Neurobiology:

Ischemia-like Oxygen and Glucose Deprivation Mediates Down-regulation of Cell Surface γ-Aminobutyric Acid$_B$ Receptors via the Endoplasmic Reticulum (ER) Stress-induced Transcription Factor CCAAT/Enhancer-binding Protein (C/EBP)-homologous Protein (CHOP)

Patrick J. Maier, Khaled Zemoura, Mario A. Acuña, Gonzalo E. Yévenes, Hanns Ulrich Zeilhofer and Dietmar Benke

J. Biol. Chem. 2014, 289:12896-12907.
doi: 10.1074/jbc.M114.550517 originally published online March 25, 2014

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 289, NO. 18, pp. 12896–12907, May 2, 2014

Ischemia-like Oxygen and Glucose Deprivation Mediates Down-regulation of Cell Surface γ-Aminobutyric Acid$_B$ Receptors via the Endoplasmic Reticulum (ER) Stress-induced Transcription Factor CCAAT/Enhancer-binding Protein (C/EBP)-homologous Protein (CHOP)*

Received for publication, January 15, 2014, and in revised form, March 24, 2014 Published, JBC Papers in Press, March 25, 2014, DOI 10.1074/jbc.M114.550517

Patrick J. Maier[‡§], **Khaled Zemoura**[‡§], **Mario A. Acuña**[‡§], **Gonzalo E. Yévenes**[‡], **Hanns Ulrich Zeilhofer**[‡§¶], **and Dietmar Benke**[‡§]

From the [‡]*Institute of Pharmacology and Toxicology, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland, the* [§]*Neuroscience Center Zurich, University of Zurich and ETH Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland, and the* [¶]*Institute of Pharmaceutical Sciences, ETH Zurich, Wolfgang-Pauli-Strasse 10, 8093 Zurich, Switzerland*

Background: ER stress associated with cerebral ischemia induces the expression of the transcription factor CHOP.
Results: Interaction with CHOP down-regulates cell surface $GABA_B$ receptors and, thus, $GABA_B$ receptor-mediated neuronal inhibition.
Conclusion: Interaction of CHOP with $GABA_B$ receptors in the ER prevents forward trafficking of the receptors.
Significance: This mechanism is expected to contribute to excitotoxicity in cerebral ischemia.

Cerebral ischemia frequently leads to long-term disability and death. Excitotoxicity is believed to be the main cause for ischemia-induced neuronal death. Although a role of glutamate receptors in this process has been firmly established, the contribution of metabotropic $GABA_B$ receptors, which control excitatory neurotransmission, is less clear. A prominent characteristic of ischemic insults is endoplasmic reticulum (ER) stress associated with the up-regulation of the transcription factor CCAAT/enhancer-binding protein-homologous protein (CHOP). After inducing ER stress in cultured cortical neurons by sustained Ca^{2+} release from intracellular stores or by a brief episode of oxygen and glucose deprivation (*in vitro* model of cerebral ischemia), we observed an increased expression of CHOP accompanied by a strong reduction of cell surface $GABA_B$ receptors. Our results indicate that down-regulation of cell surface $GABA_B$ receptors is caused by the interaction of the receptors with CHOP in the ER. Binding of CHOP prevented heterodimerization of the receptor subunits $GABA_{B1}$ and $GABA_{B2}$ and subsequent forward trafficking of the receptors to the cell surface. The reduced level of cell surface receptors diminished $GABA_B$ receptor signaling and, thus, neuronal inhibition. These findings indicate that ischemia-mediated up-regulation of CHOP down-regulates cell surface $GABA_B$ receptors by preventing their trafficking from the ER to the plasma membrane. This mechanism leads to diminished neuronal inhibition and may contribute to excitotoxicity in cerebral ischemia.

$GABA_B$ receptors are $G_{i/o}$ protein-coupled receptors composed of the two obligatory and functionally distinct subunits $GABA_{B1}$ and $GABA_{B2}$. $GABA_{B1}$ harbors the binding site for orthosteric ligands, whereas $GABA_{B2}$ contains a binding site for allosteric modulators, recruits the G protein, and is required for trafficking of the receptors from the endoplasmic reticulum (ER)[2] to the plasma membrane (reviewed in Ref. 1). $GABA_{B1}$ contains an ER retention signal in the C-terminal domain that retains unassembled $GABA_{B1}$ in the ER. Heterodimerization with $GABA_{B2}$ masks the ER retention signal and permits ER export of the receptor heterodimers (2–4). $GABA_B$ receptors are abundantly expressed throughout the mammalian central nervous system, where they mediate slow and persistent inhibition. According to their prominent role in regulating neuronal excitability, $GABA_B$ receptors have been implicated in a variety of neurological disorders, including cerebral ischemia.

In cerebral ischemia, excessive glutamatergic neurotransmission eventually leads to neuronal death (5). Decreased GABAergic activity appears to contribute to neuronal overexcitation (6), and there are indications that $GABA_B$ receptors are down-regulated under ischemic conditions (7–10). This suggests that impaired $GABA_B$ receptor signaling contributes to excitotoxicity. In line with these findings, enhancing $GABA_B$ receptor activity during ischemic insults by application of the $GABA_B$ receptor agonist baclofen has been reported to be neuroprotective *in vitro* and *in vivo* (10–19).

Cerebral ischemia induces ER stress, which is characterized by the accumulation of proteins in the ER, leading to the acti-

* This study was supported by Swiss National Science Foundation grants 31003A_121963 and 31003A_138382 (to D. B.).
[1] To whom correspondence should be addressed: Institute of Pharmacology and Toxicology, University of Zurich, Winterthurerstr. 190, 8057 Zurich, Switzerland. Tel.: 41-44-635-5930; Fax.: 41-44-635-6874; E-mail: benke@pharma.uzh.ch.

[2] The abbreviations used are: ER, endoplasmic reticulum; CHOP, CCAAT/enhancer-binding protein-homologous protein; C/EBP, CCAAT/enhancer-binding protein; PLA, proximity ligation assay; PDI, protein disulfide isomerase; sPSC, spontaneous postsynaptic current; OGD, oxygen and glucose deprivation; GM, Golgi apparatus marker; EGFP, enhanced GFP; E18, embryonic day 18.

vation of several pathways to restore normal ER function (20). If, however, ER function is severely impaired and cellular homeostasis cannot be restored, apoptosis of the neuron is induced. ER stress triggers the activation and expression of a number of proteins, including up-regulation of the transcription factor CHOP (C/EBP-homologous protein, also known as C/EBPζ, growth arrest- and DNA damage-inducible gene 153 (GADD153) or DNA-damage inducible transcript 3 (DDIT3)). CHOP belongs to the C/EBP transcription factor family and has been shown to trigger apoptosis (21).

Besides its function as a transcription factor, there is evidence that CHOP interacts with $GABA_B$ receptors to regulate their cell surface expression. We showed previously that CHOP interacts with its C-terminal leucine zipper, with the leucine zipper present in the C-terminal domain of $GABA_{B2}$ and with its N-terminal domain with an as yet unidentified site in $GABA_{B1}$ (22). Upon coexpression in HEK 293 cells, CHOP induced the intracellular accumulation of $GABA_B$ receptors and a significant reduction of cell surface receptors (22). However, the mechanism behind this down-regulation of cell surface receptors as well as its physiological role remained unclear. Because ischemic conditions cause ER stress and up-regulate CHOP, we aimed, in this study, to elucidate the mechanism of CHOP-induced down-regulation of cell surface $GABA_B$ receptors in neurons and its putative role in cerebral ischemia. We found that ER stress-induced CHOP interacts with $GABA_B$ receptors in the ER to disrupt $GABA_B$ receptor heterodimerization. This prevents forward trafficking of the receptors to the plasma membrane and, thus, leads to the down-regulation of cell surface receptors and reduced $GABA_B$ receptor signaling. This mechanism is operative in an *in vitro* model of cerebral ischemia.

EXPERIMENTAL PROCEDURES

Antibodies—The following primary antibodies were used: rabbit $GABA_{B1a,b}$ directed against the C terminus of $GABA_{B1}$ and rabbit $GABA_{B1b}$ directed against the N terminus of $GABA_{B1b}$ (affinity-purified, 1:200 for immunofluorescence, 1:40 for *in situ* PLA, custom-made by GenScript) (23) as well as rabbit $GABA_{B2}$ directed against the N terminus of $GABA_{B2}$ (24) (affinity-purified, 1:500 for immunofluorescence, 1:40 for *in situ* PLA, custom-made by GenScript), guinea pig $GABA_{B2}$ (1:50, Chemicon International, catalog no. AB5394), rabbit $GABA_A$ receptor α1 subunit (25) (affinity-purified, 1:100 for *in situ* PLA), mouse $GABA_A$ receptor β2/3 subunits (25) (1:500 for in *situ* PLA), mouse GADD153/CHOP (1:100, Santa Cruz Biotechnology, catalog no. sc-7351), mouse GADD153/CHOP (1:100 for immunofluorescence, 1:30 for *in situ* PLA, Cell Signaling Technology, catalog no. 2895), rabbit Anti-MAP Kinase (ERK1, ERK2, 1:500, Sigma-Aldrich, catalog no. M5670), mouse anti-MAP kinase-activated (diphosphorylated ERK1 and ERK2, 1:250, Sigma-Aldrich, catalog no. M9692), rabbit NeuN (1:500, Millipore AG, catalog no. ABN78), goat PDI (1:150, Santa Cruz Biotechnology, catalog no. sc-17222), mouse GM130 (1:1000, Abcam, catalog no. ab32337), rabbit HA (1:200, Santa Cruz Biotechnology, catalog no. sc-805), and rabbit c-myc (1:500, Santa Cruz Biotechnology, catalog no. sc-789). Secondary antibodies were either coupled to Alexa Fluor 488 (1:1000, Invitrogen), Cy-3 (1:500, Jackson ImmunoResearch Laboratories), or Cy-5 (1:300, Jackson ImmunoResearch Laboratories).

Plasmids—Plasmids containing full-length cDNA of human CHOP and deletion mutants of CHOP (C-terminal deletion (ΔLZ) and N-terminal deletion (ΔN)) have been described previously (22). Plasmids containing wild-type and mutant rat $GABA_{B1}$ and rat $GABA_{B2}$ were provided by Bernhard Bettler (University of Basel, Basel, Switzerland) and are described in Ref. 3.

Drugs—The following drugs were used: thapsigargin (1 μM, Sigma-Aldrich), (R)-baclofen (100 μM, Tocris Bioscience), and CPG 56999A (10 μM, a gift from Novartis, Basel, Switzerland).

Cell Culture—Primary cortical neurons were prepared from E18 embryos of time-pregnant Wistar rats as described previously (26). Briefly, minced E8 cortex was incubated for 15 min with papain solution (0.5 mg/ml PBS, 1 mg/ml BSA, 10 mM glucose, and 10 μg/ml DNase I), washed with Dulbecco's modified Eagle's medium containing 10% fetal calf serum and titrated using a Pasteur pipette. Neurons were plated at a density of 120,000 cells onto poly-L-lysine coverslips (12 mm) in 24-well plates and kept in culture at 37 °C and 5% CO_2 for 11–15 days.

Neurons in neuron-glia cocultures were transfected with plasmids using magnetofection, exactly as described in Ref. 27. 60,000 cells were plated on 18-mm coverslips and kept in culture for 11–15 days at 37 °C and 5% CO_2. Magnetofection was performed for 30 min on a prewarmed magnetic plate.

Immunocytochemistry and Confocal Laser-scanning Microscopy—Multiplex-labeling immunocytochemistry was performed as described previously (26). For the visualization of cell surface $GABA_B$ receptors, living neurons were incubated with primary antibodies for 2 h at 4 °C in ACSF (2 mM $CaCl_2$, 2 mM $MgCl_2$, 30 mM L-glucose, 5 mM KCl, 119 mM NaCl, and 25 mM HEPES (pH 7.4)) containing 10% normal goat serum. For staining of intracellularly localized proteins, neurons were subsequently fixed with 4% paraformaldehyde for 15 min at room temperature and permeabilized for 6 min with 0.2% Triton X-100. Neurons were then incubated with primary antibodies for 1 h (in PBS/10% normal goat serum) at room temperature, washed four times for 5 min with PBS, and incubated with secondary antibodies for 1 h. After four washes with PBS, neurons were mounted in fluorescence mounting medium and analyzed by confocal laser-scanning microscopy (LSM510 Meta, Zeiss). Images were acquired using a Zeiss ×100 plan apochromat oil differential interference contrast objective (1.4 numerical aperture) at 512 × 512 pixel resolution for fluorescence intensity measurements or 1024 × 1024 pixel resolution for colocalization studies. For each neuron, five optical sections spaced by 0.4 μm were taken.

Fluorescence intensity measurements were performed using the Mac Biophotonics ImageJ software (version 1.41n). For analysis of cell surface protein expression, cells were outlined carefully, and the mean fluorescence intensity of the soma was subtracted. For total protein expression analysis, somata of neurons were outlined carefully, and the mean intensity of the fluorescence signals was measured. An area of each image con-

taining no specific signals was selected for determining background staining and was subtracted from the image.

Colocalization studies were performed using Imaris (version 7.1.1, Bitplane, Zurich, Switzerland). Images were smoothed using the median filter tool (filter size, $3 \times 3 \times 1$) and processed further by setting threshold cutoffs for each channel to exclude background staining. Colocalization channels were built (colocalization intensity 255, constant value), and protein clusters (>15 pixels) as well as colocalized clusters were counted within a randomly selected, 30-μm^2 area of the somata.

Gene Expression Assays—$GABA_{B1}$, $GABA_{B2}$, and CHOP RNA levels were determined in cortical primary neurons using real-time PCR. Total RNA was extracted from neurons using the GenElute mammalian total RNA miniprep kit (Sigma-Aldrich) according to the recommendations of the manufacturer. Reverse transcription was performed with the QuantiTec reverse transcription kit (Qiagen). Quantitative real-time PCR (7900HT fast real-time PCR system, Applied Biosciences) was done using the prepared cDNA and TaqMan gene expression assays (Applied Biosciences) for $GABA_{B1}$ (Gabbr1, assay ID Rn00578911_m1), $GABA_{B2}$ (Gabbr2, assay ID Rn00582550_m1), CHOP (Ddit3, assay ID Rn00492098_g1), and β-actin (Actb, assay ID Mm00607939_s1) as a control. Quantification of RNA levels was done using the $\Delta\Delta$Ct method.

Baclofen-induced ERK1/2 Phosphorylation—$GABA_B$ receptor activity was indirectly determined by measuring the levels of baclofen-induced ERK1/2 phosphorylation (28–31). Cortical neurons were incubated with the $GABA_B$ receptor agonist baclofen for 10 min at 37 °C, 5% CO_2 or were left untreated for controls. Subsequently, the cultures were placed on ice, fixed with 4% paraformaldehyde for 15 min at 4 °C, and permeabilized with 0.2% Triton X-100 for 6 min. For determination of ERK1/2 phosphorylation, cultures were incubated overnight at 4 °C with antibodies directed against total ERK1/2 as well as with antibodies against diphosphorylated ERK1/2. After washing with PBS, secondary antibodies were added for 1 h at room temperature, and neurons were analyzed by measuring fluorescence intensities of total and diphosphorylated ERK1/2 levels using confocal laser-scanning microscopy. Levels of phosphorylated ERK1/2 were normalized to total ERK1/2 levels. The specificity of the baclofen-induced ERK1/2 phosphorylation was determined using the $GABA_B$ receptor antagonist CPG 56999A.

In Situ PLA—The *in situ* PLA is a highly sensitive antibody-based method for the visualization of protein-protein interactions and posttranslational modification in cultured cells and tissue sections (32, 33). This method employs two primary antibodies detecting the proteins of interest raised in different species and corresponding secondary antibodies (PLA probes) tagged with oligonucleotides. Only when the proteins of interest are in close proximity (<30 nm), specific connector oligonucleotides can be hybridized and ligated to the oligonucleotides attached to the secondary antibodies, forming a circular oligonucleotide. Rolling circle amplification then creates a large DNA strand to which numerous fluorophore-labeled oligonucleotides (detection probes) are hybridized. This generates a bright fluorescent spot that can be easily detected by microscopy. Quantification is done by counting the number of spots.

Here we used *in situ* PLA for analyzing the interaction of CHOP with $GABA_B$ receptors and the heterodimerization of $GABA_{B1}$ and $GABA_{B2}$. *In situ* PLA was performed using the Duolink kit (Olink Bioscience) according to the protocol of the manufacturer, as described previously (34). The specificity of the PLA signal was verified for both pairs of antibodies in HEK 293 cells expressing or not expressing one of the interaction partners. Furthermore, leaving out one of the primary antibodies completely prevented PLA signals. For *in situ* PLA, neurons were fixed and permeabilized as described above and incubated with primary antibodies (in PBS/10% normal goat serum) overnight at 4 °C. After *in situ* PLA, neurons were analyzed by confocal laser-scanning microscopy as described above. Protein-protein interactions were quantified by counting signal dots using the ImageJ software. Image stacks (five optical sections spaced by 0.4 μm) of individual neurons were merged, visualizing maximum intensities, and the number of maxima per area was determined.

Forward Trafficking Assay—For visualization of the amount of $GABA_B$ receptors inserted into the plasma membrane within a time period of 16 h, neurons were incubated in culture medium with antibodies directed against $GABA_{B2}$ for 2 h at 37 °C. Following washes with ACSF to remove unbound primary antibody, cells were incubated for 2 h with a large excess of Alexa Fluor 488-conjugated secondary antibody to label (*i.e.* mask) the existing pool of cell surface receptors. After washing, the neurons were further incubated in culture medium for 16 h at 37 °C to allow neosynthesis of $GABA_B$ receptors and forward trafficking of receptors to the plasma membrane. Neurons were then placed on ice and stained at 4 °C for receptors newly inserted into the plasma membrane using antibodies directed against $GABA_{B2}$ and Cy3-conjugated secondary antibodies. Neurons were then processed for confocal laser-scanning microscopy. Controls for judging the efficiency of labeling (*i.e.* masking) the pool of cell surface receptors were treated in exactly the same manner but kept at 4 °C for the 16-h incubation period.

Electrophysiology—Neurons of thapsigargin-treated or untreated control cultures were recorded in the whole-cell voltage clamp configuration at room temperature. Spontaneously occurring postsynaptic currents (sPSCs) were recorded before, during, and after the application of 50 μM baclofen at a holding potential of −60 mV. Patch electrodes were pulled from borosilicate glass and filled with 120 mM CsCl, 10 mM EGTA, 10 mM HEPES (pH 7.4), 4 mM $MgCl_2$, 0.5 mM GTP, and 2 mM ATP. The external solution contained 140 mM NaCl, 10 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES (pH 7.4), and 10 mM glucose. Recordings were performed with a HEKA EPC-7 amplifier and Patch Master v2.11 software (HEKA Elektronik, Germany). Baclofen (50 μM) was applied locally using an outlet tube (inner diameter, 200 μm) of a custom-designed, gravity-fed microperfusion system positioned 50–100 μm of the recorded neuron. All synaptic events displaying amplitudes above the background noise (5–12 pA) were identified and analyzed offline using the MiniAnalysis 6.0.7 software (Synaptosoft). Mean amplitudes and frequency values were obtained from 1-min-long recordings for each experimental condition and normalized.

Oxygen and Glucose Deprivation (OGD) Model of Ischemia—Primary cortical neurons were washed twice with DMEM without glucose or with DMEM containing glucose for controls. For OGD, neurons were incubated for 10 min at 37 °C in 95% N_2/5% CO_2 prebubbled, glucose-free DMEM in an airtight box filled with 95% N_2/5% CO_2. Control cultures were incubated in DMEM containing glucose at 37 °C in a physiological oxygen-containing environment. Subsequently, cells were washed twice with DMEM containing glucose and incubated for 24 h in their original culture medium.

RESULTS

CHOP Interacts with GABA$_B$ Receptors and Down-regulates Cell Surface Receptors in Neurons—We have previously shown that the stress-induced transcription factor CHOP, which is only marginally expressed under normal physiological conditions, interacts with GABA$_B$ receptors by binding to the coiled coil motif in the C-terminal domain of GABA$_{B2}$ and with a so far unidentified site in GABA$_{B1}$ (22). Upon overexpression in HEK 293 cells, the interaction of CHOP with GABA$_B$ receptors resulted in the intracellular accumulation and a reduced cell surface expression of the receptors by an as yet unknown mechanism (22). To confirm an interaction of GABA$_B$ receptors with CHOP in neurons, we overexpressed CHOP in cultured cortical neurons and tested for an interaction with GABA$_B$ receptors using the *in situ* PLA (32, 33). In non-transfected neurons, only very few PLA dots were detected, in line with a low expression level of CHOP under normal physiological conditions. However, overexpression of CHOP dramatically increased PLA signals, indicating numerous GABA$_B$ receptor-CHOP interactions (Fig. 1*A*).

Because ER stress-induced up-regulation of CHOP has been shown to play an important role in ischemia-induced neuronal death (35–42), we then analyzed the mechanism of CHOP-induced down-regulation of cell surface GABA$_B$ receptors by exposing cultured cortical neurons to ER stress. ER stress was induced by treating neurons for 2 h with the sarco/endoplasmic reticulum Ca^{2+}-ATPase blocker thapsigargin. After a 16-h recovery period, neurons were analyzed for CHOP and GABA$_B$ receptor interaction using an *in situ* PLA. Under control conditions, *i.e.* in untreated neurons, only few interactions were observed (Fig. 1*B*). However, upon up-regulation of CHOP with thapsigargin, numerous interactions with GABA$_B$ receptors were detected (454 ± 53% of control, $n = 30$, $p < 0.001$, Fig. 1*B*). Thus, up-regulation of CHOP by ER stress resulted in an interaction with GABA$_B$ receptors. Under these conditions, no *in situ* PLA signals were generated using CHOP and GABA$_A$ receptor antibodies, documenting the specificity of the CHOP-GABA$_B$ receptor interaction (Fig. 1*C*).

An analysis of the protein expression levels revealed that CHOP was vastly up-regulated in thapsigargin-treated neurons (706 ± 65% of control, $n = 57$, $p < 0.001$; Fig. 2*A*), whereas the cell surface staining of GABA$_{B1}$ and GABA$_{B2}$ was decreased significantly (GABA$_{B1}$, 67 ± 4%; GABA$_{B2}$, 50 ± 3% of control, $n = 50–55$, $p < 0.001$, Fig. 2*A*). No colocalization of CHOP with cell surface GABA$_B$ receptors was detected. In contrast to cell surface receptors, total GABA$_{B1}$ and GABA$_{B2}$ expression levels remained unchanged in thapsigargin-treated neurons (Fig. 2*B*).

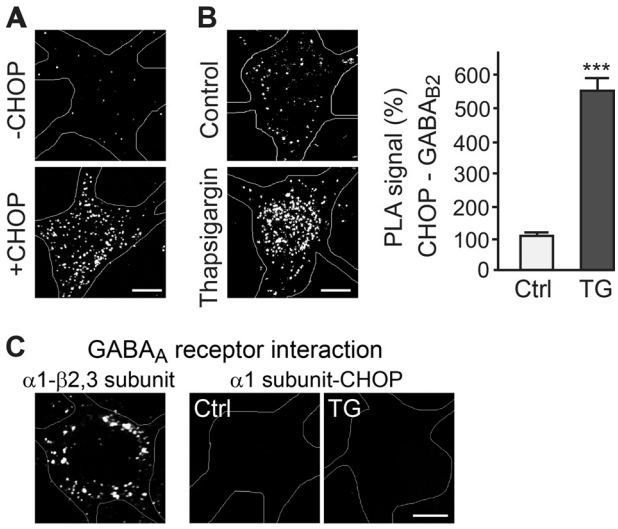

FIGURE 1. **Up-regulated CHOP interacts with GABA$_B$ receptors in neurons.** *A*, CHOP overexpressed in neurons interact with GABA$_B$ receptors. Cortical neurons were left non-transfected (−*CHOP*) or were transfected with CHOP (+*CHOP*) and analyzed for interaction with GABA$_B$ receptors by *in situ* PLA using antibodies directed against CHOP and GABA$_{B2}$. Because under normal physiological conditions CHOP expression is low, only few interactions (*white dots* in representative images) were detected. However, overexpression of CHOP resulted in numerous CHOP-GABA$_B$ receptor interactions. The images represent merged Z-stack reconstructions of five optical section spaced by 0.4 μm. *Scale bar* = 5 μm. *B*, thapsigargin-induced CHOP interacts with GABA$_B$ receptors. Cortical neurons were incubated with 2 μM thapsigargin (*TG*) for 2 h and allowed to recover in their original culture medium for 16 h. The interaction of CHOP with GABA$_B$ receptors was determined by *in situ* PLA using antibodies directed against CHOP and GABA$_{B2}$ (*white dots* in representative images, *left panel*, *scale bar* = 5 μm). *Right panel*, quantification of *in situ* PLA signals. Data are means ± S.E., 30 neurons from four experiments. ***, $p < 0.001$, Student's *t* test. *Ctrl*, control. *C*, CHOP does not interact with GABA$_A$ receptors. Cortical neurons were treated (*TG*) or not treated (*Ctrl*) with 2 μg of thapsigargin for 2 h and allowed to recover in their original culture medium for 16 h. The interaction of CHOP with GABA$_A$ receptors was determined by *in situ* PLA using antibodies directed against CHOP and the α1 subunit of GABA$_A$ receptors. No interactions were observed (*right panel*). As a positive control, the interaction of the GABA$_A$ receptor subunits α1 and β2/3 was tested. Numerous interactions were detected in untreated neurons (*white dots* in representative image, *left panel*). The images represent merged Z-stack reconstructions of five optical section spaced by 0.4 μm. *Scale bar* = 5 μm.

These findings suggest that the interaction of CHOP with GABA$_B$ receptors caused a down-regulation of the receptors from the cell surface and their intracellular accumulation.

To verify that the loss of cell surface receptors was caused by the interaction of CHOP with GABA$_B$ receptors, neurons were transfected with mutant forms of CHOP that are unable to interact with GABA$_{B1}$ (CHOPΔN, N-terminal deletion (22)) or GABA$_{B2}$ (CHOPΔLZ, deletion of C-terminal leucine zipper motif (22)). Neurons overexpressing wild-type CHOP displayed a significantly reduced level of cell surface GABA$_{B2}$ compared with control neurons transfected with EGFP (63 ± 4%, $n = 82$, $p < 0.001$, Fig. 2*C*). However, overexpressing either of the CHOP mutants did not affect cell surface expression of GABA$_B$ receptors (CHOPΔLZ, 92 ± 5%, $p > 0.05$, $n = 79$; CHOPΔN, 103 ± 5%, $n = 101$, $p > 0.05$), suggesting that down-regulation of cell surface receptors was mediated by its interaction with CHOP.

To rule out that up-regulation of CHOP affects transcription of GABA$_{B1}$ and GABA$_{B2}$, their mRNA levels were quantified in neurons treated with thapsigargin using real-time PCR (Fig. 2*D*).

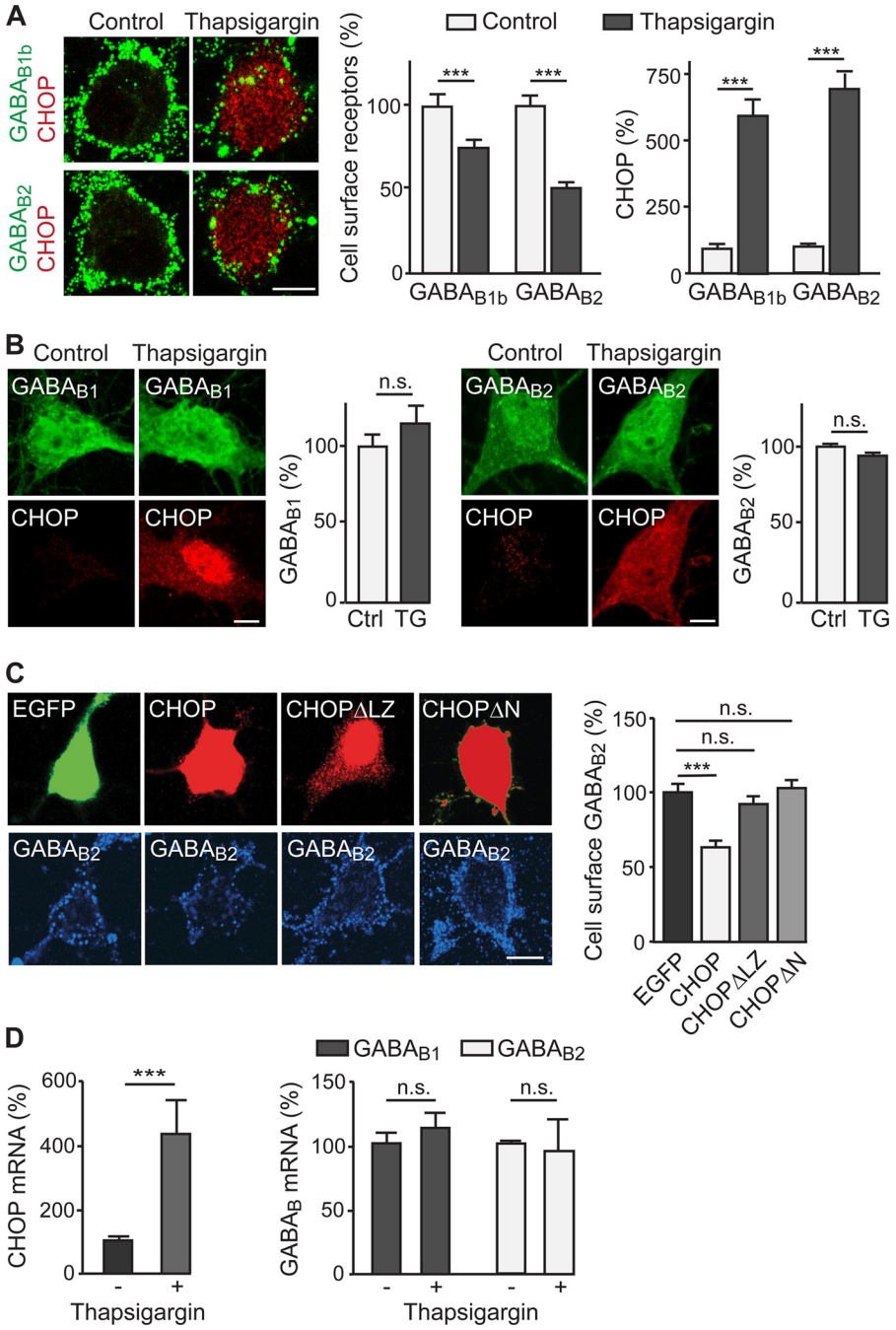

FIGURE 2. **Up-regulated CHOP in neurons mediates down-regulation of cell surface GABA$_B$ receptors.** *A*, up-regulation of CHOP down-regulates cell surface GABA$_B$ receptors. Neurons were treated with thapsigargin and stained for surface GABA$_{B1}$ and GABA$_{B2}$ (*green*). Subsequently, cells were fixed, permeabilized, and stained for CHOP (*red*). Neurons not treated with thapsigargin served as controls. *Left panel*, representative images. *Scale bar* = 7 μm. *Right panel*, quantification of cell surface GABA$_{B1}$, GABA$_{B2}$, and CHOP. Data are means ± S.E., 50–55 neurons from five experiments. ***, $p < 0.001$, Student's *t* test. *B*, up-regulation of CHOP does not affect total GABA$_B$ receptor expression levels. Neurons were treated with thapsigargin (*TG*) and stained for total GABA$_{B1}$ and GABA$_{B2}$ (*green*) and CHOP (*red*). Neurons not treated with thapsigargin served as controls (*Ctrl*). *Scale bar* = 10 μm. *Bar graphs* show the quantification of total expression levels of GABA$_{B1}$ and GABA$_{B2}$. Data are means ± S.E., 30 neurons from three experiments. *n.s.*, not significant, $p > 0.05$, Student's *t* test. *C*, mutant forms of CHOP that cannot bind to GABA$_B$ receptors do not mediate down-regulation of cell surface receptors. Neurons were cotransfected with EGFP and CHOP or mutant forms of CHOP lacking its interaction site with GABA$_{B2}$ (CHOPΔLZ) or its interaction site with GABA$_{B1}$ (CHOPΔN). After 48 h, transfected neurons were stained for cell surface GABA$_{B2}$ (*green*) and CHOP (*red*). *Left panel*, representative images. *Scale bar* = 10 μm. *Right panel*, quantification of cell surface GABA$_{B2}$ levels in neurons expressing either CHOP, CHOPΔLZ, or CHOPΔN. Data are means ± S.E.; 82 (EGFP and CHOP), 79 (CHOPΔLZ), and 101 (CHOPΔN) neurons from three experiments. ***, $p < 0.001$; one-way analysis of variance, Bonferroni's multiple comparison test. *D*, CHOP does not regulate GABA$_B$ receptor mRNA levels. Total mRNA was isolated from neuronal cultures treated or not treated (controls) with thapsigargin. CHOP, GABA$_{B1}$, and GABA$_{B2}$ mRNA was quantified using real-time PCR. Data are means ± S.E., four (CHOP) and five (GABA$_{B1}$ and GABA$_{B2}$) individual cultures were analyzed. ***, $p < 0.001$; *n.s.*, not significant ($p > 0.05$); Student's *t* test.

CHOP mRNA levels were increased drastically in thapsigargin-treated neurons (432 ± 54% of control, $n = 4$, $p < 0.001$), but GABA$_{B1}$ and GABA$_{B2}$ mRNA levels remained unchanged (GABA$_{B1}$, 113 ± 6% of control, $n = 5$, $p > 0.05$; GABA$_{B2}$, 94 ± 12% of control, $n = 5$, $p > 0.05$). Thus, CHOP does not regulate GABA$_B$ receptor expression at the transcriptional level.

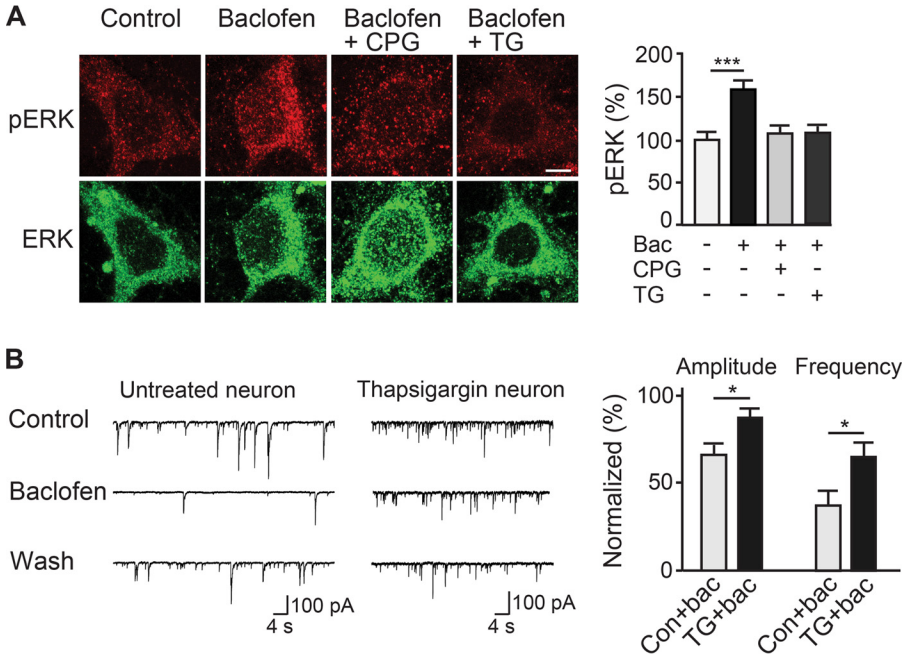

FIGURE 3. **CHOP-mediated down-regulation of cell surface GABA$_B$ receptors impairs GABA$_B$ receptor-mediated downstream signaling.** *A*, GABA$_B$ receptor-induced phosphorylation of ERK1/2 was prevented by the GABA$_B$ receptor antagonist CPG 56999A (*CPG*) and by thapsigargin-induced up-regulation of CHOP. Neurons were treated with thapsigargin (+*TG*) to up-regulate CHOP, whereas control cultures remained untreated. Subsequently, neurons were incubated for 10 min with 100 μM baclofen (*Bac*) in the presence or absence of 10 μM CPG 56999A (+*CGP*, GABA$_B$ receptor antagonist). After fixation and permeabilization, neurons were stained for total ERK1/2 (*ERK*, *green*) and diphosphorylated ERK1/2 (*pERK*, *red*). *Left panel*, representative images. *Scale bar* = 5 μm. *Right panel*, quantification of pERK levels. Data are means ± S.E., 26 (*Baclofen*, *Baclofen + CPG*) and 28 (*Baclofen + TG*) neurons from three experiments. ***, $p < 0.001$, one-way analysis of variance, Bonferroni's multiple comparison test. *B*, up-regulation of CHOP diminished baclofen-induced inhibition of sPSCs. *Left panel*, representative current traces depicting spontaneous sPSCs recorded from untreated control neurons ($n = 9$) or from neurons treated with thapsigargin for up-regulation of CHOP ($n = 5$). *Right panel*, normalized amplitude and frequency values of the sPSCs. Mean amplitudes and frequency values were normalized to the control condition of the individual neuron. *Con.*, control. Data are mean ± S.E. *, $p < 0.05$; Student's *t* test.

CHOP-mediated Down-regulation of Cell Surface Receptors Reduces GABA$_B$ Receptor Signaling—It is well established that GABA$_B$ receptors activate extracellular-signal-regulated kinase 1/2 (ERK1/2) in neurons (28–31). To analyze the functional consequences of CHOP-mediated down-regulation of GABA$_B$ receptors, receptor activity was assayed on the basis of baclofen-induced ERK1/2 phosphorylation. Activation of GABA$_B$ receptors with 100 μM baclofen in control neurons significantly increased ERK1/2 phosphorylation (159 ± 10%, $n = 26$, $p < 0.001$, Fig. 3*A*), which was abolished by preincubation of neurons with the GABA$_B$ receptor antagonist CPG 56999A (106 ± 10%, $n = 26$, $p > 0.05$; Fig. 3*A*). However, in thapsigargin-treated neurons, no increase of ERK1/2 phosphorylation was observed (108 ± 8% of control, $n = 28$, $p > 0.05$, Fig. 3*A*), indicating that CHOP-mediated down-regulation of cell surface GABA$_B$ receptors inhibits downstream signaling.

To test whether CHOP-induced down-regulation of GABA$_B$ receptors affects GABA$_B$ receptor-mediated neuronal inhibition, spontaneous synaptic activity was measured in electrophysiological experiments using the whole-cell voltage clamp configuration. In untreated control neurons, application of baclofen considerably decreased the amplitude (66 ± 6% of control, $n = 9$) and the frequency (38 ± 8% of control, $n = 5$) of sPSCs (Fig. 3*B*). However, after up-regulation of CHOP by treating cultures with thapsigargin, baclofen-induced inhibition of sPSCs was strongly reduced (amplitude, 87 ± 6% of control, $n = 9$, $p < 0.05$; frequency, 65 ± 9% of control, $n = 5$, $p < 0.05$; Fig. 3*B*). This finding is consistent with an up-regulation of CHOP and, subsequently, reduced levels of functional GABA$_B$ receptors available for neuronal inhibition.

CHOP Interacts with GABA$_B$ Receptors in the ER and Interferes with Receptor Heterodimerization—Next, we investigated the mechanism of CHOP-mediated down-regulation of cell surface GABA$_B$ receptors. We envisioned two scenarios. 1) CHOP disrupts functional GABA$_B$ receptor heterodimers on the cell surface, which would lead to their internalization and degradation, or 2) CHOP interacts with GABA$_B$ receptors in the ER and inhibits receptor heterodimerization and, consequently, forward transport to the cell surface. To test these two scenarios, we analyzed the colocalization of CHOP and GABA$_B$ receptor subunits in different cellular compartments in untreated control neurons and neurons treated with thapsigargin to up-regulate CHOP. In thapsigargin-treated neurons, GABA$_B$ receptors accumulated in the ER, as indicated by an increased colocalization of GABA$_{B1}$ (control, 10 ± 0.7 clusters, $n = 20$; thapsigargin, 15 ± 0.8 clusters, $n = 21$, $p < 0.001$) and GABA$_{B2}$ (control, 7 ± 0.7 clusters, $n = 32$; thapsigargin, 13 ± 0.7 clusters, $n = 32$, $p < 0.001$) with the ER marker PDI (Fig. 4, *A* and *B*). Hardly any CHOP was observed in the ER under control conditions, whereas CHOP accumulated in the ER after thapsigargin treatment (GABA$_{B1}$-stained neurons, control, 2 ± 0.4 clusters, $n = 20$; thapsigargin, 9 ± 0.9 clusters, $n = 21$, $p < 0.001$; Fig. 4*A*; GABA$_{B2}$-stained neurons, control, 1.5 ± 0.4 clusters, $n = 32$; thapsigargin, 9 ± 0.6 clusters, $n = 32$, $p < 0.001$; Fig. 4*B*). Triple colocalization of GABA$_B$-CHOP-PDI was basically absent under control conditions but increased

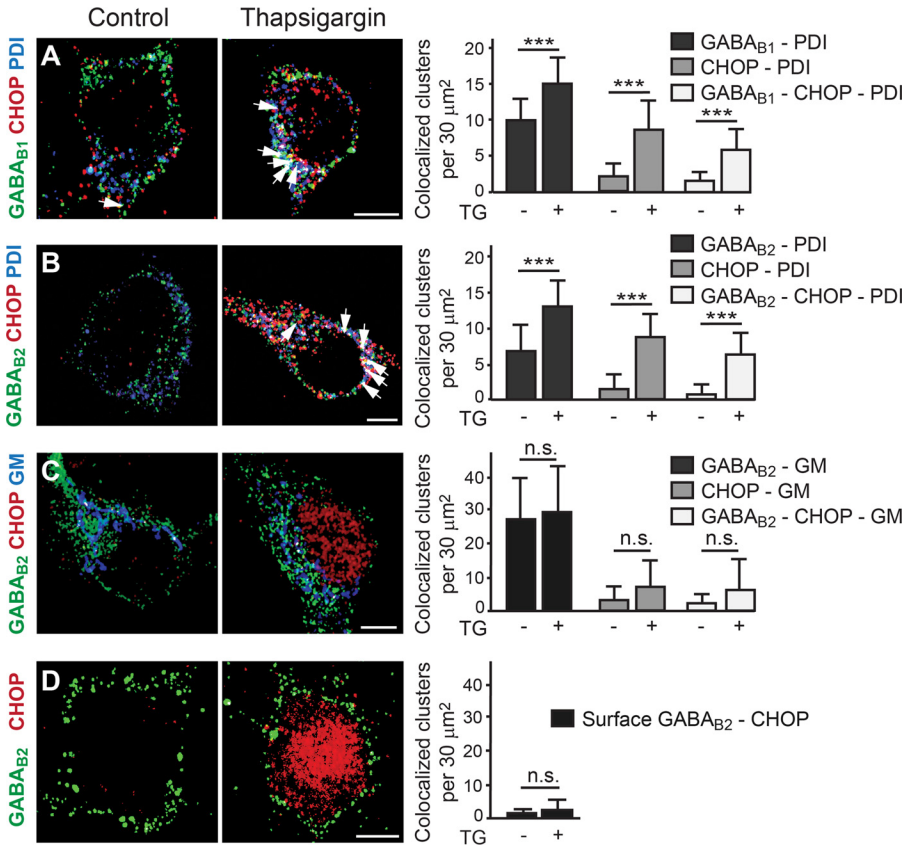

FIGURE 4. **Up-regulated CHOP colocalizes with GABA$_B$ receptors in the ER and induces their intracellular accumulation.** *A* and *B*, GABA$_B$ receptors accumulate in the ER and colocalize with CHOP. Neurons were treated with thapsigargin (*TG*) to up-regulate CHOP and stained for GABA$_{B1}$ (*A*) or GABA$_{B2}$ (*B*) (*green*), CHOP (*red*), and the ER marker PDI (*blue*). Cultures not treated with thapsigargin served as controls. *Left panel*, representative images depicting GABA$_{B1}$/$_{B2}$-CHOP-PDI colocalization (*white dots* marked with *arrows*, *scale bar* = 5 µm). *Right panel*, quantification of colocalization. Data are means ± S.D., 20–32 neurons/colocalization from three experiments. ***, $p < 0.001$, Student's *t* test. *C*, GABA$_B$ receptors do not accumulate or colocalize with CHOP in the Golgi apparatus. Neurons were treated with thapsigargin to up-regulate CHOP and stained for GABA$_{B2}$ (*green*), CHOP (*red*), and the Golgi marker GM130 (*blue*). Cultures not treated with thapsigargin served as controls. *Left panel*, representative images. *Scale bar* = 5 µm. *Right panel*, quantification of colocalization. Data are means ± S.D., 19 neurons/colocalization from three experiments. *n.s.*, not significant ($p > 0.05$), Student's *t* test. *D*, GABA$_B$ receptors do not colocalize with CHOP on the cell surface. Neurons were treated with thapsigargin to up-regulate CHOP and then stained for cell surface GABA$_{B2}$ (*green*) as well as for CHOP (*red*). Cultures not treated with thapsigargin served as controls. *Left panel*, representative images. *Scale bar* = 5 µm. *Right panel*, quantification of colocalization. Data are means ± S.D., 44 (control) and 45 (thapsigargin) neurons from four experiments. *n.s.*, not significant ($p > 0.05$), Student's *t* test.

significantly after up-regulation of CHOP by thapsigargin (Fig. 4, *A* and *B*; GABA$_{B1}$-CHOP-PDI, control, 1.4 ± 0.3 clusters, $n = 20$; thapsigargin, 6 ± 0.7 clusters, $n = 21$, $p < 0.01$; GABA$_{B2}$-CHOP-PDI, control, 0.7 ± 0.3 clusters, $n = 32$; thapsigargin, 6 ± 0.5 clusters, $n = 32$, $p < 0.001$). These findings indicate that GABA$_B$ receptors interact with CHOP in the ER, which leads to the accumulation of GABA$_{B1}$ and GABA$_{B2}$ in the ER.

In contrast, GABA$_B$ receptors did not display increased colocalization with the Golgi apparatus marker (GM) GM130 in thapsigargin-treated neurons (control, 27 ± 3 clusters, $n = 19$; thapsigargin, 30 ± 3 clusters, $p > 0.05$, $n = 19$; Fig. 4*C*), indicating no accumulation of GABA$_B$ receptors in the Golgi after up-regulation of CHOP. In line with this finding, there was no statistically significant increase in CHOP-GM colocalization control (3 ± 1 clusters, $n = 19$; thapsigargin, 7 ± 2 clusters, $p > 0.05$, $n = 19$, $p > 0.05$; Fig. 4*C*) nor in GABA$_{B2}$-CHOP-GM (control, 2 ± 0.7 clusters, $n = 19$; thapsigargin, 6 ± 2 clusters, $n = 19$, $p > 0.05$; Fig. 4*C*), indicating marginal interactions between CHOP and GABA$_B$ receptors in the Golgi after thapsigargin-induced up-regulation of CHOP. In addition, there was no significant colocalization of CHOP with GABA$_B$ receptors at the cell surface (control, 1.3 ± 0.2 clusters, $n = 45$; thapsigargin, 2.3 ± 0.5 clusters, $n = 44$, $p > 0.05$; Fig. 4*D*), indicating that GABA$_B$ receptors do not interact with CHOP in the plasma membrane.

To demonstrate that the accumulation of GABA$_B$ receptors in the ER is mediated by direct interaction with CHOP, we expressed in neurons a mutant form of GABA$_{B2}$ lacking its leucine zipper (GABA$_{B2}$ΔLZ) and, thus, its CHOP interaction site, together with GABA$_{B1}$ containing a mutated ER retention signal (GABA$_{B1}$ASA) to ensure forward trafficking of the receptors. GABA$_{B1}$ASA was required because deletion of the GABA$_{B2}$ leucine zipper unmasks the ER retention signal in the C terminus of GABA$_{B1}$ and, therefore, prevents forward trafficking of the receptors. To verify normal trafficking behavior of the GABA$_{B1}$ASA/GABA$_{B2}$ΔLZ heterodimers, we analyzed the colocalization of GABA$_{B1}$ASA and GABA$_{B2}$ΔLZ with PDI and CHOP in thapsigargin-treated and untreated neurons. In thapsigargin-treated neurons, GABA$_{B1}$ASA (control, 10 ± 3 clusters, $n = 12$; thapsigargin, 15 ± 5 clusters, $n = 19$, $p < 0.01$, Fig. 5*A*), GABA$_{B2}$ (control, 13 ± 5 clusters, $n = 17$; thapsigargin, 17 ± 4 clusters, $n = 15$, $p < 0.01$, Fig. 5*B*), and CHOP (Fig. 5*A*, control, 1.3 ± 1.4 clusters, $n = 13$; thapsigargin, 11 ±

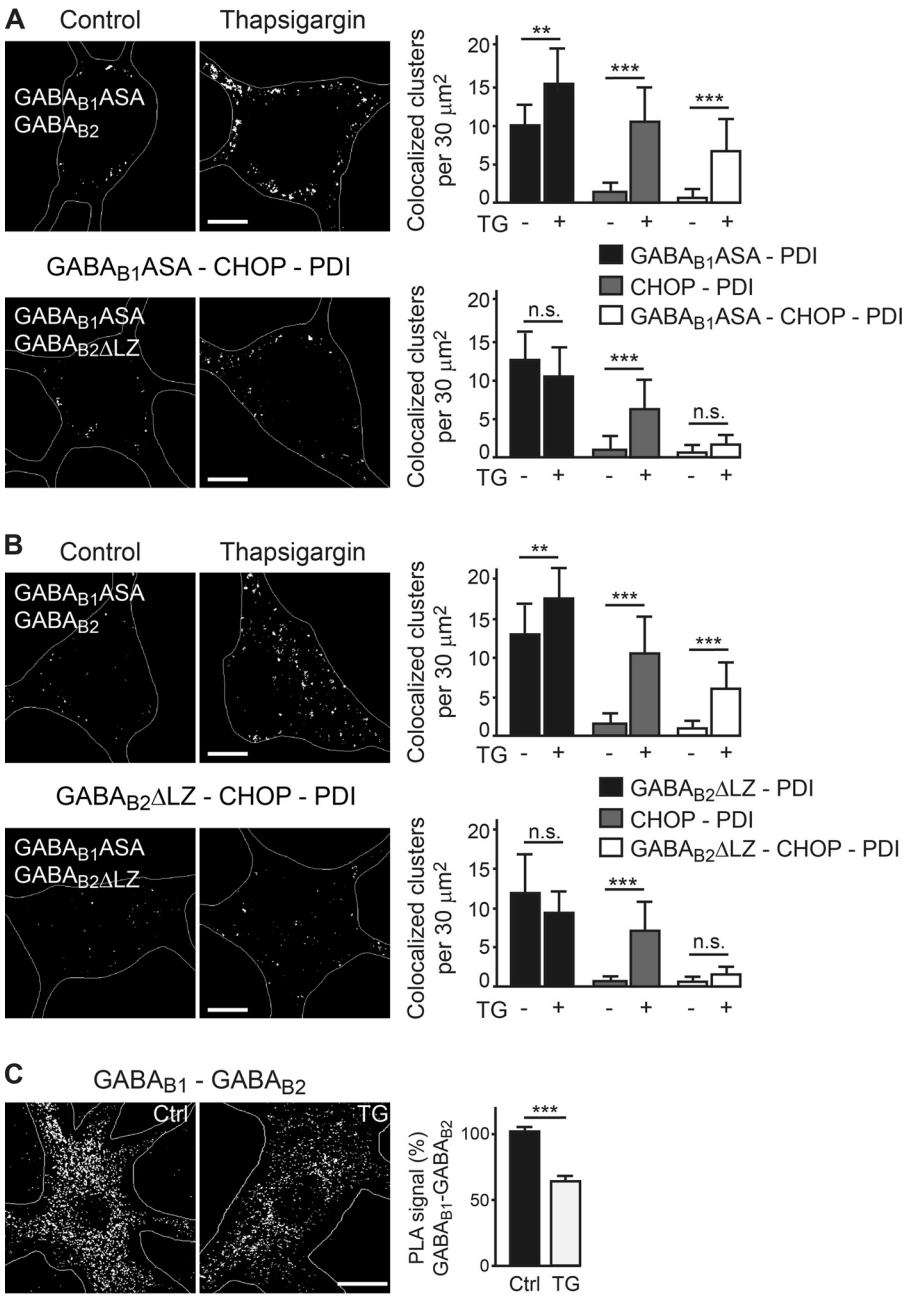

FIGURE 5. **Accumulation of GABA$_B$ receptors in the ER is mediated by interaction with CHOP.** *A* and *B*, neurons were transfected with EGFP in combination with GABA$_{B1}$ASA/GABA$_{B2}$ or GABA$_{B1}$ASA/GABA$_{B2}$ΔLZ. After 48 h, neurons were treated with thapsigargin (*TG*) to up-regulate CHOP and then stained for GABA$_{B1}$ASA (*A*) or GABA$_{B2}$ΔLZ (*B*), CHOP, and the ER marker PDI. Cultures not treated with thapsigargin served as controls. *Left panels*, representative images depicting colocalization of GABA$_{B1}$-CHOP-PDI and GABA$_{B2}$-CHOP-PDI (*white dots*). *Scale bars* = 5 µm). *Right panels*, quantification of colocalization. Data are means ± S.D., 11–22 neurons/colocalization from two experiments. **, $p < 0.01$;***, $p < 0.001$, *n.s.*, not significant ($p > 0.05$); Student's *t* test. *C*, Up-regulation of CHOP interferes with GABA$_B$ receptor heterodimerization. Neurons were treated with thapsigargin to up-regulate CHOP and tested for GABA$_{B1}$/GABA$_{B2}$ heterodimerization by *in situ* PLA using antibodies directed against GABA$_{B1}$ and GABA$_{B2}$ (*white dots* in representative images, *left panel*, *scale bar* = 5 µm). Cultures not treated with thapsigargin served as controls (*Ctrl*). The images represent merged Z-stack reconstructions of five optical section spaced by 0.4 µm. Right *panel*, quantification of *in situ* PLA signals. Data are means ± S.E., 32 neurons from three experiments. ***, $p < 0.001$; Student's *t* test.

5 clusters, $n = 20$, $p < 0.001$; Fig. 5*B*, control, 1.4 ± 1.8 clusters, $n = 17$; thapsigargin, 11 ± 5 clusters, $n = 16$, $p < 0.001$) showed increased colocalization with the ER marker PDI. Also, increased colocalization of GABA$_{B1}$ or GABA$_{B2}$ with CHOP and PDI (GABA$_{B1}$/CHOP/PDI and GABA$_{B2}$/CHOP/PDI) was detected in thapsigargin-treated neurons (GABA$_{B1}$ASA, control, 0.7 ± 1.1 clusters, $n = 13$; thapsigargin, 7 ± 4 clusters, $n = 20$, $p < 0.001$; GABA$_{B2}$, control, 0.8 ± 1.1 clusters, $n = 17$; thapsigargin, 6 ± 3 clusters, $n = 16$, $p < 0.001$; Fig. 5, *A* and *B*). The identical colocalization patterns of GABA$_{B1}$ASA/GABA$_{B2}$ receptors and endogenous GABA$_B$ receptors indicate normal trafficking capabilities of the mutant receptors.

However, transfection of neurons with GABA$_{B1}$ASA/GABA$_{B2}$ΔLZ receptors, which lack the CHOP interaction site in GABA$_{B2}$, fully abolished the increased colocalization of GABA$_{B1}$-PDI (control, 13 ± 4 clusters, $n = 14$; thapsigargin, 10 ± 4 clusters, $n = 20$, $p > 0.05$), GABA$_{B2}$-PDI (control, 12 ± 5 clusters, $n = 14$; thapsigargin, 9 ± 3 clusters, $n = 11$, $p > 0.05$), GABA$_{B1}$-CHOP-

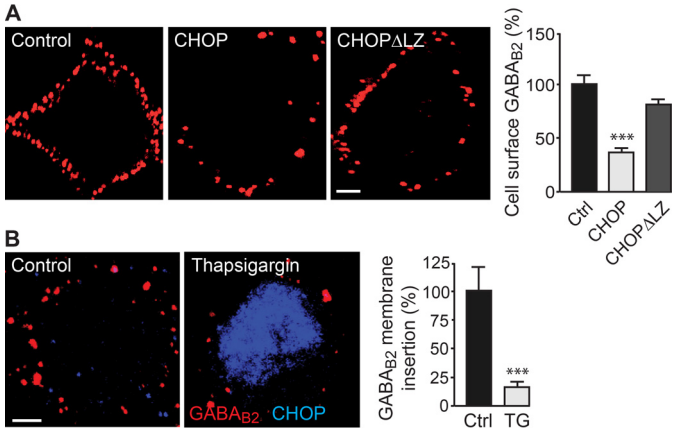

FIGURE 6. **Up-regulated CHOP impairs forward trafficking of GABA$_B$ receptors.** *A*, neurons were transfected with either EGFP and HA-tagged GABA$_{B2}$ (*Control*), EGFP, HA-GABA$_{B2}$ and CHOP, or EGFP HA-GABA$_{B2}$ and CHOPΔLZ (CHOPΔLZ). Twenty-four hours after transfection, GABA$_B$ receptors newly inserted into the plasma membrane were visualized by staining with HA-antibodies. *Left panel*, representative images. *Scale bar* = 5 μm. *Right panel*, quantification of GABA$_B$ receptor membrane insertion. Data are means ± S.E., 25 neurons/condition from three experiments. ***, $p < 0.001$; one-way analysis of variance, Dunnett's post-hoc test. *B*, neurons were treated with thapsigargin (*TG*) to up-regulate CHOP und subjected to an immunofluorescence-based forward trafficking assay to determine the amounts of GABA$_B$ receptors inserted into the plasma membrane within a time period of 16 h (*red clusters* in representative images, *scale bar* = 5 μm). CHOP expression is depicted in *blue*. Cultures not treated with thapsigargin served as controls. *Right panel*, quantification of GABA$_B$ receptor membrane insertion. Data are means ± S.E., 38 (control) and 33 (thapsigargin) neurons from three experiments. ***, $p < 0.001$; Student's *t* test.

PDI (control, 0.5 ± 1.1 clusters, $n = 15$; thapsigargin, 1.5 ± 1.5 clusters, $n = 22$, $p > 0.05$), and GABA$_{B2}$-CHOP-PDI (control, 0.3 ± 0.8 clusters, $n = 14$; thapsigargin, 1.3 ± 1.2 clusters, $n = 12$, $p > 0.05$; Fig. 5, *A* and *B*) after induction of CHOP with thapsigargin. This result verified that CHOP directly interacts with GABA$_B$ receptors to induce their accumulation in the ER.

Because GABA$_{B1}$ and GABA$_{B2}$ interact via the leucine zippers in their C-terminal domains, binding of CHOP to GABA$_{B2}$ may prevent GABA$_B$ receptor heterodimerization and, thus, inhibit forward trafficking of the receptors. Therefore, we tested whether up-regulation of CHOP interferes with heterodimerization of GABA$_{B1}$ and GABA$_{B2}$ using *in situ* PLA. In thapsigargin-treated neurons, we observed a significant reduction of *in situ* PLA signals (64 ± 5% of control, $n = 32$, $p < 0.001$, Fig. 5*C*), indicating the presence of considerably less GABA$_{B1}$/GABA$_{B2}$ heterodimers compared with untreated control neurons. This result suggests that the interaction of CHOP with GABA$_B$ receptors disrupts or prevents the heterodimerization of GABA$_{B1}$ and GABA$_{B2}$.

These findings suggest a mechanism in which CHOP interacts with GABA$_B$ receptors in the ER after cellular stress to prevent heterodimerization of GABA$_{B1}$ and GABA$_{B2}$. Because only heterodimerized GABA$_B$ receptors can leave the ER (2–4), this mechanism is expected to impede forward trafficking of newly synthesized receptors to the plasma membrane.

Up-regulation of CHOP Prevents Forward Trafficking of GABA$_B$ Receptors to the Cell Surface—To test whether up-regulation of CHOP impairs forward trafficking of GABA$_B$ receptors to the cell surface, we transfected neurons with HA-tagged GABA$_{B2}$ and CHOP. Twenty-four hours after transfection, newly synthetized HA-tagged receptors inserted into the plasma membrane were detected using an anti-HA antibody. In control neurons only transfected with HA-GABA$_{B2}$, strong cell surface expression of HA-tagged GABA$_{B2}$ was detected (Fig. 6*A*). However, in neurons transfected with CHOP, staining for cell surface HA-GABA$_{B2}$ was strongly reduced (36 ± 4% of control, $n = 25$, $p < 0.001$, Fig. 6*A*). This reduction of cell surface HA-GABA$_{B2}$ was not observed in neurons expressing a mutant of CHOP (CHOPΔLZ) that is unable to bind to GABA$_{B2}$ (80 ± 7% of control, $n = 25$, $p > 0.05$, Fig. 6*A*). These results suggest that the interaction of CHOP with GABA$_{B2}$ impairs forward trafficking of GABA$_B$ receptors to the cell surface.

This result was confirmed for native GABA$_B$ receptors with an immunofluorescence-based forward trafficking assay using untreated control neurons and thapsigargin-treated neurons. After masking the existing receptor pool on the cell surface with primary and secondary antibodies, the insertion of new receptors into the plasma membrane was tested after 16 h. Membrane insertion of GABA$_B$ receptors was reduced drastically in neurons expressing high levels of CHOP (16 ± 6% of control, $n = 33$, $p < 0.001$, Fig. 6*B*).

Down-regulation of Cell Surface GABA$_B$ Receptors by CHOP in the OGD Model of Ischemia—Next, we tested whether up-regulation of CHOP under ischemic conditions mediates down-regulation of cell surface GABA$_B$ receptors. We used the OGD *in vitro* model of ischemia, in which cultured cortical neurons were deprived of oxygen and glucose for 10 min, followed by a recovery period of 24 h. 24 h after OGD, CHOP expression in neurons was increased significantly (157 ± 5% of control, $n = 311$, $p < 0.001$, Fig. 7*A*). As expected, cell surface GABA$_B$ receptors decreased considerably 24 h after OGD in CHOP-expressing neurons (44 ± 3% of control, $n = 49$, $p < 0.001$, Fig. 7*B*).

To test whether the interaction of CHOP with GABA$_B$ receptors is responsible for the down-regulation of cell surface receptors after OGD, we overexpressed CHOPΔLZ in cortical neurons. CHOPΔLZ is a mutant form of CHOP that is not able to interact with GABA$_{B2}$ and does not affect cell surface numbers of GABA$_B$ receptors (Fig. 1*C*). Endogenous CHOP was significantly up-regulated after OGD in transfected (154 ± 8% of control, $n = 20$, $p < 0.001$) and in non-transfected neurons (167 ± 12% of control, $n = 22$, $p < 0.001$, Fig. 8). In non-transfected neurons, GABA$_{B2}$ receptors were down-regulated after OGD (60 ± 7% of control, $n = 27$, $p < 0.001$, Fig. 8), whereas in neurons overexpressing CHOPΔLZ, despite similar up-regulation of endogenous CHOP, the down-regulation of GABA$_B$ receptors was completely prevented (113 ± 7% of control, $n = 45$, $p > 0.05$,; Fig. 8). These results verify that down-regulation of cell surface GABA$_B$ receptors after ischemic conditions is mediated by their interaction with CHOP.

DISCUSSION

GABA$_B$ receptor-mediated neuronal inhibition critically depends on the availability of receptors in the plasma membrane. Receptor numbers might be altered under pathological conditions and a loss of receptors resulting in diminished neuronal inhibition is expected to contribute to the disease state.

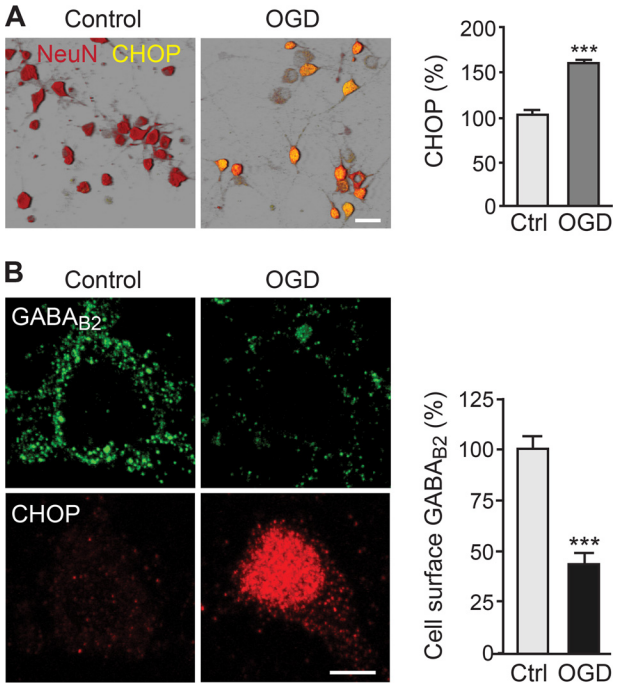

FIGURE 7. **CHOP is up-regulated and GABA$_B$ receptors are down-regulated in the OGD model of cerebral ischemia.** *A*, OGD in neurons up-regulates CHOP. Neurons were subjected to OGD for 10 min, followed by a recovery period of 24 h. Subsequently, neurons were stained for CHOP (*green*) and for NeuN to visualize the neurons (*red*). CHOP expression in neurons is depicted in *yellow*. Cultures not subjected to OGD served as controls (*Ctrl*). *Left panel*, representative images. *Scale bar* = 30 μm. *Right panel*, quantification of CHOP expression in neurons. Data are means ± S.E., 271 (control) and 311 (thapsigargin) neurons from three experiments. ***, $p < 0.001$; Student's *t* test. *B*, cell surface GABA$_B$ receptors are down-regulated following OGD. Neurons were subjected to OGD for 10 min, followed by a recovery period of 24 h, and were then stained for cell surface GABA$_{B2}$ (*green*) and CHOP (*red*). Cultures not subjected to OGD served as controls. *Left panel*, representative images. *Scale bar* = 5 μm. *Right panel*, quantification of cell surface GABA$_B$ receptor expression. Data are means ± S.E., 30 (control) and 49 (thapsigargin) neurons from three experiments. ***, $p < 0.001$; Student's *t* test.

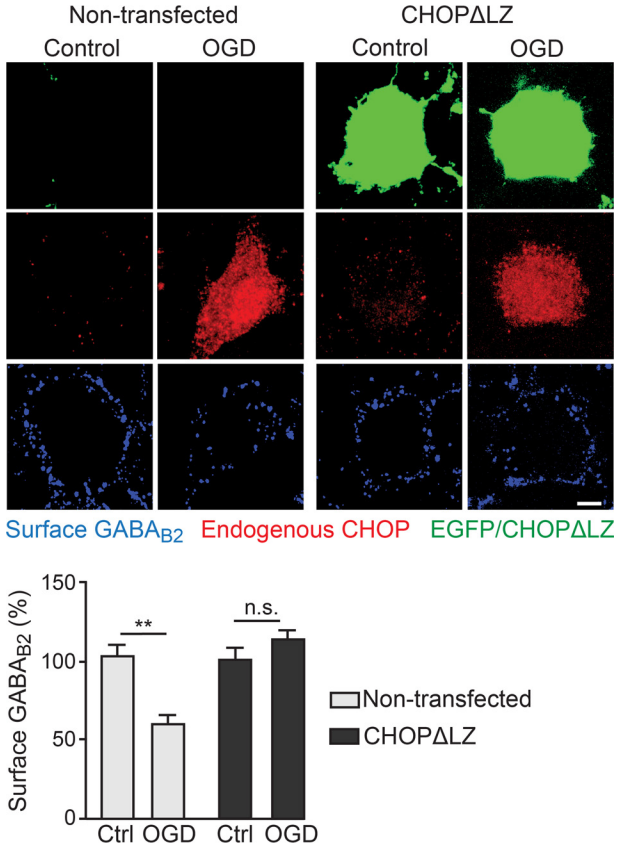

FIGURE 8. **Interaction of CHOP with GABA$_B$ receptors is responsible for OGD-induced down-regulation of cell surface receptors.** Neurons were transfected with a CHOP mutant that is not able to interact with GABA$_{B2}$ (*CHOPΔLZ*) and EGFP. After 24 h, neurons were subjected to OGD for 10 min, followed by a recovery period of 24 h. Subsequently, neurons were stained for cell surface GABA$_B$ receptors (*blue*) and endogenous CHOP (*red*). CHOPΔLZ-overexpressing neurons were selected by means of EGFP expression. Cultures not subjected to OGD served as controls. *Top panel*, representative images. *Scale bar* = 5 μm. *Bottom panel*, quantification of cell surface GABA$_B$ receptors. Data are means ± S.E.; 38 (control, non-transfected), 27 (OGD, non-transfected), 36 (control, transfected), and 45 (OGD, transfected) neurons from three experiments. **, $p < 0.01$; n.s., not significant ($p > 0.05$); Student's *t* test.

We showed previously that the ER stress-induced transcription factor CHOP interacts with GABA$_B$ receptors, causing their down-regulation from the cell surface upon coexpression in HEK 293 cells (22). This finding suggests that CHOP, besides its function as transcription factor, may regulate GABA$_B$ receptor-mediated neuronal inhibition by affecting the availability of cell surface receptors. Because CHOP is expressed at very low levels under normal physiological conditions but is highly up-regulated after induction of ER stress (21, 43–45), CHOP-induced down-regulation of cell surface GABA$_B$ receptors may be a contributing factor to neurological disease states associated with ER stress, such as stroke, Alzheimer and Parkinson disease, or bipolar disorders (20).

In this study, we verified that ER stress-induced CHOP expression also down-regulates cell surface GABA$_B$ receptors in neurons and disclosed the mechanism underlying this effect. An *in vitro* model of ischemia suggests that this mechanism is operative in cerebral ischemia.

We induced up-regulation of CHOP in cultured neurons by inhibition of the sarco/endoplasmic reticulum Ca^{2+}-ATPase with thapsigargin, which leads to the depletion of Ca^{2+} from the ER, thereby causing ER stress (46). Thapsigargin up-regulated CHOP in neurons and considerably down-regulated GABA$_B$ receptors from the cell surface. The reduced level of cell surface receptors consequently affected downstream signaling of GABA$_B$ receptors, as shown by impaired ERK1/2 phosphorylation and reduced baclofen-induced inhibition of spontaneous neuronal activity.

Down-regulation of cell surface GABA$_B$ receptors was mediated by the interaction of the receptors with CHOP because overexpression in neurons of mutant forms either lacking the site binding to GABA$_{B1}$ or the site binding to GABA$_{B2}$ prevented down-regulation of the receptors. Most importantly, up-regulation of CHOP did not affect the mRNA levels of GABA$_{B1}$ and GABA$_{B2}$, ruling out a contribution of impaired subunit transcription (Fig. 2*D*).

Up-regulated CHOP selectively accumulates together with GABA$_B$ receptors in the ER, suggesting that interaction with CHOP retains GABA$_B$ receptors in the ER and prevents their forward trafficking to the cell surface. This conclusion is supported by the finding that mutant GABA$_B$ receptors not containing the CHOP binding site in GABA$_{B2}$ do not accumulate in the ER and that the insertion of new receptors into the plasma

membrane is strongly reduced upon up-regulation of CHOP. The mechanism that interferes with forward trafficking of the receptors appears to involve prevention or disruption of receptor heterodimerization, as indicated by our *in situ* PLA experiments. It is currently not clear whether CHOP prevents heterodimerization of newly synthesized $GABA_{B1}$ and $GABA_{B2}$ by binding to $GABA_{B1}$ and $GABA_{B2}$ or directly disrupts already existing heterodimers. In either case, preventing heterodimerization exposes the ER retention signal in the C-terminal domain of $GABA_{B1}$, which prohibits ER exit of $GABA_{B1}$ (2–4). In addition, $GABA_{B2}$ contains a C-terminal sequence (amino acids 841–862) that is important for forward trafficking (47). Binding of CHOP to the leucine zipper domain of $GABA_{B2}$, upstream of this motif, might sterically interfere with the function of this motif and prevent ER export of $GABA_{B2}$.

The mechanism of CHOP-induced down-regulation of cell surface receptors appears to be operative under ischemic conditions. This is not surprising because cerebral ischemia has been shown to be associated with ER stress and the profound up-regulation of CHOP (35–42). Using the OGD *in vitro* model of ischemia, we found that up-regulated CHOP mediates down-regulation cell surface $GABA_B$ receptors, which depended on the interaction of CHOP with the receptors.

So far, the effect of cerebral ischemia on $GABA_B$ receptor expression levels has been rarely investigated, and the results are difficult to correlate because different animal species and experimental conditions were used. *In vivo* models of ischemia demonstrated a loss of $GABA_B$ receptors 1–4 days after the insult (7–9). Because considerable ischemia-induced neuronal death occurs during this time period, the loss of $GABA_B$ receptors might be due, at least in part, to a loss of $GABA_B$ receptor-expressing neurons. A recent *in vitro* study on cultured hippocampal slices using a similar OGD paradigm as in this study detected down-regulation of total $GABA_{B2}$ but no effect on the expression of total $GABA_{B1}$ (10). In this study, we detected down-regulation of both $GABA_{B1}$ and $GABA_{B2}$ selectively from the cell surface but did not observe changes in total receptor expression. The reason for this discrepancy is not clear yet, but it may be caused by different experimental conditions used (organotypic hippocampal slices, 45-min OGD *versus* cultured cortical neurons, 10-min OGD).

Cerebral ischemia is associated with excessive glutamate release, and overstimulation of glutamate receptors triggers a signaling cascade leading to neuronal death (48). The enhanced neuronal activity also increases extracellular levels of GABA (6), which, in turn, should activate $GABA_B$ receptors located at glutamatergic synapses to counteract, at least partially, the increased neuronal excitation to reduce excitotoxicity. The down-regulation of functional $GABA_B$ receptors from the cell surface might, therefore, be one factor that fosters excitotoxicity. It is well established that sustained activation of $GABA_B$ receptors (by application of baclofen) under ischemic conditions reduces neuronal cell death (10–19). In this regard, it is conceivable that restoring normal cell surface expression of $GABA_B$ receptors under ischemic conditions would also reduce excitotoxicity. This view is supported by a recent study showing that mild hypothermia reverses down-regulation of total $GABA_{B1}$ after cerebral ischemia by an unknown mechanism and reduces neuronal death (8).

One opportunity to restore normal cell surface $GABA_B$ receptor levels would be the application of small synthetic peptides interfering with the CHOP-$GABA_B$ receptor interaction. Further experiments are required to identify small peptide sequences that inhibit the interaction of CHOP with $GABA_B$ receptors but do not interfere with receptor heterodimerization. This approach provides the opportunity to unambiguously test the hypothesis whether CHOP-induced down-regulation of cell surface $GABA_B$ receptors promotes excitotoxicity. If this is the case, small interfering peptides may be of potential therapeutic use to limit neuronal death under ischemic conditions. Such an intervention would be highly specific because CHOP is significantly expressed only upon ER stress, and it targets a specific protein-protein interaction in response to the ischemic insult and is, thus, expected to be associated with a low risk of side effects.

Acknowledgments—We thank Dr. J.-M. Fritschy for support with confocal microscopy and for providing E18 rat cortex, C. Sidler and G. Bosshard for preparation of E18 rat cortex, and T. Grampp for technical assistance. We also thank L. Scheurer for help with real-time PCR.

REFERENCES

1. Bettler, B., Kaupmann, K., Mosbacher, J., and Gassmann, M. (2004) Molecular structure and physiological functions of $GABA_B$ receptors. *Physiol. Rev.* **84,** 835–867
2. Margeta-Mitrovic, M., Jan, Y. N., and Jan, L. Y. (2000) A trafficking checkpoint controls $GABA_B$ receptor heterodimerization. *Neuron* **27,** 97–106
3. Pagano, A., Rovelli, G., Mosbacher, J., Lohmann, T., Duthey, B., Stauffer, D., Ristig, D., Schuler, V., Meigel, I., Lampert, C., Stein, T., Prezeau, L., Blahos, J., Pin, J., Froestl, W., Kuhn, R., Heid, J., Kaupmann, K., and Bettler, B. (2001) C-terminal interaction is essential for surface trafficking but not for heteromeric assembly of $GABA_B$ receptors. *J. Neurosci.* **21,** 1189–1202
4. Calver, A. R., Robbins, M. J., Cosio, C., Rice, S. Q., Babbs, A. J., Hirst, W. D., Boyfield, I., Wood, M. D., Russell, R. B., Price, G. W., Couve, A., Moss, S. J., and Pangalos, M. N. (2001) The C-terminal domains of the $GABA_B$ receptor subunits mediate intracellular trafficking but are not required for receptor signaling. *J. Neurosci.* **21,** 1203–1210
5. Lipton, P. (1999) Ischemic cell death in brain neurons. *Physiol. Rev.* **79,** 1431–1568
6. Schwartz-Bloom, R. D., and Sah, R. (2001) γ-Aminobutyric acid(A) neurotransmission and cerebral ischemia. *J. Neurochem.* **77,** 353–371
7. Vollenweider, F., Bendfeldt, K., Maetzler, W., Otten, U., and Nitsch, C. (2006) $GABA_B$ receptor expression and cellular localization in gerbil hippocampus after transient global ischemia. *Neurosci. Lett.* **395,** 118–123
8. Kim, J. Y., Kim, N., Yenari, M. A., and Chang, W. (2011) Mild hypothermia suppresses calcium-sensing receptor (CaSR) induction following forebrain ischemia while increasing $GABA_B$ receptor 1 ($GABA_B$-R1) expression. *Transl. Stroke Res.* **2,** 195–201
9. Francis, J., Zhang, Y., Ho, W., Wallace, M. C., Zhang, L., and Eubanks, J. H. (1999) Decreased hippocampal expression, but not functionality, of $GABA_B$ receptors after transient cerebral ischemia in rats. *J. Neurochem.* **72,** 87–94
10. Cimarosti, H., Kantamneni, S., and Henley, J. M. (2009) Ischaemia differentially regulates $GABA_B$ receptor subunits in organotypic hippocampal slice cultures. *Neuropharmacology* **56,** 1088–1096
11. Babcock, A. M., Everingham, A., Paden, C. M., and Kimura, M. (2002) Baclofen is neuroprotective and prevents loss of calcium/calmodulin-dependent protein kinase II immunoreactivity in the ischemic gerbil hippocampus. *J. Neurosci. Res.* **67,** 804–811

12. Dave, K. R., Lange-Asschenfeldt, C., Raval, A. P., Prado, R., Busto, R., Saul, I., and Pérez-Pinzón, M. A. (2005) Ischemic preconditioning ameliorates excitotoxicity by shifting glutamate/γ-aminobutyric acid release and biosynthesis. *J. Neurosci. Res.* **82,** 665–673
13. Jackson-Friedman, C., Lyden, P. D., Nunez, S., Jin, A., and Zweifler, R. (1997) High dose baclofen is neuroprotective but also causes intracerebral hemorrhage: a quantal bioassay study using the intraluminal suture occlusion method. *Exp. Neurol.* **147,** 346–352
14. Kulinskii, V. I., and Mikhel'son, G. V. (2000) Additivity and independence of neuroprotective effects of $GABA_A$ and $GABA_B$ receptor agonists in complete global cerebral ischemia. *Bull. Exp. Biol. Med.* **130,** 772–774
15. Han, D., Zhang, Q. G., Yong-Liu, Li C., Zong, Y. Y., Yu, C. Z., Wang, W., Yan, J. Z., and Zhang, G. Y. (2008) Co-activation of GABA receptors inhibits the JNK3 apoptotic pathway via the disassembly of the GluR6-PSD95-MLK3 signaling module in cerebral ischemic-reperfusion. *FEBS Lett.* **582,** 1298–1306
16. Lal, S., Shuaib, A., and Ijaz, S. (1995) Baclofen is cytoprotective to cerebral ischemia in gerbils. *Neurochem. Res.* **20,** 115–119
17. Xu, J., Li, C., Yin, X. H., and Zhang, G. Y. (2008) Additive neuroprotection of $GABA_A$ and $GABA_B$ receptor agonists in cerebral ischemic injury via PI-3K/Akt pathway inhibiting the ASK1-JNK cascade. *Neuropharmacology* **54,** 1029–1040
18. Zhou, C., Li, C., Yu, H. M., Zhang, F., Han, D., and Zhang, G. Y. (2008) Neuroprotection of γ-aminobutyric acid receptor agonists via enhancing neuronal nitric oxide synthase (Ser847) phosphorylation through increased neuronal nitric oxide synthase and PSD95 interaction and inhibited protein phosphatase activity in cerebral ischemia. *J. Neurosci. Res.* **86,** 2973–2983
19. Zhang, F., Li, C., Wang, R., Han, D., Zhang, Q. G., Zhou, C., Yu, H. M., and Zhang, G. Y. (2007) Activation of GABA receptors attenuates neuronal apoptosis through inhibiting the tyrosine phosphorylation of NR2A by Src after cerebral ischemia and reperfusion. *Neuroscience* **150,** 938–949
20. Kim, I., Xu, W., and Reed, J. C. (2008) Cell death and endoplasmic reticulum stress: disease relevance and therapeutic opportunities. *Nat. Rev. Drug. Discov.* **7,** 1013–1030
21. Oyadomari, S., and Mori, M. (2004) Roles of CHOP/GADD153 in endoplasmic reticulum stress. *Cell Death Differ.* **11,** 381–389
22. Sauter, K., Grampp, T., Fritschy, J. M., Kaupmann, K., Bettler, B., Mohler, H., and Benke, D. (2005) Subtype-selective interaction with the transcription factor CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP) regulates cell surface expression of $GABA_B$ receptors. *J. Biol. Chem.* **280,** 33566–33572
23. Benke, D., Honer, M., Michel, C., Bettler, B., and Mohler, H. (1999) γ-Aminobutyric acid type B receptor splice variant proteins GBR1a and GBR1b are both associated with GBR2 *in situ* and display differential regional and subcellular distribution. *J. Biol. Chem.* **274,** 27323–27330
24. Benke, D., Michel, C., and Mohler, H. (2002) Structure of $GABA_B$ receptors in rat retina. *J. Recept. Signal Transduct. Res.* **22,** 253–266
25. Benke, D., Mertens, S., Trzeciak, A., Gillessen, D., and Mohler, H. (1991) $GABA_A$ receptors display association of γ 2-subunit with α1- and β2/3-subunits. *J. Biol. Chem.* **266,** 4478–4483
26. Grampp, T., Notz, V., Broll, I., Fischer, N., and Benke, D. (2008) Constitutive, agonist-accelerated, recycling and lysosomal degradation of $GABA_B$ receptors in cortical neurons. *Mol. Cell. Neurosci.* **39,** 628–637
27. Buerli, T., Pellegrino, C., Baer, K., Lardi-Studler, B., Chudotvorova, I., Fritschy, J. M., Medina, I., and Fuhrer, C. (2007) Efficient transfection of DNA or shRNA vectors into neurons using magnetofection. *Nat. Protoc.* **2,** 3090–3101
28. Vanhoose, A. M., Emery, M., Jimenez, L., and Winder, D. G. (2002) ERK activation by G-protein-coupled receptors in mouse brain is receptor identity-specific. *J. Biol. Chem.* **277,** 9049–9053
29. Ren, X., and Mody, I. (2003) γ-Hydroxybutyrate reduces mitogen-activated protein kinase phosphorylation via $GABA_B$ receptor activation in mouse frontal cortex and hippocampus. *J. Biol. Chem.* **278,** 42006–42011
30. Tu, H., Rondard, P., Xu, C., Bertaso, F., Cao, F., Zhang, X., Pin, J. P., and Liu, J. (2007) Dominant role of $GABA_{B2}$ and $G\beta\gamma$ for $GABA_B$ receptor-mediated-ERK1/2/CREB pathway in cerebellar neurons. *Cell Signal.* **19,** 1996–2002
31. Im, B. H., and Rhim, H. (2012) $GABA_B$ receptor-mediated ERK1/2 phosphorylation via a direct interaction with $Ca_V1.3$ channels. *Neurosci. Lett.* **513,** 89–94
32. Leuchowius, K. J., Jarvius, M., Wickström, M., Rickardson, L., Landegren, U., Larsson, R., Söderberg, O., Fryknäs, M., and Jarvius, J. (2010) High content screening for inhibitors of protein interactions and post-translational modifications in primary cells by proximity ligation. *Mol. Cell Proteomics* **9,** 178–183
33. Söderberg, O., Gullberg, M., Jarvius, M., Ridderstråle, K., Leuchowius, K. J., Jarvius, J., Wester, K., Hydbring, P., Bahram, F., Larsson, L. G., and Landegren, U. (2006) Direct observation of individual endogenous protein complexes in situ by proximity ligation. *Nat. Methods* **3,** 995–1000
34. Zemoura, K., and Benke, D. (2014) Proteasomal degradation of γ-aminobutyric $acid_B$ receptors is mediated by the interaction of the $GABA_{B2}$ C terminus with the proteasomal ATPase Rtp6 and regulated by neuronal activity. *J. Biol. Chem.* **289,** 7738–7746
35. Hossmann, K. A. (1993) Disturbances of cerebral protein synthesis and ischemic cell death. *Prog. Brain Res.* **96,** 161–177
36. Paschen, W., and Doutheil, J. (1999) Disturbance of endoplasmic reticulum functions: a key mechanism underlying cell damage? *Acta Neurochir. Suppl.* **73,** 1–5
37. Osada, N., Kosuge, Y., Kihara, T., Ishige, K., and Ito, Y. (2009) Apolipoprotein E-deficient mice are more vulnerable to ER stress after transient forebrain ischemia. *Neurochem. Int.* **54,** 403–409
38. Osada, N., Kosuge, Y., Ishige, K., and Ito, Y. (2010) Characterization of neuronal and astroglial responses to ER stress in the hippocampal CA1 area in mice following transient forebrain ischemia. *Neurochem. Int.* **57,** 1–7
39. Tajiri, S., Oyadomari, S., Yano, S., Morioka, M., Gotoh, T., Hamada, J.-I., Ushio, Y., and Mori, M. (2004) Ischemia-induced neuronal cell death is mediated by the endoplasmic reticulum stress pathway involving CHOP. *Cell Death Differ.* **11,** 403–415
40. Paschen, W., Gissel, C., Linden, T., Althausen, S., and Doutheil, J. (1998) Activation of Gadd153 expression through transient cerebral ischemia: evidence that ischemia causes endoplasmic reticulum dysfunction. *Mol. Brain Res.* **60,** 115–122
41. Oida, Y., Shimazawa, M., Imaizumi, K., and Hara, H. (2008) Involvement of endoplasmic reticulum stress in the neuronal death induced by transient forebrain ischemia in gerbil. *Neuroscience* **151,** 111–119
42. Morimoto, N., Oida, Y., Shimazawa, M., Miura, M., Kudo, T., Imaizumi, K., and Hara, H. (2007) Involvement of endoplasmic reticulum stress after middle cerebral artery occlusion in mice. *Neuroscience* **147,** 957–967
43. Fornace, A. J., Jr., Alamo, I., Jr., and Hollander, M. C. (1988) DNA damage-inducible transcripts in mammalian cells. *Proc. Natl. Acad. Sci. U.S.A.* **85,** 8800–8804
44. Oyadomari, S., Araki, E., and Mori, M. (2002) Endoplasmic reticulum stress-mediated apoptosis in pancreatic β-cells. *Apoptosis* **7,** 335–345
45. Zinszner, H., Kuroda, M., Wang, X., Batchvarova, N., Lightfoot, R. T., Remotti, H., Stevens, J. L., and Ron, D. (1998) CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. *Genes Dev.* **12,** 982–995
46. Lytton, J., Westlin, M., and Hanley, M. R. (1991) Thapsigargin inhibits the sarcoplasmic or endoplasmic reticulum Ca-ATPase family of calcium pumps. *J. Biol. Chem.* **266,** 17067–17071
47. Pooler, A. M., Gray, A. G., and McIlhinney, R. A. (2009) Identification of a novel region of the $GABA_{B2}$ C-terminus that regulates surface expression and neuronal targeting of the $GABA_B$ receptor. *Eur. J. Neurosci.* **29,** 869–878
48. Kostandy, B. B. (2012) The role of glutamate in neuronal ischemic injury: the role of spark in fire. *Neurol. Sci.* **33,** 223–237