad) and kept frozen at −30 °C until use. For protein transfer, the frozen nitrocellulose sheets containing the brain sections were moistened with 39 mM glycine, 48 mM Tris, 20% methanol, 2% SDS and incubated for 5 min at room temperature. After 2 h of incubation in TBST, 5% blocker, the blots were washed with TBST and...
incubated overnight with gentle agitation in 0.1 M Tris, pH 6.8, 2% SDS, 0.1 M β-mercaptoethanol at room temperature to remove the brain tissue and denature the proteins bound to the nitrocellulose membranes. Following washing with TBST, histoblots were processed for immunostaining with the GBR1a-, GBR1b-, GBR1a/b-, and GBR2-selective antisera as described for Western blotting.

Cell Culture and Transfection—Human embryonic kidney cells (ATCC CRL 1573, 293 cells) were grown on culture dishes (9 cm) in minimal essential medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum and 1% gentamycin. Cells were transfected with 30 μg of GBR1a or GBR1b variant expression constructs using the calcium phosphate precipitation technique. For harvesting, culture dishes were washed with ice-cold buffer (10 mM Tris/HCl, pH 7.4, 0.32 M sucrose, 5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride) followed by scraping the cells in buffer and centrifugation at 5,000 × g for 10 min. The cell pellets were stored at −80 °C until used. For Western blotting, the cells were thawed, resuspended in 50 mM Tris/HCl, pH 7.4, 150 mM KCl, homogenized, and washed twice with buffer.

RESULTS

Generation and Characterization of Splice Variant-selective Antisera—Affinity purified polyclonal antisera directed against the GABAB receptor splice variants GBR1a and GBR1b were generated using synthetic peptides derived from the N-terminal domain, which differs between the two splice variants (GBR1a-(83–107) and GBR1b-(1–18)). In addition, an antiserum was raised against the C terminus common to both splice variants (GBR1a,b-(922–944)). On Western blots of crude rat brain membranes, the GBR1a-(83–107) antiserum detected a broad protein band of 100 kDa, and the GBR1b-(1–18) antiserum was specific for the GABAB receptors’ splice variants GBR1a and GBR1b and lacked any cross-reactivity. The selectivity of the GBR2 antiserum was described previously (7).

Native GABAB Receptor Subtypes Are Heterodimers—Recent studies on mainly recombinant receptors indicated that fully functional GABAB receptors are heterodimers assembled from GBR1 and GBR2 (7–10). To test whether an association of GBR1 with GBR2 also holds for the native GABAB receptor variants, immunopurification experiments were performed. As a prerequisite, optimal solubilization conditions were determined. Crude brain membranes were treated with different detergents followed by Western blot analysis of both the extract and insoluble material using the GBR1a,b-(922–944) antiserum. Among the detergents tested, the ionic detergent deoxycholate (0.5%) and the detergent mixture RIPA (0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS) solubilized both GABAB receptor variants most efficiently (Fig. 2). All non-ionic and zwiterionic detergents tested (1% Triton X-100, 2% CHAPS, 2% Big-CHAP, and 2% octyl-β-D-glucopyranoside) were considerably less effective in solubilization (Fig. 2). There was no apparent difference in the efficiency of extracting GBR1a compared with GBR1b. Thus, deoxycholate was the detergent of choice for solubilization of both GABAB receptors. Increasing the deoxycholate concentration to 1.5% and the ionic strength to 1 M NaCl did not improve the solubilization efficiency (not shown). Quantification of the amount of solubilized GABAB receptor protein by densitometric analysis of Western blots revealed that 58 ± 5% (n = 6) of the GBR1a variant and 56 ± 5% (n = 6) of the GBR1b variant were solubilized by 0.5% deoxycholate.

The ability of GBR1a and GBR1b to associate with GBR2 was analyzed by immunoprecipitating GABAB receptors from deoxycholate extracts of brain membranes using the GBR2 antiserum. In the well washed immunoprecipitate an IR for both GBR1a and GBR1b was detected on Western blots with the GBR1a,b-(922–944) antisera, whereas no IR was found with the GBR1b-(1–18) antiserum (Fig. 1B). Conversely, in HEK 293 cells expressing the GBR1b variant, a 100-kDa protein was detected with the GBR1b-(1–18) and GBR1a,b-(922–944) antisera, but no IR was observed with the GBR1a-(83–107) antiserum (Fig. 1B). In controls with non-transfected cells none of the antisera showed an IR. Thus, the GBR1a-(83–107) and GBR1b-(1–18) antisera were specific for the GABAB receptors’ splice variants GBR1a and GBR1b and lacked any cross-reactivity. The selectivity of the GBR2 antiserum was described previously (7).
GABAB receptors, in addition to forming heteromers, may also assemble into native forms—

To assess whether the constituent proteins of the molecular forms of GABAB receptors were also detected by immuno precipitation and immunoaffinity purification. A, GABA<sub>B</sub> receptors were immunoprecipitated from deoxycholate extracts of whole rat brain membranes with the GBR2 antiserum followed by Western blot analysis using GBR1a,b-(922–944) and GBR2 antiserum. IR of GBR1a, GBR1b, and GBR2 is indicated by 1a, 1b, and 2, respectively. B, deoxycholate extracts of whole rat brain membranes were applied to a column containing the GBR1b-(1–18) antibodies coupled to protein A-Sepharose. After washing, the eluates were analyzed for the presence of GBR1a, GBR1b, and GBR2 proteins by Western blotting using the GBR1a-(83–107), GBR1a,b-(922–944), GBR1b-(1–18), and GBR2 antiserum. +P indicates co-incubation of the antiserum with 10 µg/ml of the respective peptide antigen. A weak nonspecifically labeled protein band at about 170 kDa was occasionally observed.

Native GABA<sub>B</sub> Receptor Variants Each Occur in Two Molecular Forms—To assess whether the constituent proteins of GABA<sub>B</sub> receptors, in addition to forming heteromers, may also occur as monomers or homo-oligomers, the molecular size of native solubilized receptors was analyzed by sucrose density gradient centrifugation. Proteins of rat brain membranes were extracted with 0.5% deoxycholate and subjected to 5–20% linear sucrose density gradient centrifugation, followed by an analysis of the pattern of distribution of GBR1a and GBR1b and GBR2. Western blotting revealed IR for GBR1a, GBR1b, and GBR2 in the same fractions, pointing to their co-sedimentation. The relative staining intensity of GBR2 roughly corresponded to that of GBR1a and GBR1b IR, in line with the association of GBR1 and GBR2 (Fig. 4). Surprisingly, the two GBR1 proteins and the GBR2 protein were present in two distinct peaks. The first broad peak, containing the majority of GBR1a, GBR1b, and GBR2 proteins, migrated to a position between that of the marker proteins aldolase (158 kDa) and catalase (232 kDa) and overlapped with GABA<sub>A</sub> receptors as marker protein (~250 kDa). The second receptor fraction migrated slightly further into the gradient than the GABA<sub>A</sub> receptor marker, suggesting a molecular size of ~250 kDa. The two molecular forms of GABA<sub>B</sub> receptors were found irrespective of the detergent used. Solubilization of GABA<sub>B</sub> receptors with Triton X-100 (1%) or CHAPS (2%) and inclusion of the respective detergent in the sucrose density gradients resulted likewise in the high and low molecular forms (not shown). Both molecular forms of GABA<sub>B</sub> receptors were also detected by size-exclusion chromatography on a Superose 6HR 10/30 column using a fast liquid chromatography system (Amersham Pharmacia Biotech) (not shown). Most importantly, since GABA<sub>B</sub> receptor IR was not eluted in the void volume of the column, it is unlikely that GABA<sub>B</sub> receptors strongly aggregate nonspecifically under the conditions used. The high molecular form of GABA<sub>B</sub> receptors is therefore not expected to represent aggregated receptor protein.

To demonstrate directly the association of GBR1 with GBR2 in both GABA<sub>B</sub> receptor peaks, GABA<sub>B</sub> receptors were immunoprecipitated from individual fractions taken from the high and low molecular weight forms (Fig. 4B). Immunoprecipitation was performed with the GBR1a,b-(922–944) antiserum, and the precipitate was tested for the presence of GBR1a, GBR1b, and GBR2 by Western blotting. In both, the high and low molecular size fractions GBR1a and GBR1b were co-precipitated with GBR2 (Fig. 4B).

Since in all fractions tested GBR1a and GBR1b were found to be associated with GBR2, it is unlikely that considerable amounts of monomeric GABA<sub>B</sub> receptors are present in brain. However, to rule out the existence of monomeric GABA<sub>B</sub> receptors, quantitative immunoprecipitation experiments were performed. GABA<sub>B</sub> receptor proteins GBR1a, GBR1b, or GBR2 were immunoprecipitated from deoxycholate extracts of brain membranes with the GBR1a,b-(922–944) or GBR2 antiserum, respectively. Following precipitation of the receptor-antibody complex with protein A-agarose the immunoprecipitation was repeated two times with the resulting supernatants to ensure quantitative depletion of either GBR1a and GBR1b or GBR2 by the respective antiserum. Western blot analysis revealed that neither in the GBR1a- and GBR1b-depleted supernatant nor in the GBR2-depleted extract either GBR2 or GBR1a/GBR1b were detectable (Fig. 5). This result indicated that no measurable population of monomeric or homo-oligomeric GBR1a, GBR1b, or GBR2 were present in the brain extracts.

Since GBR1a and GBR1b do not appear to heteromize (Fig. 3), the high molecular weight form of GABA<sub>B</sub> receptors (>250 kDa) identified in the sucrose density gradients is unlikely be due to the existence of multimeric complexes built up of GBR1a, GBR1b, and GBR2. It was therefore tested whether the differences in size of GABA<sub>B</sub> receptors is due to the formation of disulfide bridges, as demonstrated for metabotropic glutamate receptors (20). However, on Western blots performed under non-reducing conditions GBR1 and GBR2 proteins migrate as monomers, arguing against a dimerization via disulfide bridges. Likewise, inclusion of dithiothreitol (10 mM) into the sucrose density gradients did not affect the migration of GBR1a, GBR1b, or GBR2 (not shown). Thus, the high molecular weight forms may either be due to variations in the stoichiometry of the receptor subunits or due to the association of at least some GABA<sub>B</sub> receptors with yet unknown proteins.

Differential Regional Distribution of GBR1a and GBR1b—On Western blots of whole brain membranes staining of the GBR1a variant was consistently weaker than that of the GBR1b variant when the GBR1a,b-(922–944) antiserum was used (Figs. 1 and 2). Since the GBR1a,b-(922–944) antiserum was raised against a peptide sequence that is identical in GBR1a and GBR1b proteins, the GBR1a,b-(922–944) antiserum permitted a direct comparison of the relative abundance of the GBR1a and GBR1b variants in brain tissue. Densitometric analysis of Western blots with increasing protein concentrations of membranes prepared from whole rat brains revealed that GBR1b is 2.1 ± 0.5 (n = 6)-fold more abundant than GBR1a (Fig. 6). Thus, the two GABA<sub>B</sub> receptor subtypes were expected to display a different distribution on the regional and cellular level in the brain. The regional distribution of GBR1a, GBR1b, and GBR2 proteins was analyzed by Western blotting using membranes prepared from seven major brain...
regions. All three GABA<sub>B</sub> receptor proteins displayed a widespread distribution, being present in all brain areas analyzed with the highest relative expression in the cerebral cortex and cerebellum (Fig. 7A). However, the presence of the GBR1 splice variants differed considerably among brain regions. Whereas GBR1a and GBR1b were expressed to a similar extent in the hippocampus, GBR1a predominated in the olfactory bulb and striatum, and GBR1b was more abundant than GBR1a in cerebral cortex, thalamus, cerebellum, and medulla. The staining intensity for GBR2, which appeared to correspond to the sum of GBR1a and GBR1b-IR in the different brain areas, was strongest in cerebral cortex, hippocampal formation, thalamus, and cerebellum, followed by the olfactory bulb, striatum, and medulla (Fig. 7A).

The distribution of GBR1a, GBR1b, and GBR2 was analyzed in more detail in sections of adult rat brain. Since the GBR1a-(83–107) antiserum failed to recognize the GBR1a protein in perfusion-fixed brain tissue processed for immunohistochemistry, the histoblot technique was used (17–19). Parasagittal cryostat-cut sections were blotched onto nitrocellulose membranes, providing a protein image of the section for immunostaining performed according to the protocol of Western blotting.

**Fig. 4. Sucrose density gradient centrifugation of native GABA<sub>B</sub> receptors.** Deoxycholate extracts of rat brain membranes (200 μl) were layered on 5–20% linear sucrose density gradients and centrifuged at 170,000 × g for 14 h at 4°C. A, after fractionation, individual fractions were analyzed for GBR1a and GBR1b IR by Western blotting using the GBR1a,b-(922–944) antiserum. For comparison, the migration of GABA<sub>A</sub> receptors (~250 kDa) was probed with a α3-subunit-specific antiserum. The position of marker proteins (bovine serum albumin, aldolase, and catalase) is indicated on the top. B, the association of GBR1 with GBR2 proteins in the high and low molecular weight peak of GBR1 and GBR2 IR observed in the sucrose density gradients was analyzed in immunoprecipitation experiments. After fractionation, individual fractions were analyzed for GBR2 IR by Western blotting followed by immunoprecipitation of GABA<sub>B</sub> receptors from fractions displaying strong IR (fractions 6–7 and 13–16) using the GBR1a,b-(922–944) antiserum. The resulting immunoprecipitates were analyzed by Western blotting with the GBR1a,b-(922–944) and the GBR2 antiserum.

**Fig. 5. Quantitative immunoprecipitation of GBR1a/GBR1b and GBR2 indicates the lack of monomeric and homo-oligomeric GABA<sub>B</sub> receptors in rat brain.** Crude membranes prepared from whole rat brains (5 mg of protein/ml) were solubilized with 0.5% deoxycholate (final concentration) and centrifuged for 60 min at 100,000 × g, and aliquots of the resulting supernatant were subjected to immunoprecipitation with the GBR1a,b-(922–944) and the GBR2 antiserum. Antigen-antibody complexes were precipitated with protein A-agarose, and the immunoprecipitation was repeated two times with the resulting supernatants using the respective antiserum to ensure quantitative depletion of GBR1a/GBR1b or GBR2. Aliquots of the supernatant after the third cycle of immunoprecipitation and aliquots of the precipitates after the first (A), second (B), and third (C) cycle of immunoprecipitation were analyzed by Western blotting with the GBR1a,b-(922–944) and the GBR2 antiserum. C, controls run in parallel in which the antiserum was replaced by buffer; lane 1, immunoprecipitation with the GBR1a,b-(922–944) antiserum; lane 2, immunoprecipitation with the GBR2 antiserum.
In order to analyze the subcellular distribution of GABAB receptor proteins, homogenates of rat brain were fractionated by differential centrifugation. Western blot analysis of the various cellular subfractions demonstrated a localization of the GABAB receptor proteins GBR1a, GBR1b, and GBR2 consistent with a synaptic membrane localization. All three proteins were enriched in fractions containing synaptic plasma membranes (LP-SPM) (Fig. 8). A potential postsynaptic membrane enrichment (Fig. 7B). The histoblot technique offered very high sensitivity and high signal to noise ratios, making it the method of choice for an analysis of the gross regional distribution of GBR proteins, although lacking cellular resolution. GBR1a-IR was observed throughout the brain with higher than average levels in the external plexiform layer of the olfactory bulb, CA1 region of the hippocampus, superior colliculus, and striatum. GBR1b was distributed as widespread as GBR1a but displayed much larger regional differences in staining intensity. Strongest GBR1b-IR was seen in the thalamus, outer layers of the cerebral cortex, and molecular layer of the cerebellum, whereas moderate staining was observed in the hippocampus and amygdala, and faint staining was observed in the olfactory bulb, striatum, basal ganglia, mesencephalon, pons, and medulla. Interestingly, the sum of GBR1a and GBR1b patterns of distribution and staining intensities, as visualized with the GBR1a,b-(922–944) antiserum, strikingly matched that of GBR2 (Fig. 7B), supporting the view that GBR1a and GBR1b are both associated with GBR2 in all brain areas to form functional GABA<sub>B</sub> receptors.

**Differential Subcellular Distribution of GBR1a and GBR1b Variants**—In order to analyze the subcellular distribution of GBR1a, GBR1b, and GBR2, rat brain tissue was subjected to subcellular fractionation (for details see "Experimental Procedures"), and aliquots (30 μg) of each fraction were analyzed by Western blotting using the GBR1a-(83–107), GBR1a,b-(922–944), GBR1a,b-(922–944), and GBR2 antisera. H, homogenate; P1, nuclear pellet; P2, crude membranes; SPM, synaptic plasma membranes; LP-SPM, lysed synaptic vesicle membranes; LS-SPM, cytosolic synaptic vesicle fractions (also contains synaptic vesicles and membranes of the endoplasmic reticulum and Golgi apparatus); P3, light membrane pellet (contains synaptic vesicles and membranes of the endoplasmic reticulum and Golgi apparatus); S3, cytosolic fraction (devoid of membranes); PSD, postsynaptic densities.

Fig. 6. Relative abundance of the GBR1a and GBR1b variants. Crude rat brain membranes containing increasing protein concentrations (2.5–40 μg) were subjected to Western blotting using the GBR1a,b-(922–944) antiserum (top). Quantification of immunoreactive bands was performed with a high resolution computer-based image analysis system (MCID M2, Imaging Research, Ontario, Canada). The densitometric analysis of the Western blots revealed that the GBR1b variant is 2.1 ± 0.5 (n = 6)-fold more abundant than the GBR1a variant.

Fig. 7. Regional distribution of GBR1a, GBR1b, and GBR2 proteins in adult rat brain. A, equal amounts of crude membranes (40 μg of protein/lane) prepared from olfactory bulb (Olf. bulb), striatum, cerebral cortex, hippocampus, thalamus, cerebellum, and medulla/pons were analyzed for GBR1a-(83–107), GBR1a,b-(922–944), GBR1a,b-(922–944), and GBR2 immunoreactivity by Western blotting. B, GBR1a, GBR1b, GBR1a/b, and GBR2-IR were visualized in parasagittal rat brain sections (16 μm) blotted onto nitrocellulose membranes followed by immunostaining with the GBR1a-(83–107), GBR1a,b-(922–944), GBR1a,b-(922–944), or GBR2 antisera. Co-incubation with the respective peptide antigens (10 μg/ml) completely prevented immunostaining, underlining its specificity (not shown).

Fig. 8. Subcellular distribution of GBR1a, GBR1b, and GBR2 proteins. Rat brain tissue was subjected to subcellular fractionation (for details see "Experimental Procedures"), and aliquots (30 μg) of each fraction were analyzed by Western blotting using the GBR1a-(83–107), GBR1b-(1–18), GBR1a,b-(922–944), and GBR2 antisera. H, homogenate; P1, nuclear pellet; P2, crude membranes; SPM, synaptic plasma membranes; LP-SPM, lysed synaptic vesicle membranes; LS-SPM, cytosolic synaptic vesicle fractions (also contains synaptic vesicles and membranes of the endoplasmic reticulum and Golgi apparatus); P3, light membrane pellet (contains synaptic vesicles and membranes of the endoplasmic reticulum and Golgi apparatus); S3, cytosolic fraction (devoid of membranes); PSD, postsynaptic densities.

were observed for the GBR1b variant (Fig. 8), suggesting that the GBR1a and GBR1b variants differ in their extent of postsynaptic localization. In addition, GBR1-IR and GBR2-IR were detected in fractions containing membranes of the endoplasmic reticulum and the Golgi apparatus (P3 and LS-SPM), pointing to the existence of an intracellular pool of GABA<sub>B</sub> receptors in neurons (Fig. 8).
Association of GABA<sub>B</sub> Receptor Subunit Proteins in Situ

DISCUSSION

For GABA<sub>B</sub> receptors, two constituent proteins with a seven-transmembrane domain topology, GBR1 and GBR2, are known, of which GBR1 exists in two splice variants (3, 7–11). In situ, the subunit composition of the two GABA<sub>B</sub> receptor variants is yet unknown. In the present study, the structural organization as well as the regional and subcellular distribution of the two GABA<sub>B</sub> receptor subtypes were analyzed in rat brain with antibodies selectively recognizing the GBR1a, GBR1b, or GBR2 proteins. The results suggest that GBR1a and GBR1b characterize two distinct receptor populations, which both contain GBR2. There was no indication for the presence of monomeric or homo-oligomeric GABA<sub>B</sub> receptor components. Most interestingly, determination of the size of native GABA<sub>B</sub> receptors pointed to the presence of yet unknown receptor associated proteins. Striking differences in the relative abundance of GBR1a and GBR1b as well as their regional and subcellular distribution suggest that they differ in their targeting specificity and may thus serve distinct functions in neuronal signaling.

Heteromerization of GBR1a and GBR1b with GBR2 in Rat Brain—To date, the only examples for a heterodimerization of G-protein-coupled receptors are opioid receptors, which are able to form functional recombinant dimeric or homo-oligomeric GABA<sub>B</sub> receptor components. Recombinant GABA<sub>B</sub> receptors are unique among seven-transmembrane domain receptors, as their targeting to the cell membrane and their efficient coupling to effector systems largely require the co-expression of the two constituent proteins GBR1 and GBR2 (7–11). In cerebral cortex both GBR1a and GBR1b were found to be associated with GBR2 as shown by immunoprecipitation of 125I-CGP 71872 photoaffinity labeled GABA<sub>B</sub> receptors (7). However, it was not clear whether GBR1a and GBR1b assemble with GBR2 in separate receptor subtypes or whether GABA<sub>B</sub> receptors consist of ternary complexes containing both GBR1a and GBR1b together with GBR2. Thus, variant-specific antisera were required for a detailed investigation of the molecular organization of native GABA<sub>B</sub> receptor subtypes.

Although their solubilization by deoxycholate was not quantitative (GBR1a, 58 ± 5%; GBR1b, 56 ± 5%), GBR1a and GBR1b were extracted to an equal extent (Fig. 2), ensuring comparable receptor concentrations in the supernatant. When solubilized GABA<sub>B</sub> receptors were immunopurified using GBR1b-(1–18) or GBR2 antibodies, both GBR1a and GBR1b were found to be associated with GBR2 (Figs. 3 and 4). Moreover, the results did not support the presence of GABA<sub>B</sub> receptors in which the two splice variants GBR1a and GBR1b were co-assembled (Fig. 3B). This result is further supported by the observation that the C termini of GBR1 and GBR2 do not form homo-oligomers in the yeast two-hybrid system (8, 10). Thus, the brain appears to largely contain two distinct receptor subtypes with the subunit combination GBR1a/GBR2 and GBR1b/GBR2. An association of GBR1a or GBR1b with GBR2 in native GABA<sub>B</sub> receptors is further substantiated by their overlapping pattern of distribution and relative abundance as detected by the histoblot technique on brain slices on a regional level (Fig. 7). The degree of GBR2 IR largely corresponded to the sum of GBR1a and GBR1b IR.

Homomeric GABA<sub>B</sub> receptors have been suggested to be functional as tested in heterologous expression systems. This was based on the observation that GBR2 expressed in HEK 293 cells induced a decrease in forskolin-stimulated cAMP production to the same extent as that detected after co-expression of GBR1 with GBR2 (8). In addition, the coupling of GBR1 to adenyl cyclase and K<sup>+</sup> channels has been detected (3, 12). However, molecular size estimation of solubilized GABA<sub>B</sub> receptors using sucrose density gradient centrifugation did not reveal an indication for the presence of monomers for GBR1a, GBR1b, or GBR2 as detected by Western blotting. Moreover, quantitative immunoprecipitation of solubilized receptors with the GBR2 or the GBR1a,b-(922–944) antiserum resulted in the precipitation of the entire population of GABA<sub>B</sub> receptors; the supernatants did not contain any measurable amounts of excess GBR1a, GBR1b, or GBR2. Thus, there was no evidence for the presence of monomeric or homo-oligomeric forms of GABA<sub>B</sub> receptors in brain (Fig. 5).

Most interestingly, the molecular size estimation of native solubilized GABA<sub>B</sub> receptors by sucrose density gradient centrifugation revealed two different molecular forms. The lower molecular weight peak, which contained the majority of GABA<sub>B</sub> receptors, can be attributed to heterodimeric receptors GBR1a/GBR2 and GBR1b/GBR2. The receptor populations with the higher molecular size, which likewise contained GBR1a and GBR1b in association with GBR2, are unlikely to consist of heterotrimeric GBR1a/GBR1b/GBR2 receptors since GBR1b-specific immunoaffinity chromatography ruled out an association of GBR1b with GBR1a (Fig. 3). Furthermore, the high molecular weight GABA<sub>B</sub> receptors are not due to a dimerization via disulfide bridges, as in the case of metabotropic glutamate receptors, which, in contrast to GABA<sub>B</sub> receptors, contain four highly conserved cysteine residues in the N-terminal domain (20). Although metabotropic glutamate receptors migrate as dimers under non-reducing conditions (20), GBR1a, GBR1b, and GBR2 migrate as monomers. Thus, the high molecular forms of GBR1a/GBR2 and GBR1b/GBR2 may rather represent complexes with yet unidentified proteins, possibly involved in the regulation or anchoring of GABA<sub>B</sub> receptors. The coiled-coil domains in the C termini of GBR1 and GBR2 (8, 10) may be preferred sites of interaction with accessory proteins (22) in addition to the putative PDZ-interacting module at the GBR1 and the GBR2 C terminus (5).

Differential Regional Distribution and Subcellular Targeting of GBR1a and GBR1b—In line with previous immunohistochemical evidence using the GBR1b-(1–18) and GBR1a,b-(922–944) antisera (4), the pattern and the sum of the staining intensities of GBR1a and GBR1b matched the distribution and staining intensity of GBR2 (Fig. 7). Furthermore, the regional pattern of the GABA<sub>B</sub> receptor proteins GBR1a, GBR1b, and GBR2 also corresponded to that of the respective mRNAs analyzed by in situ hybridization histochemistry (7–9). Finally, the distribution of GABA<sub>B</sub> receptor-binding sites detected autoradiographically (23–25) corresponded to the pattern of GABA<sub>B</sub> receptor proteins. Thus, the vast majority of GABA<sub>B</sub> receptors appear to consist of two isoforms with the subunit combinations GBR1a/GBR2 and GBR1b/GBR2. However, since GBR1a and GBR1b variants represent distinct GABA<sub>B</sub> receptor subtypes, they were expected to differ in their regional distribution. In general, the GBR1b variant was more abundant than the GBR1a variant, although the latter predominated in some brain areas (e.g., olfactory bulb, striatum, and CAI region of the hippocampus; Fig. 7).

In some brain areas (olfactory bulb, striatum, and hypothalamus), the relative signal intensity of GBR2 mRNA in in situ hybridization histochemistry was considerably lower than that of GBR1 mRNA (7–9). This finding led to the suggestion that not all GBR1 proteins may be associated with GBR2. However, on the protein level, the ratio of GBR1 to GBR2 was not different from that in other brain areas (Fig. 7). The discrepancy between the mRNA and protein ratios suggests that GABA<sub>B</sub> receptors may be subject to regionally different transcriptional and/or post-transcriptional regulation. It will therefore be of interest to determine whether GABA<sub>B</sub> receptors are subject to regionally distinctive translational control.
Presynaptic GABA_B receptors have been reported to be pharmacologically distinct from those of postsynaptic receptors in several brain areas (26–29). It has been speculated that GBR1a and GBR1b might be the structural correlates for post- and presynaptic receptors, respectively (3). Indeed, GBR1a appears to be preferentially located postsynaptically as shown by their prevalence in purified PSDs. Conversely, GBR1b IR, which was only rarely present in the PSD fraction (Fig. 8), may be preferentially localized at pre- and extrasynaptic sites. A differential localization of GBR1a and GBR1b may be mediated via the distinctive extracellular N-terminal domain, e.g. by the sushi repeats for extracellular protein contacts present exclusively by the sushi repeats for extracellular protein contacts present exclusively.

In conclusion, the results indicate that the vast majority of GABA_B receptors are represented by two populations of heterodimeric receptors GBR1a/GBR2 and GBR1b/GBR2. These two distinct receptor subtypes are localized to mainly distinct subcellular sites, with GBR1b being rarely located in postsynaptic densities. There was no indication for the presence of monomers of GBR1a, GBR1b, or GBR2 in the brain or a trimeric population of receptors containing GBR1a/GBR1b/GBR2.

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