Subtype-selective Interaction with the Transcription Factor CCAAT/Enhancer-binding Protein (C/EBP) Homologous Protein (CHOP) Regulates Cell Surface Expression of GABA\(_B\) Receptors*

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The metabotropic \(\gamma\)-aminobutyric acid, type B (GABA\(_B\)) receptors mediate the slow component of GABAergic transmission in the brain. Functional GABA\(_B\) receptors are heterodimers of the two subunits GABA\(_{B1}\) and GABA\(_{B2}\), of which GABA\(_{B1}\) exists in two main isoforms, GABA\(_{B1a}\) and GABA\(_{B1b}\). The significance of the structural heterogeneity of GABA\(_B\) receptors, the mechanism leading to their differential targeting in neurons as well as the regulation of cell surface numbers of GABA\(_B\) receptors, is poorly understood. To gain insights into these processes, we searched for proteins interacting with the C-terminal domain of GABA\(_{B2}\). Here, we showed that the transcription factor CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP) directly interacts with GABA\(_B\) receptors in a subtype-selective manner to regulate cell surface expression of GABA\(_{B1a}/BABA_{B2}\) receptors in HEK293 cells. The interaction of CHOP with GABA\(_{B1a}/GABA_{B2}\) receptors resulted in their intracellular accumulation and in a reduced number of cell surface receptors. This regulation required the interaction of CHOP via two distinct domains with the heterodimeric receptor; its C-terminal leucine zipper associates with the leucine zipper present in the C-terminal domain of GABA\(_{B2}\), and its N-terminal domain associates with an as yet unidentified site on GABA\(_{B1a}\). In conclusion, the data indicated a subtype-selective regulation of cell surface receptors by interaction with the transcription factor CHOP.

GABA\(_B\) receptors are G-protein-coupled receptors that play an important role in the control of inhibitory neurotransmission. Presynaptically, GABA\(_B\) receptors modulate neurotransmitter release by inhibiting Ca\(^{2+}\) channels, whereas postsynaptically, they induce slow inhibitory potentials by activating K\(^+\) channels. Structurally, functional GABA\(_B\) receptors require the heterodimerization of the two subunits GABA\(_{B1}\) and GABA\(_{B2}\). Heterodimerization is in part mediated by leucine zipper motifs present in the intracellular C-terminal domains of GABA\(_{B1}\) and GABA\(_{B2}\), forming a coiled-coiled structure. Although GABA\(_{B1}\) harbors the ligand binding site, GABA\(_{B2}\) contains all the molecular determinants for G-protein coupling and is necessary for trafficking of GABA\(_B\) receptors to the cell surface by shielding the endoplasmic reticulum retention signal of GABA\(_{B1}\) (for a review, see Refs. 1–4). Two main GABA\(_B\) receptor subtypes, GABA\(_{B1a}/GABA_{B2}\) and GABA\(_{B1b}/GABA_{B2}\), are expressed in the brain, as defined by the presence of alternative transcription sites in the GABA\(_B2\) gene and by differential promoter usage (5). GABA\(_{B1a}\) and GABA\(_{B1b}\) display strikingly distinct spatial and temporal expression patterns, with GABA\(_{B1a}\) being the predominant isoform in the developing brain and GABA\(_{B1b}\) being prevalent in the adult brain. However, the functional significance of GABA\(_B\) receptor subtypes is not yet understood since no unambiguous differences in function or pharmacology have been detected so far (reviewed in Refs. 1–4).

The molecular mechanisms leading to differential targeting and anchoring of GABA\(_B\) receptor subtypes to specific synaptic and extrasynaptic sites as well as the regulation of the cell surface number of GABA\(_B\) receptors are poorly understood. Accessory proteins interacting with GABA\(_B\) receptors in a temporally and spatially defined manner may be involved in these processes, and distinct GABA\(_B\) receptor subtypes may be regulated differentially by specific sets of interacting proteins. Extensive searches for GABA\(_B\) receptor-associated proteins using the yeast two-hybrid system yielded a number of potential interacting proteins. Most interestingly, the transcription factor CREB2/ATF4 was shown to directly interact with the leucine zipper of GABA\(_{B1}\), suggesting a novel G-protein-independent mechanism of signal transduction (6–8). Furthermore, GABA\(_{B1}\) associates with 14-3-3 proteins via a site that overlaps with its coiled-coil domain (9). Since 14-3-3 competes with GABA\(_{B2}\) for binding to GABA\(_{B1}\) in vitro, it may regulate GABA\(_B\) receptor heterodimerization (9). Recently, Marlin-1, a novel brain-specific RNA-binding protein, was shown to interact with GABA\(_{B1}\) via the coiled-coil domain (10). Marlin-1 was proposed to regulate the cellular levels of GABA\(_{B2}\) and thereby to affect the number of functional GABA\(_B\) receptors (10). These findings demonstrate that in particular, the coiled-coiled domain of the GABA\(_B\) receptor subunits provides a site of interaction for a variety of different protein partners. However, the precise physiological relevance of these interactions remains to be determined. In addition, none of these proteins were shown to interact with specific GABA\(_B\) receptor subtypes. In this regard, the presence of two sushi repeats (complement control protein modules) in the extracellular domain of GABA\(_{B1a}\) but not in GABA\(_{B1b}\) and their capability to bind
the extracellular matrix protein fibulin-2 in vitro (11), provide evidence for the existence of subtype-selective interacting proteins.

We used the yeast two-hybrid system to search for novel GABA<sub>B</sub> receptor-interacting proteins and identified the transcription factor CHOP as a potential GABA<sub>B</sub>-associated protein. CHOP (also designated as gadd153 or C/EBP<sub>B</sub>) belongs to the CCAAT/enhancer-binding protein (C/EBP) family of transcription factors, which all contain a characteristic basic-leucine zipper motif in their extreme C-terminal domain, whereby the basic region serves for DNA binding and the leucine zipper as a protein-protein interaction module (12). CHOP is strongly up-regulated by factors that interfere with normal function of the endoplasmic reticulum (13) and has been implicated in the control of endoplasmic reticulum stress-induced apoptosis associated with diabetes (14–16). In addition, CHOP also appears to be involved in ischemia-induced apoptosis since CHOP knock-out mice exhibit strongly reduced ischemia-induced neuronal cell death (17).

Here, we identified CHOP as a novel GABA<sub>B</sub> receptor-interacting protein and demonstrate its co-localization in cultured hippocampal neurons as well as its physical association with GABA<sub>B</sub> receptors in brain extracts by co-immunoprecipitation. Co-expression in HEK 293 cells revealed that CHOP interacts selectively with GABA<sub>B1α</sub>/GABA<sub>B2</sub> receptors via two distinct binding sites, which results in an intracellular accumulation and thereby in a reduced cell surface expression of the receptors.

**EXPERIMENTAL PROCEDURES**

*Antibodies*—The following primary antibodies were used: rabbit GABA<sub>B1α</sub> (18) (1:250 for Western blotting (WB)), guinea pig GABA<sub>B1α</sub> (Chemicon; 1:5000 for immunofluorescence), rabbit GABA<sub>B2α</sub> (19) (1:100 for WB, 1:500 for immunofluorescence), guinea pig GABA<sub>B2α</sub> (Chemicon; 1:5000 for WB), mouse CHOP (B3, Santa Cruz Biotechnology; 1:1000 for WB, 1:500 for immunofluorescence), mouse hemagglutinin tag (12CA5, gift from G. Radzizzwil, Zurich, Switzerland; 1:1000 for WB, 1:500 for immunofluorescence). In addition, a polyclonal antisera directed against recombinant CHOP protein (amino acids 35–170) overexpressed in *Escherichia coli* was generated by immunizing rabbits. CHOP antibodies were affinity-purified using the recombinant protein covalently attached to Affi-Gel 15 (Bio-Rad). Specificity of the CHOP antibodies was assessed by Western blotting (1:750) and immunocytochemistry (1:50) using HEK 293 cells expressing CHOP as a negative control and non-transfected HEK 293 cells.

*Plasmids*—Expression plasmids containing GABA<sub>B1α</sub>, GABA<sub>B2α</sub>, GABA<sub>B1α</sub>LZ2, GABA<sub>B2α</sub>LZ1 and GABA<sub>B2α</sub>LZ2, and GABA<sub>B2α</sub>LZ2, and GABA<sub>B2α</sub>LZ2 were described previously (20–22). Full-length cDNA of GABA<sub>B1α</sub>LZ1 (named GABA<sub>B1α</sub>LZ1) were described previously (20–22). Full-length cDNA of GABA<sub>B2α</sub> was kindly provided by R. Nehring (Göttingen, Germany). Deletion mutants of CHOP were generated by PCR and inserted into pcDNA3.1-TOPO CHOP<sub>ΔN</sub> lacking the last 108 nucleotides (forward primer, 5′-GAGAATGAAAGGAGGATTGCGACAG-3′ and reverse primer, 5′-GTGCCATTCTTCCTATCTTC-3′) and CHOP<sub>ΔN</sub> lacking the first 42 amino acids (forward primer, 5′-ACCAT-GAATGAGGAGGAAATCAAAA-3′ and reverse primer, 5′-TGC-TTGTGCAAGTCATCCATC-3′).

*Yeast Two-hybrid Assay*—The yeast two-hybrid screen was performed by Invitrogen. The entire C-terminal domain of human GABA<sub>B2α</sub> (accession number AF056085, amino acid sequence LILRT…VMVSG) was used to screen a human brain cDNA library.

*Cell Culture*—HEK 293 cells were maintained in minimum Eagle’s medium containing 10% (v/v) fetal calf serum, 2 mM glutamine and transfected with appropriate plasmids by calcium phosphate precipitation. Transfected cells were used 2 days after transfection for subsequent immunofluorescence or immunoprecipitation studies.

Primary cultured neurons were prepared from rat embryos taken at embryonic day 19 from pregnant Wistar rats anesthetized with ether. The hippocampus was dissected on ice in PBS containing 5.5 mM glucose. The tissue was dissociated with trypsin and resuspended in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and plated to a density of 50,000 cells/cm² onto poly-L-lysine-coated coverslips and transferred to 12-well plates containing glial feeder cells in a defined serum-free medium (Dulbecco’s modified Eagle’s medium without phenol red containing B27 supplement, 250 µg/ml AlbuMAX, 11 mg/ml sodium-pyruvate, 5 mM glutamine, and 50 µg/ml gentamycin).

*Immunoprecipitation*—Transfected HEK 293 cells were harvested and homogenized in lysis buffer (PBS containing 5 mM EDTA, 5 mM EGTA, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 25 µg/ml aprotinin, 1 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride). After homogenization, cells were solubilized by the addition of 1% Triton X-100 (final concentration) for 30 min on ice followed by centrifugation at 100,000 × g for 30 min. The supernatant was subjected to immunoprecipitation using the respective GABA<sub>B</sub> receptor antibodies covalently immobilized to protein A-agarose or protein G-agarose, respectively. Immunoprecipitation was performed for 4 h at 4 °C. Immune complexes were collected by centrifugation and extensively washed with lysis buffer containing 1% Triton X-100. Bound proteins were released with 2× sample buffer for SDS-polyacrylamide gel electrophoresis, heated, and analyzed by Western blotting. Immunoprecipitation of GABA<sub>B</sub> receptors from rat brain tissue was performed essentially as described previously (18).

*Immunocytochemistry*—Double and triple labeling immunocytochemistry was performed with transiently transfected HEK 293 cells and primary cultured neurons. HEK 293 cells transfected with the appropriate plasmids were fixed with 4% paraformaldehyde in 150 mM phosphate buffer, pH 6.8–7.5, permeabilized for 5 min with 0.5% Triton X-100 in PBS and incubated with the primary antibodies diluted to the appropriate concentration (see above) in PBS containing 10% normal goat serum. After washing extensively with PBS, the cells were incubated with secondary antibody coupled to Alexa Fluor 488 (1:1000, Molecular Probes), Cy-3 (1:500, Jackson ImmunoResearch), and/or Cy-5 (1:1000, Jackson ImmunoResearch). After three washes for 10 min with PBS, the cells were mounted in 90% glycerol containing 0.1% phenylenediamine, 50 mM Tris, pH 7.5, and 10 mM NaCl. Cells were analyzed by confocal laser scanning microscopy (LSM 510 Meta, Zeiss), and images were processed using Imaris (Bitplane, Zurich, Switzerland).

*Cell Surface Enzyme-linked Immunosorbent Assay*—For labeling of cell surface receptors, living HEK 293 cells transiently transfected with GABA<sub>B1α</sub>/GABA<sub>B2α</sub> or GABA<sub>B1α</sub>/GABA<sub>B1α</sub>/GABA<sub>B2α</sub>/CHOP plated on 96-well plates were incubated with an antibody directed against the N terminus of GABA<sub>B1α</sub> (18) in buffer A (25 mM HEPES 7.4, 2 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 30 mM glucose, 5 mM KCl, 119 mM NaCl) containing 450 mM sucrose and 10% normal goat serum for 40 min at room temperature. After washing the cells extensively with buffer A, the cells were fixed with 4% paraformaldehyde in 150 mM phosphate buffer, pH 6.8–7.5, and incubated with horseradish peroxidase-conjugated anti-rabbit antibodies for 60 min. After extensive washing, horseradish peroxidase activity was determined by tetramethylbenzidine as substrate (100 µl/well 0.24 mg/ml tetramethylbenzidine, 0.2 mM sodium citrate, pH 3.95, 0.006% H<sub>2</sub>O<sub>2</sub>). The color reaction was terminated by the addition of 100 µl of 1 M H<sub>2</sub>SO<sub>4</sub>, and the optical density was recorded at 450 nm in a microplate reader (Synergy HT, Biotek). The abundance of total receptors was determined in parallel cultures after fixation and permeabilization of the
cells with 0.5% Triton X-100. Nonspecific antibody reaction was determined in parallel cultures of non-transfected cells.

RESULTS

Interaction of CHOP with GABA<sub>B</sub> Receptors in Neurons—Screening with the yeast two-hybrid system of a brain cDNA expression library using the entire intracellular C-terminal domain of GABA<sub>B2</sub> yielded the transcription factor CHOP as a putative GABA<sub>B</sub> receptor-associated protein. An association of CHOP with native GABA<sub>B</sub> receptors was tested by co-immunoprecipitation of CHOP with GABA<sub>B</sub> receptors from brain extracts and by determination of the immunocytochemical distribution of CHOP and GABA<sub>B</sub> receptors in primary cultured hippocampal neurons. GABA<sub>B</sub> receptors were immunoprecipitated with antibodies recognizing either GABA<sub>B1</sub> or GABA<sub>B2</sub> and analyzed for the presence of CHOP. In both GABA<sub>B</sub> receptor immunoprecipitates, CHOP was detected, demonstrating the physical association of CHOP with native GABA<sub>B</sub> receptors (Fig. 1A). In agreement with their co-immunoprecipitation, double labeling immunofluorescence microscopy of primary cultured hippocampal neurons revealed an extensive overlap in the subcellular distribution of GABA<sub>B</sub> receptors and CHOP (Fig. 1B). Both GABA<sub>B1</sub> and GABA<sub>B2</sub> were distributed in the soma and neurites of the neurons. Similar to GABA<sub>B</sub> receptors, CHOP exhibited a widespread distribution in the soma and neurites (Fig. 2B). Apparent co-localization of CHOP with GABA<sub>B</sub> receptors was observed in the soma and in large patches on the neurites (Fig. 1B). In conclusion, these experiments demonstrated that CHOP interacts with GABA<sub>B</sub> receptors in their native environment, i.e. in the brain.

Interaction of CHOP with GABA<sub>B2</sub> in HEK 293 Cells—To analyze the interaction of CHOP with GABA<sub>B</sub> receptors in more detail, the effect of co-expression of HEK 293 cells of CHOP with GABA<sub>B2</sub> in the absence of GABA<sub>B1</sub> was tested. In contrast to GABA<sub>B2</sub>, CHOP was trafficked to the cell surface on its own. Immunocytochemistry revealed that in the absence of GABA<sub>B2</sub>, CHOP was specifically localized to the nucleus (Fig. 2A, a). However, co-expression of CHOP with GABA<sub>B2</sub> resulted in a cytoplasmic distribution of CHOP, where it co-localized extensively with GABA<sub>B2</sub> (Fig. 2A, b). The redistribution of CHOP upon co-expression with GABA<sub>B2</sub> argues for a direct interaction of CHOP and GABA<sub>B2</sub>.

Since CHOP was isolated with the C-terminal domain of GABA<sub>B2</sub>, the specificity of the CHOP-GABA<sub>B</sub> interaction was determined using a deletion mutant of GABA<sub>B2</sub> lacking its entire C-terminal domain (GABA<sub>B2ΔC</sub>). Upon co-expression with the truncated GABA<sub>B2</sub>, the redistribution of CHOP to cytoplasmic sites was prevented, and consequently, no co-localization of CHOP with GABA<sub>B2</sub> was observed (Fig. 2A, c).

The physical association of CHOP and GABA<sub>B2</sub> in HEK 293 cells was verified by co-immunoprecipitation using an antibody directed against the C terminus of GABA<sub>B2</sub>. By immunoblotting, CHOP was detected in the GABA<sub>B2</sub> immunoprecipitate, demonstrating that CHOP associates with GABA<sub>B2</sub> (Fig. 2B).

Isosubunit-specific Interaction of CHOP with GABA<sub>B2</sub> in HEK 293 Cells—To investigate whether CHOP interacts with GABA<sub>B1</sub> receptors in a subunit-specific manner, a potential association of CHOP with the two main GABA<sub>B1</sub> isoforms was tested. CHOP was co-expressed in HEK 293 cells with GABA<sub>B1α</sub> or GABA<sub>B1β</sub> respectively, and analyzed for physical association by immunoprecipitation. Surprisingly, CHOP co-
Thus, CHOP was not only able to interact with GABAB2 but also with GABAB1a (Fig. 4). Thus, the CHOP-GABAB2 interaction was not only able to interact with GABAB1a but not with GABAB1b (Fig. 3A). Thus, CHOP was not only able to interact with GABAB2, but also with GABAB1a.

At the subcellular level, double labeling immunofluorescence microscopy revealed a widespread distribution of GABAB1a throughout the cell with the exception of the nucleus (Fig. 3B, a). This distribution was in line with the endoplasmic reticulum retention of GABAB1 when expressed in the absence of GABAB2 (22–24). Co-expression of GABAB1a with CHOP resulted in a predominant perinuclear localization of CHOP, whereas CHOP was localized to the nucleus (Fig. 3B, b). These observations demonstrated that the interaction of CHOP with GABAB1a is restricted to the C-terminal domains.

Identification of the CHOP-GABAB Receptor Interaction Domains—The presence of a leucine zipper motif in the C-terminal domains of both CHOP and GABAB2 argued for an association via these structures. Co-expression of CHOP with a GABAB2 deletion mutant lacking its leucine zipper (GABAB2LZ) resulted in the loss of co-localization and redistribution of CHOP (Fig. 4A). Accordingly, co-expression of full-length GABAB2, with a CHOP deletion mutant lacking its leucine zipper (CHOPΔLZ) resulted likewise in the loss of co-localization and redistribution of CHOP (Fig. 4B). Thus, the CHOP-GABAB2 interaction was mediated by the leucine zipper motifs present in their C-terminal domains.

Since GABAB1 also contains a leucine zipper motif in its C-terminal domain, it was tested whether the leucine zipper of GABAB1 also interacts with CHOP. Co-expression of CHOP with a GABAB1a insertion mutant in which the leucine zipper of GABAB2 was replaced by the wild-type GABAB2ΔLZ resulted in the loss of co-localization and redistribution of CHOP (Fig. 4C). These findings demonstrated that CHOP associates specifically with the leucine zipper of GABAB1a, but not with the leucine zipper of GABAB2.
minal domain with an as yet unidentified site on GABAB1a (Fig. 4f). Thus, CHOP interacted via different domains with GABAB1a and GABAB2.

Cell Surface GABAB1a/GABAB2 Receptors Are Regulated by CHOP—

The previous results demonstrated that CHOP interacts with GABAB2 and GABAB1a but not with GABAB1b. Since the expression of functional GABA<sub>a</sub> receptors requires the heterodimerization of both GABAB1a and GABAB2, the effect of co-expression of CHOP with heterodimeric GABA<sub>a</sub> receptors was analyzed. When co-expressed in HEK 293 cells, GABAB<sub>1a</sub> and GABAB<sub>2</sub> were targeted to the cell surface, where they are co-localized (Fig. 5a, a). In contrast, co-expression of CHOP with GABAB<sub>1a</sub> and GABAB<sub>2</sub> resulted in a dramatic alteration of receptor distribution. In the presence of CHOP, GABAB<sub>1a</sub> and GABAB<sub>2</sub> displayed, besides their normal cell surface expression, an unusual intracellular localization (Fig. 5a, b). Co-localization of all three proteins was occasionally detected at cytoplasmic and perinuclear sites. In addition, GABAB<sub>2</sub> but not GABAB<sub>1a</sub> was often found in inclusions in or in the vicinity of the nucleus. CHOP staining was detected in the nucleus and cytoplasmic sites and was partially co-localized with GABAB<sub>1a</sub> and GABAB<sub>2</sub> at cytoplasmic and perinuclear sites (Fig. 5a, c). This is in contrast to the abundant co-localization and association of CHOP with either GABAB<sub>2</sub> alone (Fig. 2) or GABAB<sub>1a</sub> alone (Fig. 3) shown above. To verify a reduced association of CHOP with heterodimeric GABAB<sub>1a</sub>/GABAB<sub>2</sub> receptors, GABAB<sub>1a</sub>/GABAB<sub>2</sub> and CHOP were co-expressed in HEK 293 cells and subjected to immunoprecipitation using GABAB<sub>1a</sub>- or GABAB<sub>2</sub>-selective antibodies. Immunoblotting revealed the lack of co-immunoprecipitation of CHOP in the GABAB<sub>2</sub> immunoprecipitate, whereas a weak signal of CHOP was detected in the GABAB<sub>1a</sub>, immunoprecipitate (Fig. 5b). The weak CHOP signal in the GABAB<sub>1a</sub>, immunoprecipitate is in line with the co-localization of CHOP and GABAB<sub>1a</sub> observed in the immunocytochemical experiment (Fig. 5a, c). The lack or strongly reduced co-immunoprecipitation of CHOP with GABAB<sub>2</sub> and GABAB<sub>1a</sub>, respectively, and the intracellular accumulation of GABAB<sub>1a</sub> and GABAB<sub>2</sub> suggested that a transient interaction of CHOP with heterodimeric GABAB<sub>1a</sub>/GABAB<sub>2</sub> receptors may induce the internalization of cell surface receptors, or alternatively, disturb the targeting of receptors to the cell surface.

To analyze whether the perturbed localization of GABAB<sub>1a</sub>/GABAB<sub>2</sub> receptors is specifically mediated by CHOP, GABAB<sub>1a</sub>/GABAB<sub>2</sub> receptors were co-expressed with CHOP lacking its leucine zipper (CHOPΔLZ) that is not able to interact with GABAB<sub>2</sub>. Indeed, co-expression of CHOPΔLZ with GABAB<sub>1a</sub>/GABAB<sub>2</sub> receptors prevented their intracellular accumulation and resulted in a normal cell surface distribution of both GABAB<sub>1a</sub> and GABAB<sub>2</sub> without the formation of GABAB<sub>2</sub>-positive inclusions (Fig. 5a, c). Interestingly, CHOPΔLZ was still co-localized with GABAB<sub>1a</sub>/GABAB<sub>2</sub> receptors at the cell surface (Fig. 5a, c). This co-localization of CHOPΔLZ with GABAB<sub>1a</sub> was most likely due to an interaction with GABAB<sub>1a</sub>, which binds to the N-terminal domain of CHOP, not involving the leucine zippers (Fig. 4). Similarly, co-expression of the deletion mutant of CHOP lacking the N-terminal amino acids 1–42 (CHOPΔN) that is not able to interact with GABAB<sub>2</sub> at the cell surface. e CHOP did not interact with GABAB<sub>1a</sub>/GABAB<sub>2</sub> receptors, as evidenced by the normal cell surface localization of GABAB<sub>1a</sub> and GABAB<sub>2</sub> and the entirely nuclear localization of CHOP. b, reduced or lack of permanent association of CHOP with heterodimeric GABAB<sub>1a</sub>/GABAB<sub>2</sub> receptors. HEK 293 cells expressing GABAB<sub>1a</sub>/GABAB<sub>2</sub> receptors and CHOP were solubilized and subjected to immunoprecipitation with GABAB<sub>1a</sub>, antibodies (left panel) or GABAB<sub>1a</sub>, antibodies followed by Western blotting for detection of GABAB<sub>1a</sub>, GABAB<sub>2</sub>, and CHOP. Only in the GABAB<sub>1a</sub>-immunoprecipitate was a faint CHOP signal detected. Extract Triton X-100 solubilized proteins; Extract depleted, extract after immunoprecipitation of GABAB<sub>1a</sub>, or GABAB<sub>2</sub>, proteins immunoprecipitated by GABAB<sub>1a</sub>, antibodies; IP, GABAB<sub>2</sub>, proteins immunoprecipitated by GABAB<sub>1a</sub>, antibodies; Control precipitation with protein A-agarose in the absence of antibodies. c, reduction of cell surface GABAB<sub>1a</sub>/GABAB<sub>2</sub> receptors upon co-expression with CHOP. Cell surface receptors of living HEK 293 cells expressing GABAB<sub>1a</sub>/GABAB<sub>2</sub> receptors in the presence and absence of CHOP were labeled with antibodies directed against the N-terminal domain of GABAB<sub>1a</sub> and quantified by enzyme-linked immunosorbent assay. The total amounts of receptors expressed were determined upon fixation and permeabilization of the cells prior to the incubation with the antibody (100%). The values represent the mean ± S.D. from three independent experiments.
To test whether CHOP induces a reduction of GABAB1a/GABAB2 receptors on the cell surface, the abundance of cell surface receptors was quantified by enzyme-linked immunosorbent assay. HEK 293 cells expressing GABAB1a/GABAB2 in the absence or presence of CHOP were seeded onto 96-well plates, and cell surface receptors were determined by incubating living cells with an antibody directed against the N terminus of GABAB2. The abundance of total receptors expressed was measured by staining fixed and permeabilized cells of parallel cultures. Quantification of the colorimetric enzymatic reactions revealed a 27 ± 9% (n = 3) reduction of cell surface receptors (Fig. 5C). This value might be underestimated since not all HEK 293 cells expressing GABAB1a/GABAB2 receptors co-express CHOP.

In contrast to the co-expression of CHOP with GABAB1a/GABAB2 receptors, co-expression of CHOP with GABAB1b/GABAB2 receptors resulted in a normal cell surface expression of GABAB1b and GABAB2 with CHOP being entirely localized to the nucleus (Fig. 5A, e). Thus, CHOP was not able interact with GABAB1b/GABAB2 receptors. Although CHOP associates with GABAB2 when expressed in the absence of GABA B1 (Fig. 2A), the presence of GABA B1 apparently prevents its interaction with GABAB2. This may be due to a competition of binding between the leucine zippers of GABA B1 and CHOP for GABAB2. This finding supported the observation that the effect of CHOP on GABAB1a/GABAB2 requires the interaction with both GABAB1a and GABAB2.

DISCUSSION

The results of the present study have provided the first example of a subtype-selective regulation of GABA<sub>B</sub> receptors by an interacting protein. Yeast two-hybrid screens identified the transcription factor CHOP as a potential interacting protein. CHOP interacted via its C-terminal leucine zipper with the leucine zipper present in the C-terminal domain of GABAB2. This interaction was highly specific since replacing the leucine zipper of GABAB2 with the leucine zipper of GABAB1 abolished the association. Unexpectedly, CHOP also interacted through its N-terminal domain with an as yet undefined site of GABA B1 but not with GABA B1s. Since GABA B1a and GABA B1s share identical amino acid sequences in their intracellular domains, GABA B1a and GABA B1s may differ in their conformation, which results in the formation of a binding site for CHOP selectively on GABA B1a. Co-expression of CHOP with heterodimeric GABA B1/GABA B2 receptors resulted in a perturbed localization of the receptors to intracellular sites along with frequent GABA B2-positive inclusions and a reduction of cell surface receptors. To exert this effect, CHOP must bind to both GABA B1a (via its N-terminal domain) and GABA B2 (via its C-terminal leucine zipper), which explains the GABA B1a/GABA B2 subtype selectivity of the interaction. The intracellular accumulation of GABA B1a/GABA B2 receptors was specifically mediated by CHOP since deletion of either the first 42 amino acids of GABAB1 or the C-terminal leucine zipper of CHOP interacting with GABAB2 abolished the perturbed localization of GABA B1a/GABA B2 receptors and the formation of GABA B2-positive inclusions. Currently, it is unclear whether CHOP induces the internalization of cell surface receptors or disturbs the targeting of receptors to the cell surface. The observation that the distribution of GABA B1a/GABA B2 receptors was not affected by co-expression of CHOP indicated that CHOP cannot interact alone via its leucine zipper with the coiled-coil domain of GABAB2 in the presence of GABA B1s. Similar findings were reported for ATF4 and 14-3-3, which interact selectively with the leucine zipper of GABA B1 solely in the absence of GABA B1s (7–9). Thus, the coiled-coil domain of GABA B1 and GABA B2 appeared to provide a site of interaction for a number of different protein partners for which binding should be mutually exclusive. Accordingly, the interaction of CHOP via two distinct sites with GABA B1a/GABA B2 receptors may at least transiently disrupt or weaken the heterodimers. The formation of the large GABA B2-positive inclusions that are largely devoid of staining for GABA B1 further supported this hypothesis. In this regard, the prominent co-localization of CHOP with GABA B1 and GABA B2 in cultured hippocampal neurons as well as their co-immunoprecipitation from brain extracts not only indicated that the interaction of CHOP with GABA B2 receptors is of physiological relevance but also suggested that GABA B1 and GABA B2 may exist in appreciable pools of unassembled subunits.

The mechanism by which CHOP induces the perturbed localization of GABA B1a/GABA B2 remains to be determined. The results of the present study fit well to a hypothetical model in which CHOP transiently interacts selectively with GABA B1a/GABA B2 receptors to induce their intracellular accumulation. This mechanism would allow selective down-regulation of the cell surface expression of a specific GABA B receptor subtype upon up-regulation of CHOP expression. In this regard, it is interesting to note that the expression of CHOP is strongly up-regulated upon endoplasmic reticulum stress (12). In neurons, CHOP expression has been shown to be induced preceding to N-methyl-D-aspartate- or dopamine-induced cytotoxicity (25) as well as transient cerebral ischemia (17, 26). In addition, CHOP was shown to be up-regulated in response to mutations in presenilin-1, which cause early onset familial Alzheimer disease (27). Therefore, induction of CHOP might be a powerful mechanism to regulate the cell surface expression of GABA B1a/GABA B2 receptors under pathological conditions.

REFERENCES
