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$GABA_A$ receptor dephosphorylation followed by internalization is coupled to neuronal death in *in vitro* ischemia

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ABSTRACT

Cerebral ischemia is characterized by an early disruption of GABAergic neurotransmission contributing to an imbalance of the excitatory/inhibitory equilibrium and neuronal death, but the molecular mechanisms involved are not fully understood. Here we report a downregulation of $GABA_A$ receptor ($GABA_AR$) expression, affecting both mRNA and protein levels of $GABA_AR$ subunits, in hippocampal neurons subjected to oxygen-glucose deprivation (OGD), an *in vitro* model of ischemia. Similar alterations in the abundance of $GABA_AR$ subunits were observed in *in vivo* brain ischemia. OGD reduced the interaction of surface $GABA_AR$ with the scaffold protein gephyrin, followed by clathrin-dependent receptor internalization. Internalization of $GABA_AR$ was dependent on glutamate receptor activation and mediated by dephosphorylation of the β3 subunit at serine 408/409. Expression of phospho-mimetic mutant $GABA_AR$ β3 subunits prevented receptor internalization and protected hippocampal neurons from ischemic cell death. The results show a key role for β3 $GABA_AR$ subunit dephosphorylation in the downregulation of GABAergic synaptic transmission in brain ischemia, contributing to neuronal death. $GABA_AR$ phosphorylation might be a therapeutic target to preserve synaptic inhibition in brain ischemia.

Introduction

The activity of neuronal networks in the CNS is mainly determined by the balance between glutamatergic and GABAergic neurotransmission, which is up- and down-regulated, respectively, during ischemic insults resulting from an insufficient blood supply to the brain (Choi, 1992; Lipton, 1999). In contrast to the role of glutamate in ischemic damage, alterations in inhibitory neurotransmission remain poorly understood.

The down regulation of GABAergic neurotransmission in the ischemic brain occurs at the pre- and post-synaptic levels (Schwartz-Bloom and Sah, 2001). Exposure of hippocampal slices to oxygen and glucose-deprivation (OGD) induces an early release of GABA by exocytosis, followed by a delayed phase of neurotransmitter release mediated by reversal of the plasma membrane transporter (Allen et al., 2004). The downregulation of vesicular GABA transporters (Gomes et al., 2011) may contribute to the delayed inhibition of the exocytotic release of GABA. $GABA_A$ receptors ($GABA_AR$) are the major players in fast synaptic inhibition in the CNS, and a downregulation of the surface expression of $GABA_A$Rs has also been shown in different models of ischemia (Schwartz-Bloom and Sah, 2001). This is likely to play an important role in the demise process since stabilization of the $GABA_AR$ surface expression is correlated with neuroprotection in neurons subjected to OGD (Liu et al., 2010; Mielke and Wang, 2005), and inhibition of AP2/clathrin-dependent internalization of $GABA_AR$ also reduces OGD-induced cell death (Smith et al., 2012). Together, these evidences indicate that the number of cell surface $GABA_AR$ and receptor internalization play a key role in ischemic cell death, but the molecular mechanisms involved in receptor internalization have not been elucidated.

It is generally assumed that the majority of $GABA_A$Rs in the brain are assembled from at least 2 α-, 2 β-, and 1 γ2-subunits (Rudolph and Mohler, 2004). $GABA_A$Rs present a dynamic mobility between synaptic and extrasynaptic localization (Thomas et al., 2005), being the accumulation of the receptor at the inhibitory synapses regulated by its scaffold protein gephyrin (Jacob et al., 2005). Although the strength of inhibitory signaling is determined by the number of $GABA_AR$ at the synapse, the internalization of these receptors occurs outside the synapse (Bogdanov et al., 2006). The rate of internalization of $GABA_A$Rs is negatively regulated by phosphorylation of β3 or γ2 $GABA_AR$ subunits on their intracellular loop (Kittler et al., 2005, 2008). Furthermore, NMDAR signaling via calcineurin and $GABA_AR$ phosphorylation also controls the stability of synaptic $GABA_A$Rs (Muir et al., 2010), but how brain ischemia affects the mechanisms of regulation of $GABA_AR$ trafficking has not been investigated.

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In the present work we investigated the molecular mechanisms underlying $GABA_AR$ downregulation during OGD, an *in vitro* model of global ischemia. Combining cell imaging and biochemical approaches we show that OGD decreases $GABA_AR$/gephyrin interaction and induces the internalization of $GABA_AR$ via clathrin-dependent endocytosis. In addition, we demonstrate that OGD-induced dephosphorylation and internalization of β3 $GABA_AR$ subunits contributes to neuronal death. Overall, we propose that $GABA_AR$/gephyrin dissociation and receptor dephosphorylation are key steps for GABAergic down-modulation during cerebral ischemia and the subsequent neuronal cell death.

Material and methods

Hippocampal cultures

Primary cultures of rat hippocampal neurons were prepared from the hippocampi of E18-E19 Wistar rat embryos, after treatment with trypsin (0.06%, 15 min, 37 °C; GIBCO Invitrogen) in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS; 5.36 mM KCl, 0.44 mM KH_2PO_4, 137 mM NaCl, 4.16 mM $NaHCO_3$, 0.34 mM $Na_2HPO_4.2H_2O$, 5 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES and 0.001% phenol red). The hippocampi were then washed with HBSS containing 10% fetal bovine serum (GIBCO Invitrogen), to stop trypsin activity, and transferred to Neurobasal medium (GIBCO Invitrogen) supplemented with B27 supplement (1:50 dilution; GIBCO Invitrogen), 25 μM glutamate, 0.5 mM glutamine and 0.12 mg/ml gentamycin. The cells were dissociated in this solution and were then plated on 6 well plates (90.0×10^3 cells/cm^2), previously coated with poly-D-lysine (0.1 mg/ml), or on poly-D-lysine coated glass coverslips, at a density of 80.0×10^3 cells/cm^2. The cultures were maintained in a humidified incubator with 5% CO_2/95% air, at 37 °C, for 15 days.

Oxygen-glucose deprivation

Hippocampal neurons (15 DIV) were incubated in a glucose-free saline buffer (116 mM NaCl, 25 mM sucrose, 10 mM HEPES, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4, 1.8 mM $CaCl_2$, 25 mM $NaHCO_3$) in an anaerobic chamber with 10% H_2, 85% N_2, 5% CO_2 (Forma Anaerobic System, Thermo Fisher Scientific), at 37 °C, for the indicated period of time. The OGD buffer was then replaced by a conditioned medium and the cultures were returned to the humidified 95% air/5% CO_2 incubator for the indicated post-incubation time period. Under control conditions (Sham) the cells were incubated in the saline buffer described above, supplemented with 25 mM glucose instead of sucrose, and kept in the humidified 95% air/5% CO_2 incubator at 37 °C. When appropriate the cells were pre-incubated with glutamate receptor or calpain inhibitors (20 μM NBQX [Tocris] and 100 μM APV [Tocris] were added 30 min before OGD; 50 μM ALLN [Calbiochem] or 50 μM MDL28170 [Calbiochem], 1 h before OGD), and the drugs were also present during and after the insult.

Western blotting

Total cell extracts were prepared after washing the cells twice with ice-cold PBS buffer. The cells were lysed with RIPA buffer (150 mM NaCl, 50 mM Tris–HCl, 5 mM EGTA, 1% Triton, 0.5% DOC and 0.1% SDS, at a final pH 7.5) supplemented with 1 mM DTT and a cocktail of protease inhibitors (0.1 mM PMSF, 1 μg/ml chymostatin, 1 μg/ml leupeptin, 1 μg/ml antipain, 1 μg/ml pepstatin; Sigma–Aldrich Química). For phosphorylation studies the lysis buffer contained 10 mM HEPES, 150 mM NaCl, 10 mM EDTA and 1% Triton (pH 7.4), and was supplemented with phosphatase inhibitors (50 mM NaF and 1.5 mM sodium orthovanadate). After centrifugation at 16,100 ×*g* for 10 min, protein levels present in the supernatants were quantified using the BCA method (Thermo Scientific). Samples were then diluted with a 2× concentrated denaturing buffer (125 mM Tris, pH 6.8, 100 mM glycine, 4% SDS, 200 mM DTT, 40% glycerol, 3 mM sodium orthovanadate, and 0.01% bromophenol blue). Protein samples were separated by SDS-PAGE, in 10% polyacrylamide gels, transferred to PVDF membranes (Millipore) and immunoblotted. Membranes were incubated with primary antibodies (overnight at 4 °C), washed and exposed to alkaline phosphatase-conjugated secondary antibodies (1:20,000 dilution; 1 h at room temperature) (GE Healthcare or Jackson ImmunoResearch). Alkaline phosphatase activity was visualized using ECF on the Storm 860 Gel and Blot Imaging System (GE Healthcare). The following primary antibodies were used: anti-Alpha1 $GABA_A$ receptor (1:1000, NeuroMab), anti-Alpha2 $GABA_A$ receptor (1:1000, Synaptic System), anti-Beta 3 $GABA_A$ receptor (1:1000, NeuroMab), anti-Phospho-$Ser^{408/409}$ Beta 3 $GABA_A$ receptor (1:1000, Symansis), anti-Gama 2 $GABA_A$ receptor (1:1000, Synaptic Systems) and anti-Gephyrin (1:1000, Synaptic Systems). Anti-Synaptophysin (1:10,000, Abcam) and anti-β-tubulin (1:300,000, Sigma) antibodies were used as loading controls.

Fluorescence assay of receptor internalization

Cultured living hippocampal neurons (15 DIV) were incubated at RT for 10 min in the presence of a high concentration (1:100) of an anti-Alpha1 $GABA_A$ receptor antibody (Millipore), directed against the N-terminus of the α1 subunits, or an anti-myc antibody (1:300, Cell Signaling). The cells were then washed with PBS at 37 °C, to remove the unbound antibody, and were further incubated in an antibody free conditioned medium at the same temperature (for different periods) to allow the internalization of antibody-bound receptors. After this incubation neurons were fixed for 15 min in 4% sucrose/paraformaldehyde. Next, neurons were exposed to a super-saturating concentration (1:300) of the first of two secondary antibodies (Alexa Fluor 488 goat anti-rabbit; Invitrogen) for 1 h at RT. After permeabilization (0.25% Triton X-100 for 5 min) the cells were incubated with the second secondary antibody (Alexa Fluor 568 goat anti-rabbit, 1:500 Invitrogen) for 1 h at RT. This strategy allows distinguishing the surface receptors from those receptors that have been internalized before fixation (Goodkin et al., 2005). The coverslips were then mounted on slides with a fluorescence mounting medium (DAKO). Images were acquired on Axio Observer 2.1 fluorescence microscope (Zeiss) coupled to an Axiocam HRm digital camera, using a 63× oil objective and were quantified using the ImageJ image analysis software. For each experiment analyzed the cells were stained and imaged using identical settings. The ratio of internalization was calculated using the internalized antibody signal/total antibody signal ratio.

Immunocytochemistry

Hippocampal neurons were fixed in 4% sucrose/paraformaldehyde (in PBS) and permeabilized with 0.3% Triton X-100 in PBS. The neurons were then incubated with 10% BSA in PBS for 30 min at 37 °C, and incubated with the primary antibody anti-myc (1:500, Cell Signaling) diluted in 3% BSA in PBS, overnight at 4 °C. The cells were washed with PBS and incubated with the secondary antibody (anti-mouse IgG) conjugated with Alexa Fluor 488 (Invitrogen), for 1 h at RT. The coverslips were mounted in a fluorescence mounting medium (DAKO, Denmark). Imaging was performed in an Axio Observer 2.1 fluorescence microscope, coupled to an Axiocam HRm digital camera, using a 63× oil objective. The cells to count were chosen by the myc (green) channel to check for the presence of transfected neurons. Measurements were performed in three independent preparations, and at least 50 cells were counted per experimental condition for each preparation.

Surface co-immunoprecipitation assay

After stimulation using the indicated experimental conditions hippocampal neurons were washed twice with ice-cold PBS and incubated

with Sulfo-NHS–SS–biotin (0.25 mg/ml in PBS; Thermo Scientific) for 15 min on ice. Cells were then washed twice with 50 mM NH_4Cl and two times more with PBS. After biotinylation, the cells were lysed with RIPA buffer and α1-containing $GABA_A$Rs were immunoprecipitated.

Protein G Plus-Agarose beads (50 μl; Santa Cruz Biotechnology) were added to Lysis buffer (1 ml) containing 5 μg of an anti-$GABA_A$R alpha 1 subunit (NeuroMab) monoclonal antibody and incubated for 2 h on a head-over-head shaker at 4 °C. Antibody excess was removed by two rinses with lysis buffer. Lysed samples (400 μg) were added to the beads and incubated for 6 h on a head-over-head shaker at 4 °C. Beads were centrifuged at 800 ×*g* to remove the antibody, and the samples were then washed three times with lysis buffer. The residual buffer was removed and bead–IP–$GABA_A$Rs were incubated with 50 μl of 1% SDS (80 min at 37 °C) to disrupt the interaction between the beads and IP–$GABA_A$Rs. Finally, beads were centrifuged at 800 ×*g*, and the supernatants were mixed with 150 μl of lysis buffer before being used in NeutAvidin pull-downs.

NeutAvidin® Plus UltraLink Resin beads (40 μl; Thermo scientific) were added to the samples and mixed on a head-over-head shaker for 4 h. Beads were then centrifuged at 800 ×*g* and washed three times with lysis buffer. The residual lysis buffer was removed and then 60 μl of 2× loading buffer was added. Samples were heated at 90 °C for 5 min and beads were centrifuged at 800 ×*g*. The bead supernatants were used for Western blot analysis.

Neuron transfection with calcium phosphate

Transfection of cultured hippocampal neurons with myc-huGABA$_A$R β3 (WT), myc-huGABA$_A$R β3 (p-mimetic) or myc-huGABA$_A$R β3 (p-null) constructs was performed by the calcium phosphate coprecipitation method. Briefly, 2 μg of plasmid DNA was diluted in Tris-EDTA (TE) pH 7.3 and mixed with 2.5 M $CaCl_2$. This DNA/TE/calcium mix was added to 10 mM HEPES-buffered saline solution (270 mM NaCl, 10 mM KCl, 1.4 mM Na_2HPO_4, 11 mM dextrose, 42 mM HEPES, pH 7.2). The precipitates were allowed to form for 30 min at room temperature, protected from light, with vortex mixing every 5 min, to ensure that the precipitates had similar small sizes. Meanwhile, cultured hippocampal neurons were incubated with a cultured-conditioned medium with 2 mM kynurenic acid (Sigma). The precipitates were added drop-wise to each well and incubated for 2 h at 37 °C, in an incubator with 95% air/5%CO_2. The cells were then washed with acidic culture medium containing 2 mM kynurenic acid and returned to the 95% air/5% CO_2 incubator for 20 min at 37 °C. Finally, the medium was replaced with the initial culture-conditioned medium, and the cells were further incubated in a 95% air/5% CO_2 incubator for 48 h at 37 °C to allow protein expression. Cell cultures were then subjected to OGD for 90 min, and 8 h after the insult the cells were fixed to proceed with the cell death assay. In the case of the fluorescence internalization assay cells were subjected to 70 min of OGD.

Mutagenesis

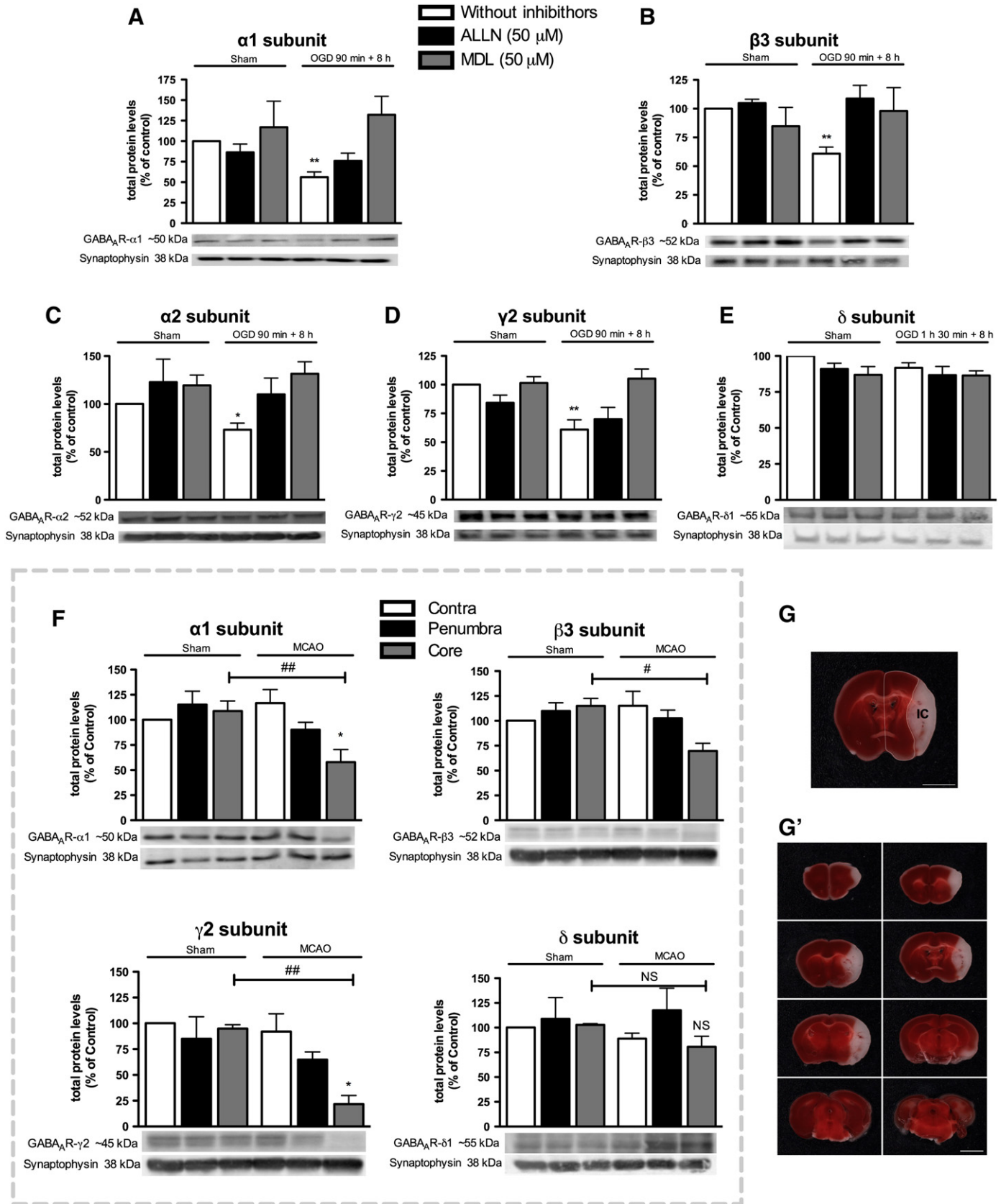
The plasmid containing the human WT $GABA_A$R β3 subunit sequence was a kind offer of Doctor Martin Wallner (Department of Molecular and Medical Pharmacology, David Geffen School of Medicine, UCLA). In the same vector, two myc-Tag sequences (GAGCAGAAGCTG ATCTCAGAGGAGGATCTGGAGC AGAAGCTGATCTCAGAGGAGGATCTG) were added between the 4th and 5th codons of human $GABA_A$R β3 cDNA that code for the amino acids belonging to the N-terminus of the protein (NZYTech). To obtain the phospho-mimetic and a phospho-null mutants of the human $GABA_A$R β3 subunit, we performed a site directed mutagenesis of the serine residues 432/433 (homologous of mouse 408/409), using QuikChange® II XL Site-Directed Mutagenesis Kit (Agilent Technology). Briefly, specific primers were designed to mutate the two serine residues to two aspartate residues (5′ gcacaagaagacccatctacggaggagggatgatcagctcaaaattaaaatacctgatctaac 3′), in the case of the phospho-mimetic mutant, and to two alanine residues (5′ gacccatctacggaggagggctgcacagctcaaaattaaaat 3′) in the case of the phospho-null mutant (primers were synthesized by Sigma–Aldrich). For each mutagenesis the reaction contained 13.5 ng of dsDNA template (myc-huGABA$_A$R β3), 5 μl of 10× reaction buffer, 125 ng of each oligonucleotide primer, 1 μl of dNTP mix, 3 μl of QuikSolution, ddH_2O to final volume of 50 μl and 1 μl of *PfuUltra* HF DNA polymerase (2.5 U/μl). The following thermal cycling was then performed: 95 °C for 1 min, 18 cycles (95 °C for 50 s, 60 °C for 50 s, 68 °C for 6 min 30 s) and 68 °C for 7 min. The parental methylated dsDNA was then digested using 1 μl of *Dpn* I enzyme (New England BioLabs) at 37 °C for 1 h. *Dpn* I digested dsDNA was used to transform *E. coli* Top 10 cells to be then amplified. The obtained plasmid DNA was extracted using Plasmid Mini Kit (Quiagen) and sequenced to confirm the mutagenesis (STABvida).

Middle cerebral artery occlusion

Focal cerebral ischemia was induced by the transient occlusion of the right middle cerebral artery (MCA), using the intraluminal filament placement technique as described previously (Nygren and Wieloch, 2005). Briefly, adult male mice were anesthetized by inhalation of 2.5% isoflurane (IsobaVet, Schering-Plough Animal Health) in O_2:N_2O (30:70). Anesthesia was subsequently reduced to 1.5–1.8% isoflurane and sustained throughout the occlusion period. Body temperature was kept at ~37 °C throughout the surgery period. To monitor regional cerebral blood flow (rCBF), an optical fiber probe (Probe 318-I, Perimed) was fixed to the skull at 2 mm posterior and 4 mm lateral to bregma, and connected to a laser Doppler flow meter (Periflux System 5000, Perimed). A filament composed of 6–0 polydioxanone suture (PSD II, Ethicon) with a silicone tip (diameter of 225–275 μm) was inserted into the external carotid artery and advanced into the common carotid artery. The filament was retracted, moved into the internal carotid artery, and advanced until the origin of the MCA, given by the sudden drop in rCBF (~70% of baseline). After 45 min, the filament was withdrawn and reperfusion observed. The animals were placed in a heating box at 37 °C for the first 2 h after surgery and thereafter transferred into a heating box at 35 °C, to avoid postsurgical hypothermia. Thirty minutes and 24 h after the onset of reperfusion, 0.5 ml of 5% glucose was administered subcutaneously. Temperature and sensorimotor deficits were assessed at 1, 2 h and 24 h after the surgery. Body weight was controlled daily. In sham surgeries, the filament was advanced up to the

Fig. 1. α1, α2, β3 and γ2 $GABA_A$R subunit protein levels are downregulated in *in vitro* (OGD) and *in vivo* (MCAO) transient brain ischemia. Cultured hippocampal neurons (15 DIV) were exposed to OGD for 90 min in the presence or in the absence of 50 μM ALLN and 50 μM MDL28170. α1 (A), β3 (B), α2 (C), and γ2 (D) $GABA_A$R subunit total protein levels were determined by Western blot analysis, 8 h after the insult, and the results were normalized with the loading control synaptophysin. (F) Effect of transient *in vivo* ischemia (MCAO) on α1, β3, γ2 (reprobing of β3 membrane) and δ $GABA_A$R subunit total protein levels, as determined 48 h after the lesion in the infarct core, penumbra and contralateral cortex. $GABA_A$R subunit protein levels were determined by Western blot as indicated above. (G) Representative image of the regions dissected from the ipsilateral brain hemisphere of C57BL/6 mice subjected to sham surgery or MCAO, considered as infarct core (IC) and penumbra (delineated). Scale bar: 3 mm. (G′) Representative image of the cerebral infarct core following a transient (45 min) occlusion of the MCA, in C57BL/6 mice, as given by lack of TTC staining in contiguous 1 mm tick coronal slices (white). Scale bar, 3 mm. Results are the mean ± SEM of at least 3 independent experiments/animals performed in different preparations, and are expressed as percentage of the control. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's or Bonferroni test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$—significantly different when compared to control conditions, as depicted in the figure. NS - not significant when compared to control conditions.

internal carotid artery, and withdrawn before reaching the MCA. The Ethics Committee for Animal Research at Lund University approved animal housing conditions, handling, and surgical procedures. Eleven to 36 week old C57BL/6J male mice (weight: 23.0 g to 37.9 g; Lund University breeding facility) were housed under diurnal conditions with ad libitum access to water and food before and after surgery.



Mice were anesthetized 48 h after MCA occlusion (MCAO) or sham surgery, by inhalation of 2.5% isoflurane, and were then perfused transcardially with 0.9% NaCl for 2 min before decapitation. Upon removal of meninges, brains were rapidly isolated and frozen by immersion in isopentane at −40 °C, further cooled down to −70 °C and stored at −80 °C. The infarct core and remaining ipsilateral tissue (designated as penumbra for simplification) were dissected, as well as the contralateral cortex, from coronal brain sections covering the majority of damage. More specifically, consecutive 2 mm, 1 mm and 2 mm thick brain sections were made, starting at 2 mm from the olfactory bulb. Dissections were performed at −15 °C, a temperature that allows an easy detachment of the infarct core and penumbra. The cortical–striatal infarcts obtained were illustrated in Inacio et al. (2011). Equivalent brain regions were dissected from sham-operated mice, which were also designated as infarct core and penumbra, and contralateral cortex. For each animal, corresponding regions from each of 3 consecutive brain sections were pulled together. Samples were then homogenized and processed for Western blotting as previously described (Inacio et al., 2011). Cellular protein extraction was performed by mechanical homogenization of the tissue and incubation in lysis buffer: 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM orthovanadate and 1 mM PMSF, supplemented with a protease inhibitor cocktail (P8340, Sigma–Aldrich). Following 30 min incubation at 4 °C, samples were centrifuged at 18,000 ×, for 15 min. Total protein concentration in lysates was determined by the Bradford assay, using bovine albumin (Sigma) as standard.

Statistical analysis

Statistical analysis was performed using one-way ANOVA analysis of variance, followed by Dunnett's or Bonferroni post-hoc test, or using Student's *t* test, as indicated in the figure captions.

Results

OGD induces cell death and downregulates GABA$_A$R subunit total protein levels by a calpain-dependent mechanism

OGD is a well-established *in vitro* model of global cerebral ischemia (Dawson et al., 1996; Goldberg and Choi, 1993; Martin et al., 1994). Exposure of cultured hippocampal neurons to OGD for 60 min-120 min induced a time-dependent cell death, as determined by analysis of nuclear morphology 7 h or 12 h after the insult (S. Fig. 1). The short periods of OGD tested, 60 min or 75 min, induced ~20% cell death, while 90 min or 120 min of OGD induced ~30% and ~40% cell death, respectively. We did not observe significant differences in cell death between the two post-incubation times used, 7 h and 12 h.

To assess the effect of OGD on GABA$_A$R subunit total protein levels, cultured hippocampal neurons were subjected to 90 min of OGD, and further incubated in the culture conditioned medium for 8 h. GABA$_A$R subunit protein levels were analyzed by Western blot using specific antibodies against α1, α2, β3, γ2 and δ subunits. α1, α2, β3, γ2 and subunits are localized preferentially at the synapses (Alldred et al., 2005; Nusser et al., 1998), mediating phasic inhibition (Brickley et al., 1996), in contrast with the δ subunit which is extrasynaptic (Nusser et al., 1998). The results show a downregulation of all the synaptic GABA$_A$R subunits, of ~40% for α1 subunits, ~20% for α2 subunits, and ~35% for β3 and γ2 subunits (Fig. 1A–D), but no effect was found for the δ subunit (Fig. 1E). Shorter periods of OGD (60 min) did not affect GABA$_A$R total protein levels (not shown). Similar to the results obtained in hippocampal neurons subjected to OGD, a downregulation of α1, β3 and γ2 subunits was observed in the infarct core of mice subjected to transient MCAO, a model of focal brain ischemia, but no effect was found for the δ subunit. No significant changes in GABA$_A$R subunit protein levels were observed in the penumbra (Fig. 1F).

The OGD-induced $[Ca^{2+}]_i$ overload activates calpains (Brorson et al., 1995; Saido et al., 1994; Vanderklish and Bahr, 2000) which cleave numerous intracellular proteins in the ischemic brain (Bevers and Neumar, 2008). To investigate whether calpains are involved in the OGD-induced downregulation of GABA$_A$R subunits, hippocampal neurons were subjected to OGD in the presence or in the absence of the chemical inhibitors ALLN or MDL28170. Western blot analysis performed 8 h after injury showed that MDL28170 fully abrogated the effect of OGD on α1, β3, α2 and γ2 GABA$_A$R subunits ($p > 0.05$) (Fig. 1A–E). Furthermore, ALLN clearly prevented the reduction of β3 and α2 subunits in hippocampal neurons subjected to OGD (Fig. 1B,C).

OGD downregulates the GABA$_A$R subunit mRNA through activation of glutamate receptors

Considering the effect of OGD on the protein levels of GABA$_A$R subunits, which is maintained 8 h after injury, we hypothesized that *in vitro* ischemia could also downregulate the mRNA for the various subunits causing a long-term effect on the synthesis of new receptors. Quantitative PCR experiments showed a decrease in the expression levels of α1, α2, β2, β3 and γ GABA$_A$R subunits in hippocampal neurons subjected to OGD for 90 min, and further incubated in the conditioned medium for 5 h (S. Fig. 2), but no effect was observed for shorter periods of *in vitro* ischemia (75 min), even when determined 7 h after the insult (S. Fig. 3A–D). The former experimental conditions led to a 70% reduction in the mRNA levels of α1, 50% in α2 and β2, 25% in β3 and 40% in γ2 subunits, which are typically found in synaptic receptors. In contrast, no changes in the mRNA levels for the extrasynaptic GABA$_A$R δ subunits were observed, even for 90 min of OGD (S. Fig. 2 F). The OGD-induced downregulation of mRNA levels for α1, α2, β2, β3 and γ2 subunits was prevented by incubation with the NMDA and non-NMDA glutamate receptors inhibitors APV (100 μM) and NBQX (20 μM) (S. Fig. 2A′–E′). We have previously shown a role for glutamate receptors in hippocampal neuronal death following OGD, as determined by nuclear morphology analysis (Caldeira et al., 2013). Although OGD decreased the mRNA levels for α1, α2, β2, β3 subunits, inhibition of transcription is unlikely to contribute to the observed downregulation of GABA$_A$R protein levels since transcription blockage with actinomycin D for 9.5 h (the maximal duration of the OGD experiments) did not affect the abundance of α1, α2, β3 and γ2 protein levels (S. Fig. 4).

Downregulation of GABA$_A$R α1 subunit/gephyrin interaction during OGD

The number of GABA$_A$R at the synapse determines the strength of inhibitory signaling. These receptors are very dynamic structures in the cell membrane, moving between synaptic and extrasynaptic sites (Thomas et al., 2005), and their accumulation at the synapse is regulated by interaction with the scaffold protein gephyrin (Jacob et al., 2005). To evaluate if GABA$_A$R/gephyrin interaction is altered in ischemic conditions, a surface co-immunoprecipitation protocol was used. Exposure of hippocampal neurons to OGD for 70 min, which does not affect total GABA$_A$R α1 subunit protein levels, significantly reduced the interaction between surface-expressed receptor subunit and gephyrin (Fig. 2A,C), as demonstrated by immunoprecipitation of surface subunits followed by Western blot with an anti-gephyrin antibody (Fig. 2C). This effect was completely inhibited by cyclosporin A (1 μM), a calcineurin inhibitor, but was insensitive to okadaic acid (0.5 μM), an inhibitor of PP1α and PP2A phosphatases. In contrast, the decrease in total surface expression of GABA$_A$R α1 subunits induced by OGD was completely abrogated in the presence of okadaic acid (Fig. 2B and C), but was insensitive to cyclosporin A (1 μM). These results indicate that different protein phosphatases mediate the effect of OGD on the dissociation of GABA$_A$R α1 subunits from gephyrin and the decrease in surface expression of GABA$_A$Rs, presumably due to an increase in the rate of internalization. Control experiments showed no changes in total gephyrin (S. Fig. 5B)

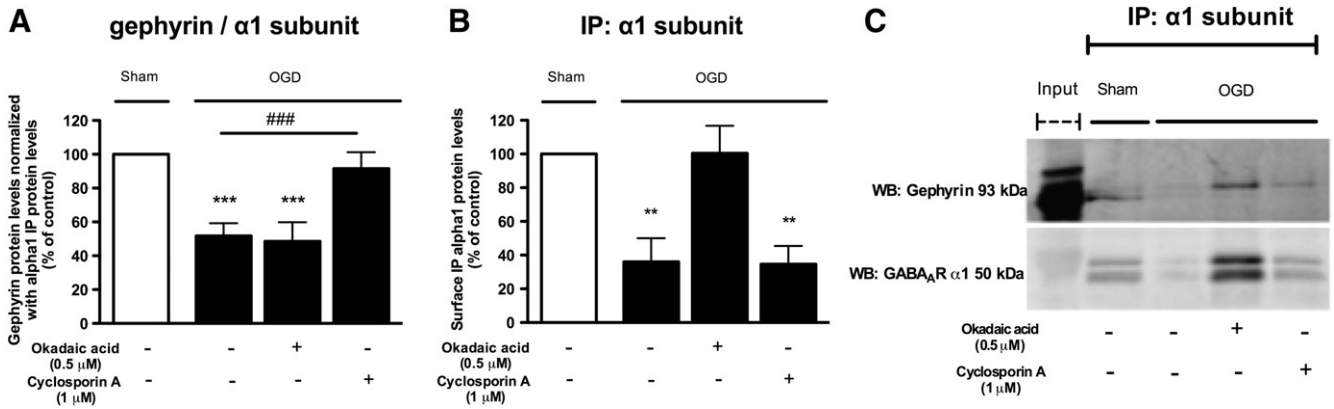


Fig. 2. OGD reduces the interaction of surface α1 $GABA_AR$ subunits with gephyrin. (A–C) A surface co-immunoprecipitation protocol was used to determine $GABA_AR$/gephyrin interaction. Cultured hippocampal neurons were exposed to OGD for 70 min and biotinylated as described in the methods section. Where indicated the cells were incubated with okadaic acid (0.5 μM) or with cyclosporin A (1 μM) during the OGD period. The surface $GABA_AR$ α1 subunits were analyzed by Western blot with a specific antibody after purification with a surface co-immunoprecipitation assay (B). The co-immunoprecipitation of gephyrin with the surface $GABA_AR$ α1 subunits was also analyzed by Western blot with a specific antibody, and the ratio between gephyrin associated with surface $GABA_AR$ α1 and the plasma membrane associated $GABA_AR$ α1 subunit is expressed in panel (A). A representative image is shown in panel (C). Results are means ± SEM of at least 3 independent experiments performed in different preparations, and expressed as percentage of the control. $GABA_AR$ α1 protein levels was used as loading control. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's or Bonferroni test. $^{**}p < 0.01$, $^{***}p < 0{,}001$, $^{\#\#}p < 0{,}01$, $^{\#\#\#}p < 0.01$—significantly different when compared to control conditions.

and $GABA_AR$ α1 (S. Fig. 5A) subunit protein levels under the experimental conditions used in these experiments.

Although okadaic acid did not affect the interaction between gephyrin and $GABA_AR$ α1 subunits, Western blot experiments showed a shift of the band corresponding to gephyrin in extracts prepared from neurons incubated with okadaic acid (S. Fig. 5B, S. Fig. 6). This shift did not correspond to an increase of total gephyrin protein levels, as confirmed by Western blot quantification (S. Fig. 5B). To investigate whether the shift in the gephyrin band was due to phoshorylation of the scaffold protein, λ-phosphatase assay was performed. Indeed, the observed shift in the gephyrin band was not observed when cell lysates were incubated with λ-phosphatase (S. Fig. 6). Also, no shift in gephyrin immunoreactivity was observed in extracts prepared from hippocampal neurons subjected to OGD, further suggesting that the phosphorylation sites regulated by protein phosphatases sensitive to okadaic acid are not involved in the regulation of the interaction between gephyrin and $GABA_AR$ α1 subunits.

OGD increases α1 $GABA_AR$ subunit internalization

From the functional point of view, it is the population of $GABA_AR$ associated with the plasma membrane that is expected to play a role in the modulation of the demise process after OGD. To determine the rate at which the cell-surface $GABA_ARs$ are internalized, we used an antibody feeding technique (Connolly et al., 1999; Lin et al., 2000) that allows distinguishing the cell-surface and the internalized pools of native $GABA_ARs$. Cell-surface $GABA_ARs$ on living cultured hippocampal neurons were labeled with an anti-$GABA_AR$-α1 (N-terminus) antibody. Control experiments showed that astrocytes present in the cultures (~10% of the cells) are not stained with the anti-$GABA_AR$-α1 (N-terminus) antibody (not shown).

Under resting conditions the $GABA_AR$ α1 subunit presented a constant rate of internalization for 30 min, when about 80% of the surface receptors were internalized. This rate of internalization, of about 10% of the surface receptors/10 min, was calculated both in the soma and neurites. In both compartments there was a pool of $GABA_AR$ α1 subunit, corresponding to about 20% of the labeled proteins, which was stable and did not undergo internalization during 60 min (Fig. 3A). Therefore, in all other experiments the internalization of $GABA_AR$ α1 subunits was followed for 10–20 min.

The effect of OGD on $GABA_AR$ α1 subunit internalization was tested in hippocampal neurons subjected to the ischemic injury for 70 min, which does not affect the total protein levels of the receptor subunit (S. Fig. 5). The experimental conditions used induce about 20% cell death as measured 7 h–12 h after the insult (S. Fig. 1). Labeling of surface receptors was performed immediately after OGD, to capture the initial alterations in the mechanisms regulating the receptor trafficking, and receptor internalization was measured for different periods of time (0–20 min). Immunocytochemistry analysis revealed ~25% increase in the ratio of α1 subunit internalization compared to the correspondent sham condition, when tested for 20 min (Fig. 3B and C), both in the soma and neurite compartments. This effect was abolished when internalization was blocked by a hyperosmolar concentration of sucrose (350 mM) (Fig. 3D and F), and with the specific dynamin inhibitor dynasor (125 μM) (Fig. 3E and G). The OGD-induced internalization of $GABA_AR$-α1 subunits was fully abrogated ($p > 0.05$) when the cells were treated with the NMDA receptor antagonist APV (100 μM), and similar results were obtained in the presence of NMDA (100 μM APV) and AMPA (20 μM NBQX) receptor antagonists (Fig. 4). The results obtained with the antibody feeding assay were similar in the soma and neurites, and show that OGD-induces the internalization of $GABA_AR$-α1 subunits by a dynamin-dependent mechanism that requires activation of NMDA receptors.

OGD-induced dephosphorylation of $GABA_AR$ β3 subunits leads to receptor internalization and mediates cell death

The internalization of $GABA_AR$ is a process negatively regulated by phosphorylation of β3 or γ2 $GABA_AR$ subunits intracellular loop (Kittler et al., 2005, 2008). The $GABA_AR$ β3 subunits are present in a large proportion of receptor subtypes in the hippocampus and cortex, regions that are particularly vulnerable to excitotoxicity (Lo et al., 2003). To evaluate if the observed increase of $GABA_AR$ internalization (Fig. 3) is mediated by receptor dephosphorylation, the levels of $GABA_AR$ β3 subunit phosphorylation were evaluated by Western blot analysis using a phospho-specific antibody against the β3 subunit serine residues 408/409 (mouse sequence) (Fig. 5). After 70 min of OGD, $GABA_AR$ β3 subunit phosphorylation was reduced by 60% (Fig. 5A,B), and a decrease in β3 subunit phosphorylation was also observed in the infarct core after transient MCAO (Fig. 5C,D). The effect of OGD on $GABA_AR$ β3 subunit phosphorylation level was reduced when the NMDA receptor inhibitor AP-5 (100 μM) was used (Fig. 5A). These observations are correlated with the role of NMDA receptors in OGD-induced internalization of $GABA_AR$ α1 subunits (Fig. 4). Furthermore, the effect of OGD on

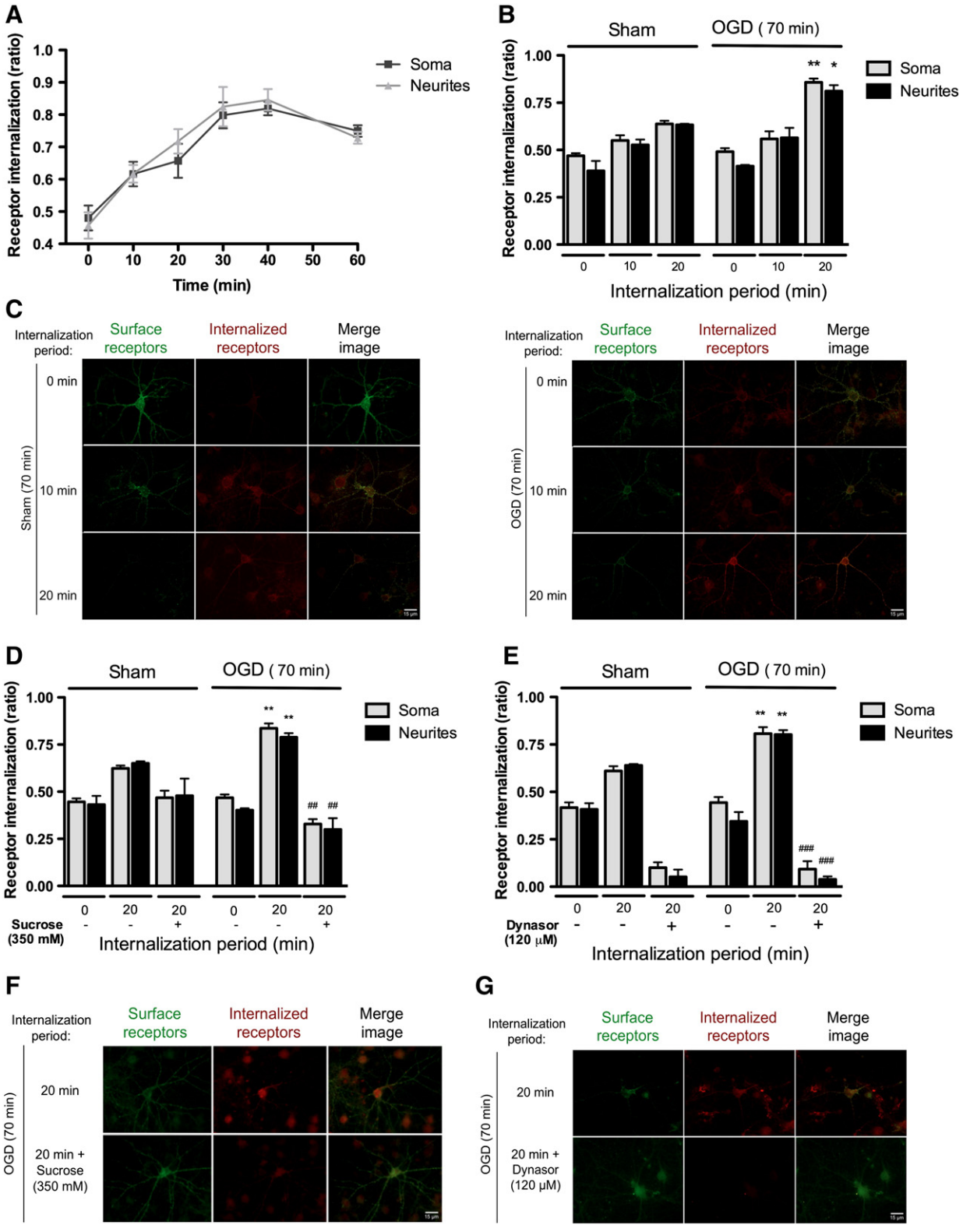


Fig. 3. OGD increases α1 $GABA_AR$ subunit internalization by clathrin-mediated endocytosis. Receptor internalization was assessed through an antibody-feeding assay and analyzed by fluorescence microscopy in cells labelled with an anti-$GABA_AR$-α1 (N-terminus) antibody. A time-course analysis of receptor internalization was performed in basal conditions (in the culture conditioned medium) to validate the method (A). After quantification of the images at the soma and dendritic compartments, the results were expressed as a ratio of internalized receptors/total receptor immunoreactivity. Different internalization periods were also tested in cells subjected to OGD (70 min) or maintained under control conditions (sham) before incubation with the anti-α1 $GABA_AR$ subunit antibody (B, C). Panels (D–G) show the effect of an hyperosmolar concentration of sucrose (350 mM) (D, F) and treatment with the dynamin inhibitor Dynasor (125 μM) (E, G) on the internalization of the α1 $GABA_AR$ subunit. Internalization of α1 $GABA_AR$ subunits was allowed for 20 min. When the effect of an hyperosmolar treatment was tested, the cells were incubated with 350 mM sucrose during the incubation period of surface receptor live staining and during the internalization period. The same strategy was adopted in the experiments with dynasor. Results are means ± SEM of at least 3 independent experiments, performed in different preparations. At least 10 cells were analyzed for each experimental condition/experiment. Internalization ratio was calculated by the ratio internalized antibody signal/total antibody signal. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's or Bonferroni test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{\#\#}p < 0{,}01$, $^{\#\#\#}p < 0.01$—significantly different when compared to control conditions.

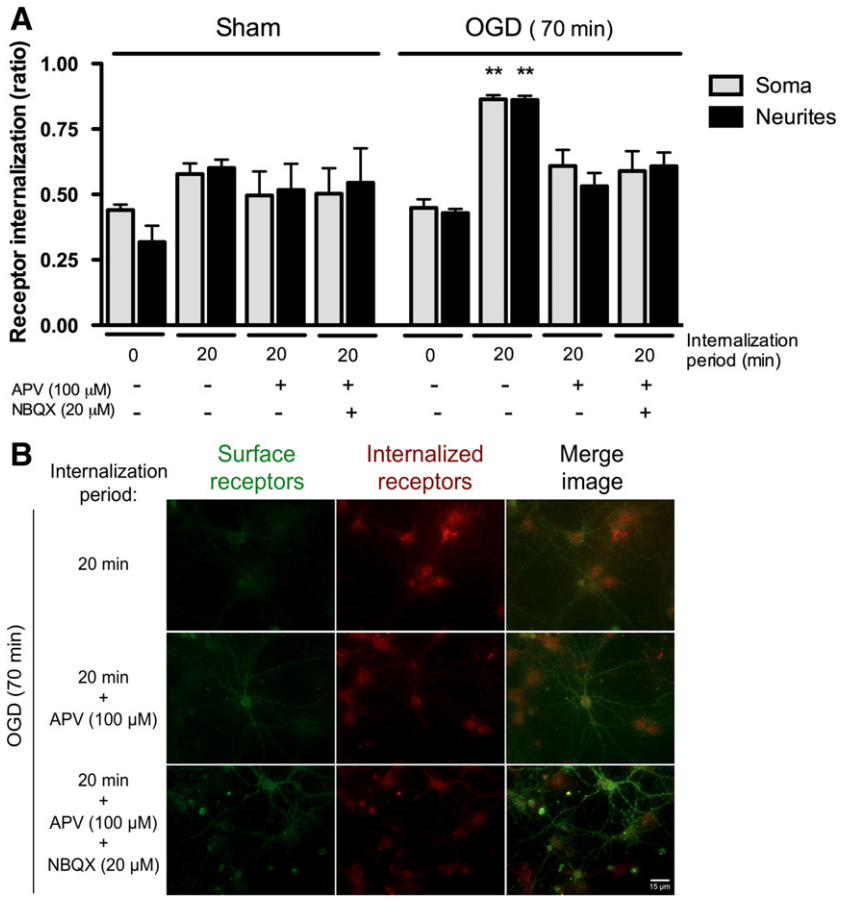


Fig. 4. OGD-induced $GABA_AR$ α1 subunit internalization is mediated by activation of glutamate receptors. Cultured hippocampal neurons were subjected to OGD (70 min) or maintained under control conditions (sham), and the internalization of $GABA_AR$ (20 min) was assessed through an antibody-feeding assay. When the effect of glutamate receptor antagonists was tested, the cells were pre-incubated (or not) with NBQX (20 μM) and AP-5 (100 μM) for 30 min before OGD, and the inhibitors were also present during the whole experimental period. Representative fluorescence images are shown in panel (B) and the results in (A) are means ± SEM of at least 3 independent experiments performed in independent preparations. At least 10 cells were analyzed in each condition per experiment. Ratio of internalization was calculated by internalized antibody signal/total antibody immunoreactivity. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's or Bonferroni test. $^{**}p < 0.01$—significantly different when compared to the sham condition.

β3 subunit dephosphorylation was prevented when neurons were incubated with 0.5 μM okadaic acid (PP1/PP2A phosphatase inhibitor) but not in the presence of 1 μ M cyclosporin A (calcineurin inhibitor) (Fig. 5B).

In vitro studies showed that phosphorylation of $GABA_AR$ β3 subunit, on serine residues 408/409, negatively regulates receptor endocytosis (Terunuma et al., 2008). These two serine residues are located in an AP2-binding motif conserved within the intracellular domain of all $GABA_AR$ subunit isoforms (KTHLRRRSSQLK) (Kittler et al., 2005). To evaluate the role of β3 subunit dephosphorylation in the increase of $GABA_AR$ internalization during OGD, and its contribution to the excitotoxicity-induced neuronal death, we made phosphomutants of the $GABA_AR$ β3 subunit. Cultured hippocampal neurons (13 DIV) were transfected with the myc-tagged wild-type or the phospho-mimetic form (SS432/433DD) (homologous of mouse 408/409) of the huGABA$_A$R β3 subunit, subjected to OGD for 90 min and further incubated in the culture conditioned medium for 12 h. The transfected cells were identified by immunocytochemistry with an anti-myc antibody (as shown in Fig. 6B), and nuclear morphology analysis of transfected cells (Fig. 6A) showed a protective effect of the phospho-mimetic form of the receptor that reduced OGD-induced cell death by about 50% when compared with the wild-type β3 subunit. In contrast, non-transfected cells in the two types of cultures exhibited a similar rate of OGD-induced neuronal death (Fig. 6A′), showing the specificity of the effects resulting from the expression of the phospho-mimetic form (SS432/433DD) of the huGABA$_A$R β3 subunit.

The surface expression of the myc-tagged $GABA_AR$ β3 subunit mutant was evaluated by immunocytochemistry with an anti-myc antibody under non-permeabilizing conditions (see Fig. 6C). The SS432/433DD mutant of the $GABA_AR$ β3 subunits presented an increased surface expression in transfected hippocampal neurons, both in control condition and after OGD (70 min), when compared to the WT $GABA_AR$ β3 subunits (Fig. 6D and D′). In contrast, transfection with the phospho-null mutant of $GABA_AR$ β3 subunits reduced the surface expression of the receptor, both in the somal (Fig. 6D) and neuritic compartments (Fig. 6D′). The total expression of the myc-tagged wild-type, phospho-mimetic (SS432/433DD) and phospho-null (SS432/433AA) forms of the $GABA_AR$ β3 subunit was evaluated by immunocytochemistry with an anti-myc antibody after permeabilization, showing similar expression level of these proteins (S. Fig. 7). The internalization rate of myc-tagged wild type, phospho-mimetic and phospho-null $GABA_AR$ β3 subunits, in cultured hippocampal neurons maintained under control conditions or subjected to OGD, was assessed using the antibody feeding technique (see Fig. 6F). A decrease in internalization ratio was observed for the phospho-mimetic mutant when compared with the WT $GABA_AR$ β3, in contrast with the phospho-null mutant which showed no alteration in internalization. Differential analysis of soma (Fig. 6E) and neurites (Fig. 6E′) showed similar results for the two cellular compartments.

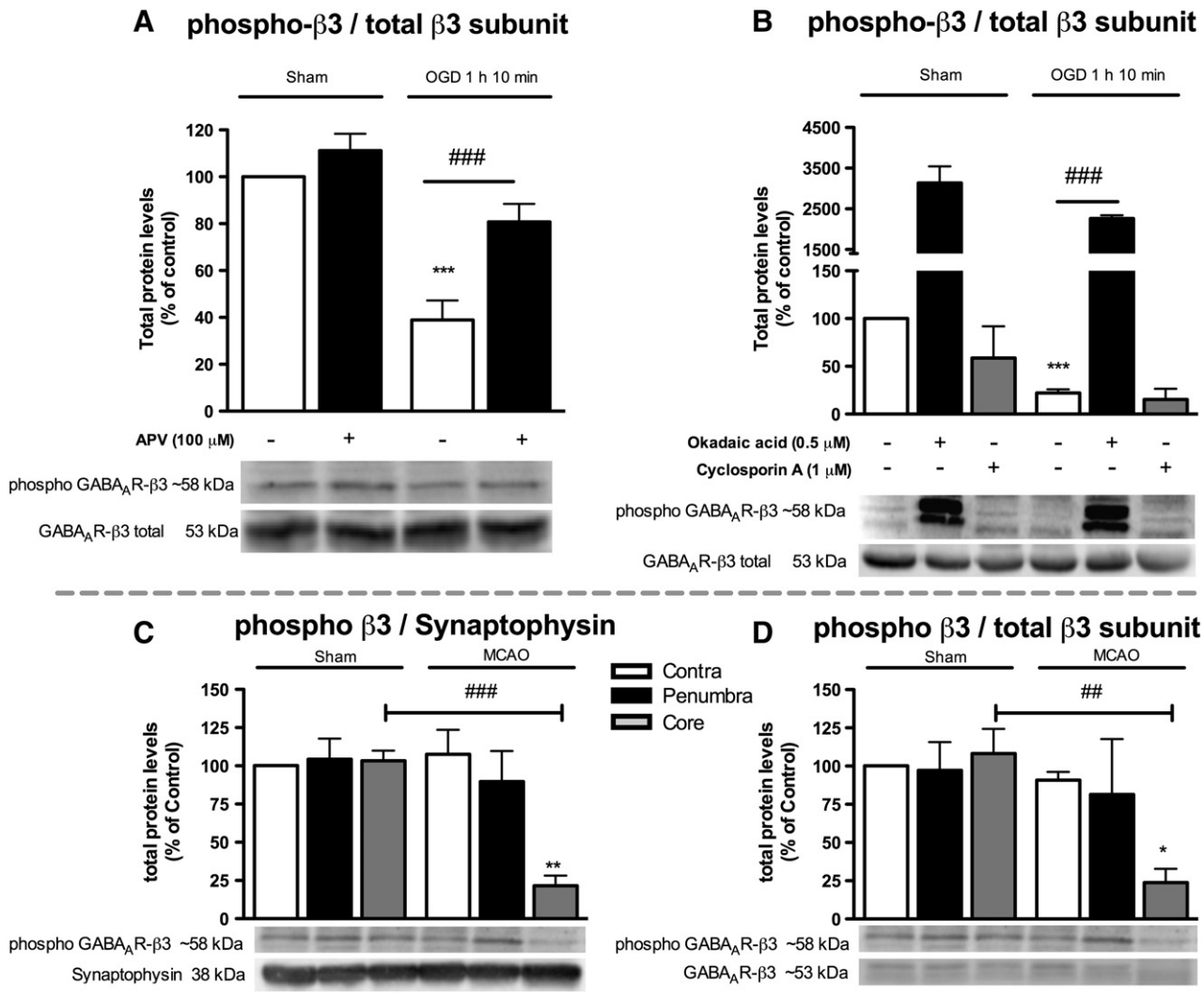


Fig. 5. OGD-induced GABA$_A$R internalization is mediated by β3 subunit dephosphorylation. GABA$_A$R β3 subunit phosphorylation was evaluated by Western blot analysis using a phospho-specific antibody against the β3 subunit serine 408/409 (A, B). The cells were subjected to OGD (70 min) or maintained under control conditions (sham), and the following inhibitors were tested: APV (100 μM) (A), okadaic acid (0.5 μM) and cyclosporin A (1 μM) (B). When the effect of glutamate receptor antagonists was tested, the cells were pre-incubated with the drugs for 30 min and they were also present during the entire experiment. GABA$_A$R β3 subunit phosphorylation was determined with a specific antibody, which binds to the phosphorylated serine 408/409. (C–D) Effect of *in vivo* ischemia (MCAO) on β3 GABA$_A$R subunit phosphorylation levels, as determined 48 h after the lesion, in the infarct core, penumbra and contralateral cortex. GABA$_A$R β3 subunit phosphorylation was evaluated by Western blot (reprobing of the membrane showing β3 subunit protein levels in Fig. 1F), as indicated above. Results were normalized to the total protein levels of GABA$_A$R β3 subunit or synaptophysin, and were expressed as percentage of control. The bars represent the means ± SEM of at least 3 independent experiments performed in different preparations. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's or Bonferroni test. $^{*}p < 0.05$ $^{**}p < 0.01$, $^{***}p < 0.001$, $^{\#\#\#}p < 0.001$—significantly different when compared to control conditions.

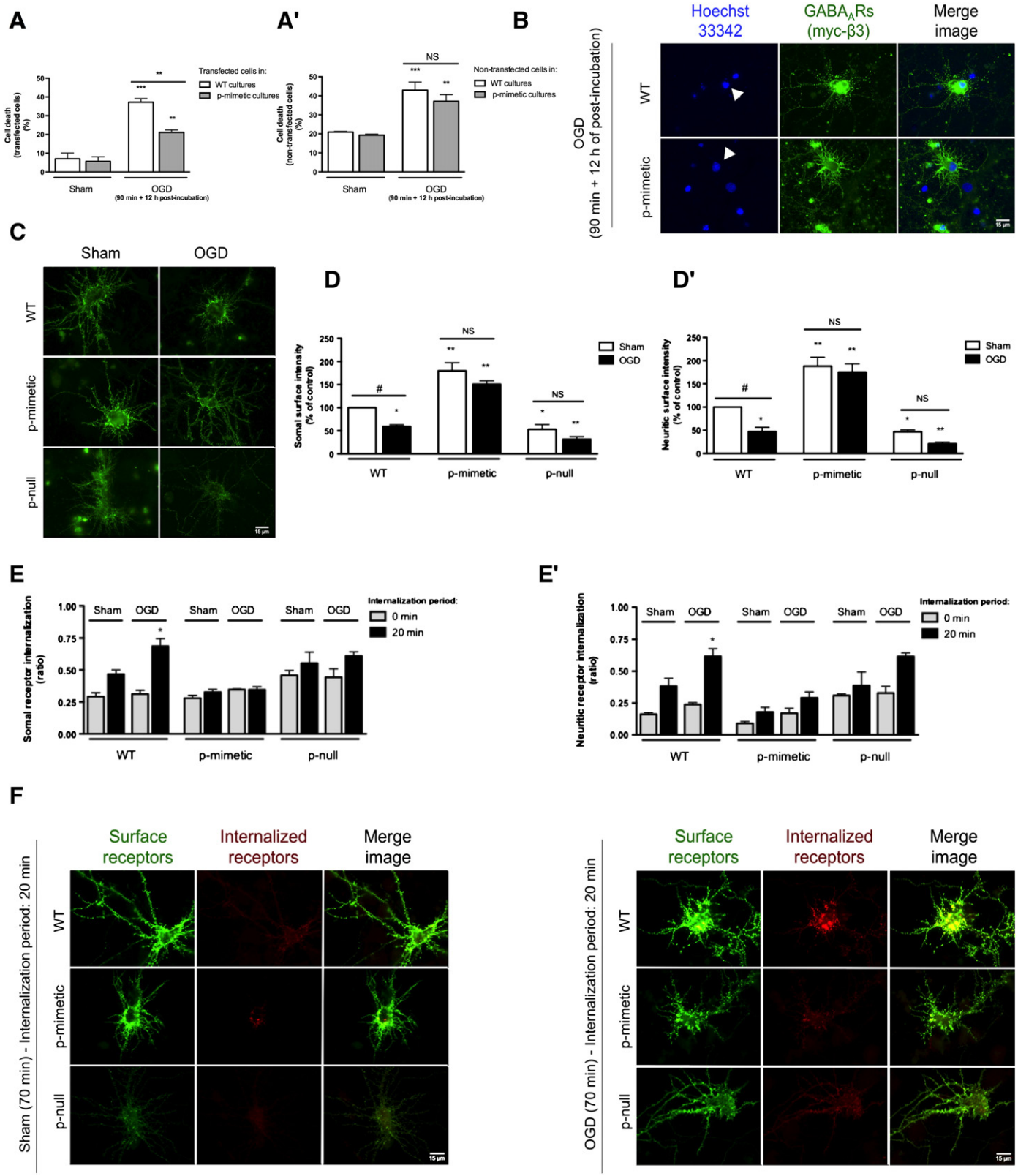
Discussion

Stroke, or cerebral ischemia, is characterized by an early disruption of GABAergic neurotransmission due to alterations at both pre- and post-synaptic sides of the GABAergic synapse (Schwartz-Bloom and Sah, 2001), but the molecular mechanisms involved are not fully understood. In this work we show a key role for protein phosphatases in the regulation of GABA$_A$R α1 subunit interaction with the anchoring protein gephyrin, which is responsible for receptor clustering at the synapse, and in the internalization of GABA$_A$R in hippocampal neurons subjected to OGD, an *in vitro* model of global ischemia. In particular, the dephosphorylation of β3 GABA$_A$Rs subunits was found to play a key

Fig. 6. OGD-induced GABA$_A$R β3 subunit dephosphorylation leads to receptor internalization and increases cell death. (A) Cultured hippocampal neurons were transfected with the myc-tagged wild-type or the phospho-mimetic form (Ser408/409) of the GABA$_A$R β3 subunit and subjected to OGD for 90 min, before incubation in the culture conditioned medium for 12 h. Where indicated (sham) the cells were treated under control conditions. The transfected cells were identified by immunocytochemistry with an anti-myc antibody, and the viability of transfected (A) and non-transfected (A′) cells was evaluated with Hoechst 33342. Representative images are shown in panel (B). The arrows point to the nuclei of hippocampal neurons transfected with the wild type or the phospho-mimetic forms of GABA$_A$R β3 subunit. Under the same conditions, hippocampal neurons transfected with the phospho-mimetic form of the GABA$_A$R β3 subunit show a decrease in cell death (the arrow in the panel B points to the nuclei of transfected cells). The effect of OGD on the surface expression of the myc-tagged GABA$_A$R β3 subunits (phospho-mimetic and phospho-null forms) was evaluated by immunocytochemistry, in the somal (D) and neuritic compartments (D′), with an anti-myc antibody under non-permeabilizing conditions. Representative images are shown in panel (C). The rate of internalization of myc-tagged wild type, phospho-mimetic and phospho-null GABA$_A$R β3 (Ser432/433) (homologous of mouse 408/409) subunits in cultured hippocampal neurons maintained under control conditions (sham) or subjected to OGD is shown in panels (E, E′) and (F). The internalization ratio, obtained by the antibody feeding assay, was calculated based on the immunoreactivity of the internalized antibody/total antibody signal. At least 10 cells were analyzed in each condition per experiment. In the case of nuclear cell morphology analysis, for each experimental condition two coverslips were analyzed and at least 40 cells were counted per coverslip. Results are means ± SEM of at least 3 independent experiments, performed in different preparations. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's or Bonferroni test. $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$—significantly different when compared to the sham condition. NS - not significant when compared to control conditions.

role in receptor internalization following OGD and the resulting loss of inhibitory activity contributes to neuronal death.

Using the antibody feeding assay we observed an increased internalization of $GABA_AR$ α1 and β3 subunits in hippocampal neurons subjected to OGD. The α1 $GABA_AR$ subunit is greatly expressed in the hippocampus (Hortnagl et al., 2013; Laurie et al., 1992; Wisden et al., 1992), a brain region that is highly vulnerable to ischemic conditions (Kirino and Sano, 1984; Schmidt-Kastner and Freund, 1991; Sugawara et al., 1999). The OGD-induced internalization of $GABA_AR$ α1 subunits was mediated by clathrin-dependent endocytosis, and required glutamate receptor activation. A similar mechanism was shown to contribute to the internalization of $GABA_AR$ in an *in vitro* model of epilepsy, a condition also characterized by excitation/inhibition imbalance (Goodkin et al., 2005).



GABA$_A$Rs are clustered at the synapse through interaction with gephyrin (Tyagarajan and Fritschy, 2010), and the GABA$_A$R α1 subunits were shown to interact directly with the scaffold protein (Mukherjee et al., 2011; Tretter et al., 2011). This is in agreement with the results obtained in the present study showing that surface GABA$_A$R α1 subunits co-immunoprecipitate with gephyrin. The interaction between α1 GABA$_A$R subunit and gephyrin promotes the receptor accumulation at inhibitory synapses, by limiting its lateral diffusion, and this interaction depends on residues 360–375 of the α1 subunit that bind directly to gephyrin (Mukherjee et al., 2011). Modulating this interaction via covalent modifications, such as phosphorylation, may be a potent mechanism to control the strength of fast GABAergic signaling. We observed that OGD significantly decreases the co-immunoprecipitation of surface GABA$_A$R α1 subunits with gephyrin, by a mechanism sensitive to calcineurin inhibition. Since calcineurin had no effect on the apparent mobility of gephyrin in SDS-PAGE, the results suggest that the phosphatase may dephosphorylate GABA$_A$R (Kapur and Lothman, 1990). The induction of long-term depression at CA1 inhibitory synapses also resulted in a reduction in the synaptic GABA$_A$R number by a calcineurin-dependent mechanism (Wang et al., 2003), while a direct effect of calcineurin on the functional properties of GABA$_A$R was proposed in a different study (Jones and Westbrook, 1997). The effect of calcineurin on the dissociation of gephyrin-GABA$_A$R α1 complexes induced by OGD clearly favors the former mechanism of action. However, at this point it is not possible to rule out an effect of OGD on the activity of protein kinase(s) responsible for the phosphorylation of the amino acid residues targeted by calcineurin (Chapell et al., 1998; Connolly et al., 1999; Filippova et al., 2000; Jovanovic et al., 2004).

The possibility that gephyrin phosphorylation might regulate GABA$_A$R binding to gephyrin, and their post-synaptic localization or trafficking, has not been investigated. Evidences available for glycine receptors (GlyR) demonstrate that proline-directed phosphorylation of gephyrin may induce a conformational change favoring GlyR binding (Tyagarajan and Fritschy, 2010; Zita et al., 2007). Several studies identified gephyrin as a target for serine/threonine directed phosphorylation (Beausoleil et al., 2006; Lundby et al., 2012), and a recent study detailed 18 different phosphorylation residues on gephyrin (Herweg and Schwarz, 2012), suggesting a key role for phosphorylation in the regulation of gephyrin function. Moreover, given that phosphorylation and $[Ca^{2+}]_i$ rise make gephyrin susceptible to proteolysis by calpain (Tyagarajan et al., 2013) the neuronal activity-driven gephyrin dynamics could very likely be phosphorylation-dependent. Accordingly, gephyrin phosphorylation on Ser268 is important for scaling (up or down) GABAergic transmission (Tyagarajan et al., 2013).

In contrast with the role of calcineurin in OGD-induced dissociation of gephyrin–GABA$_A$R α1 complexes, the dephosphorylation of GABA$_A$R β3 subunits under the same conditions is mediated by okadaic acid-sensitive protein phosphatases (PP1 and/or PP2A). Recruitment of GABA$_A$Rs into the endocytic pathway is facilitated via the interactions of the intracellular domains of β1–3 and γ2 subunits with μ2-AP2 (Kittler et al., 2005, 2008). This motif incorporates the major sites of phosphorylation for PKC and PKA, corresponding to serine residues S408 and S409 in the case of the GABA$_A$R β3 subunit (mouse sequence) (Brandon et al., 2002; McDonald and Moss, 1997). Phosphorylation of these sites has been shown to impair GABA$_A$R endocytosis by preventing the interaction of the β3 subunit with AP2 (Jacob et al., 2009; Kittler et al., 2005). The role of GABA$_A$R β3 dephosphorylation in receptor internalization and neuronal death in hippocampal neurons subjected to OGD is supported by the following evidences: i) OGD reduced the phosphorylation of GABA$_A$R β3 subunit by a mechanism sensitive to okadaic acid, as determined by Western blot with a phosphospecific antibody against serine residues 408/409; ii) the phospho-mimetic mutant of GABA$_A$R β3 subunit (SS432/433AA) was accumulated at cell surface and showed no OGD-induced

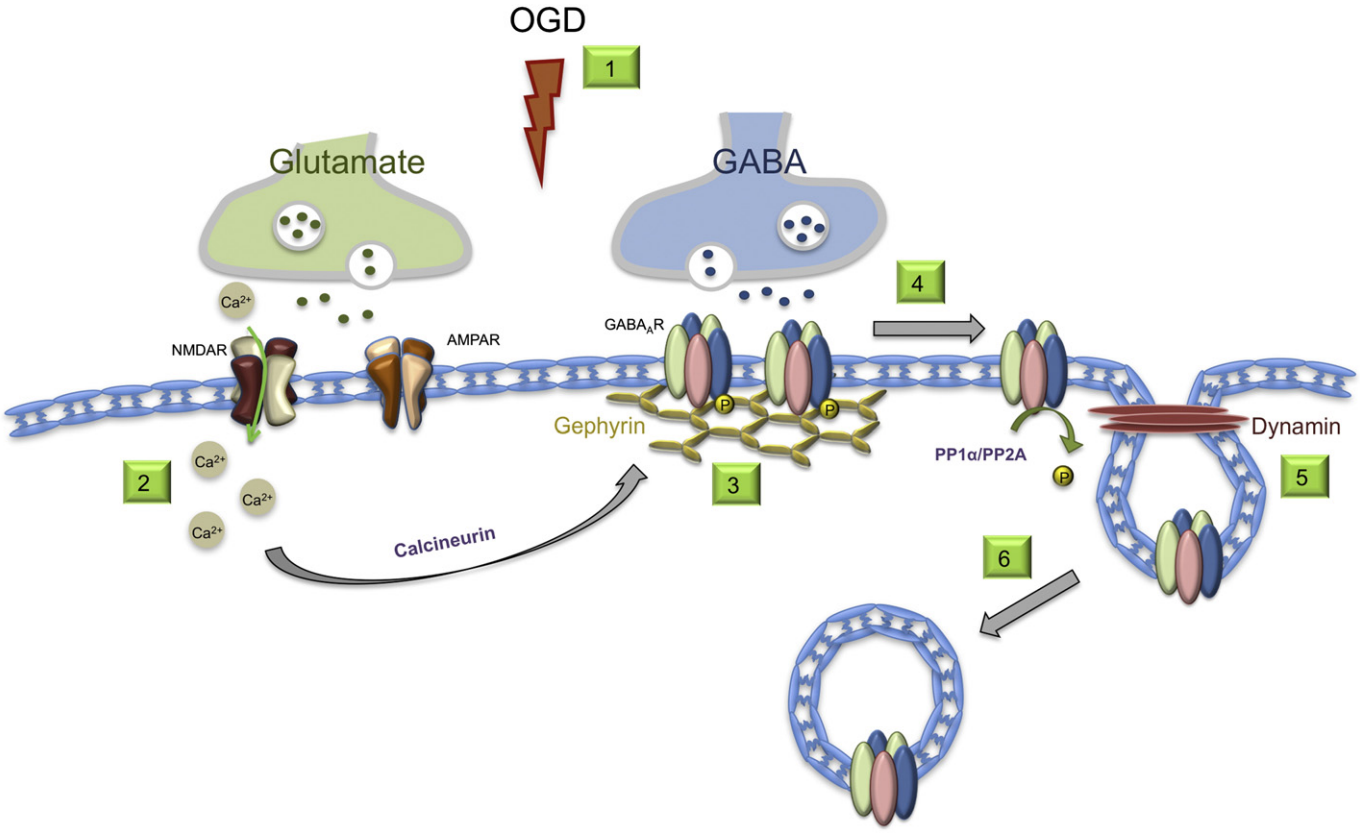


Fig. 7. Model of GABA$_A$R internalization during cerebral ischemia. Ischemic insult (1) over-activates NMDAR signaling (2) and the resulting activation of calcineurin decreases GABA$_A$R/gephyrin interaction (3). In parallel, OGD reduces phosphorylation of the GABA$_A$R β3 subunit by a mechanism sensitive to okadaic acid (4), inducing the internalization of GABA$_A$R via clathrin dependent endocytosis (5, 6).

internalization; iii) the same mutation reduced significantly OGD-induced cell death. The neuroprotection provided by the phosphomimetic mutant of $GABA_AR$ β3 subunit, resulting from receptor activation by endogenous GABA, is highly remarkable considering that less than 10% of the cells present in the culture are GABAergic (Baptista et al., 2010). The triple arginine motif of the β3 $GABA_AR$ subunit that mediates direct binding of the receptor to the clathrin adaptor protein AP2 plays a key role in regulating the surface distribution of the receptor (Smith et al., 2012). A peptide overlapping the AP2 binding region in the β3 subunit, to compete the β3/AP2 interaction, was shown to decrease OGD-induced cell death. However, this strategy does not rule out non-specific effects on the internalization of other plasma membrane proteins, which may interact with AP2 on the same binding motif. This evidence together with our result showing the protective effect of $GABA_AR$ stabilization in the membrane, strongly support the idea that $GABA_AR$ internalization is a potential target for ischemia therapy and the use of a molecule/peptide interacting with the β3 subunit residue responsible for the receptor internalization could be a good strategy to test *in vivo* for the treatment of cerebral ischemia.

In addition to the effect on the surface expression of $GABA_ARs$, OGD also downregulated the total protein levels and mRNA for several $GABA_AR$ subunits, which is likely to have a delayed and long-lasting effect on GABAergic synaptic transmission. At least some of the effects of OGD on $GABA_AR$ subunits (e.g. α2 and β3) are mediated by calpains. In addition to the effect on $GABA_AR$ subunits, the activation of calpains in ischemic condition results in the cleavage of a number of neuronal substrates that affect neuronal structure and function, leading to inhibition of neuronal survival mechanisms (Vosler et al., 2008). The OGD-induced downregulation of the mRNA levels for α1, α2, β2, β3 and γ2 $GABA_A$ receptor subunits was mediated by activation of glutamate receptors and would prevent the replenishment of the $GABA_AR$ pool degraded in response to the injury. Interestingly, under the OGD conditions used the mRNA levels for the $GABA_AR$ δ subunit were not significantly altered. Considering that this subunit is found at extrasynaptic regions (Nusser et al., 1998), the results suggest that OGD has differential effects on the synaptic and extrasynaptic pools of $GABA_ARs$.

In conclusion, we showed that the (de)phosphorylation site of $GABA_AR$ β3 subunits on serines 408/409 (mouse sequence) is a master regulator of $GABA_AR$ surface localization in ischemic conditions, and receptor internalization contributes to the death of hippocampal neurons after transient OGD. Recruitment of $GABA_AR$ for internalization is induced by glutamate receptor activation and follows the impairment in their interaction with the scaffold protein gephyrin, by a mechanism that is also regulated by protein phosphatases (Fig. 7). The degradation of $GABA_ARs$ and the downregulation of their mRNAs may further reduce GABAergic synaptic transmission. Taken together, these results suggest that modulation of $GABA_AR$ phosphorylation might be a therapeutic target to preserve synaptic inhibition in brain ischemia.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nbd.2014.01.019.

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