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Ischaemia differentially regulates GABA_B receptor subunits in organotypic hippocampal slice cultures

Helena Cimarosti¹, Sriharsha Kantamneni¹, and Jeremy M. Henley^{*}

Department of Anatomy, MRC Centre for Synaptic Plasticity, School of Medical Sciences, University Walk, University of Bristol, Bristol, BS8 1TD, UK

Abstract

Reduced synaptic inhibition due to dysfunction of ionotropic GABA_A receptors has been proposed as one factor in cerebral ischaemia-induced excitotoxic cell death. However, the participation of the inhibitory metabotropic GABA_B receptors in these pathological processes has not been extensively investigated. We used oxygen–glucose deprivation (OGD) and NMDA-induced excitotoxicity as models to investigate whether ischaemia-like challenges alter the protein levels of GABA_{B1} and GABA_{B2} receptor subunits in rat organotypic hippocampal slice cultures. Twenty-four hours after the insult both OGD and NMDA produced a marked decrease in the total levels of GABA_{B2} (~75%), while there was no significant change in the levels of GABA_{B1} after OGD, but an increase after NMDA treatment (~100%). The GABA_B receptor agonist baclofen (100 μM) was neuroprotective following OGD or NMDA treatment if added before or during the insult. GABA_B receptors comprise heterodimers of GABA_{B1} and GABA_{B2} subunits and our results suggest that the separate subunits are independently regulated in response to extreme neuronal stress. However, because GABA_{B2} is required for functional surface expression, down-regulation of this subunit removes an important inhibitory feedback mechanism under pathological conditions.

Keywords

Cerebral ischaemia; GABA_B receptors; Baclofen; Organotypic hippocampal slice cultures; Oxygen–glucose deprivation; NMDA-induced excitotoxicity

1. Introduction

Cerebral ischaemia/stroke is a major cause of death and severe long-term disability due to excessive neuronal excitation and consequent cell death (Choi, 1992). The involvement of excitatory glutamate receptors in ischaemic cell death has been extensively investigated but the contributions of inhibitory gamma-aminobutyric acid (GABA) receptors are less well established (Schwartz-Bloom and Sah, 2001). GABA acts at three main classes of receptors in the mammalian brain, the GABA_A, GABA_B and GABA_C receptors. Both GABA_A and GABA_C receptors are ligand gated Cl[−]-permeable ion channels, while GABA_B receptors are G-protein-coupled receptors that exert much longer lasting synaptic inhibition. GABA_B receptors require two subunits, GABA_{B1} and GABA_{B2}, to form a functional receptor and they are present at both post- and presynaptic compartments. GABA_B receptors act presynaptically by inhibiting Ca²⁺ channels, and postsynaptically by activating inwardly

rectifying K⁺ channels (GIRKs). Changes in the number, activity and/or localisation of GABA_B receptors can dramatically alter the level of synaptic inhibition (Bettler and Tiao, 2006). Thus, GABA_B receptors seem likely candidates to play a role in balancing excessive glutamatergic excitation that occurs during ischaemia. For example, activation of presynaptic GABA_B receptors that can down-regulate glutamate release might provide a mechanism to counteract excitotoxic neuronal cell death.

The GABA_B receptor agonist baclofen inhibits glutamate release (Huston et al., 1995) and has been reported to be neuroprotective *in vivo* (Babcock et al., 2002; Jackson-Friedman et al., 1997; Lal et al., 1995; Ouyang et al., 2007) and in organotypic hippocampal slice cultures during oxygen–glucose deprivation (OGD) (Dave et al., 2005). It has also been reported that both GABA_A and GABA_B receptor agonists can protect neurons against death induced by ischaemia/reperfusion *in vivo* via a mechanism involving inhibition of NMDA receptor-mediated nitric oxide (NO) production by neuronal NO synthase (nNOS) (Zhou et al., 2008). Interestingly, GABA_B receptors are stable at the plasma membrane showing little basal down-regulation or baclofen induced internalisation making them an attractive therapeutic target (Fairfax et al., 2004). Indeed, baclofen has been used for many years primarily as a muscle relaxant to treat spasticity (Bowery, 2006).

To gain insight into the potential roles of GABA_B receptors in ischaemia we investigated changes in GABA_{B1} and GABA_{B2} protein levels in organotypic hippocampal slice cultures exposed to OGD or NMDA-induced excitotoxicity, two models widely used to elicit neuronal cell death. In addition, we tested the neuroprotective effects of baclofen in these models. Our data suggest that the individual subunits are differentially regulated following ischaemic insult. The total levels of GABA_{B2} are dramatically reduced whereas GABA_{B1} levels remain unchanged. Surprisingly, we also found that although baclofen was neuroprotective it did not prevent the ischaemia-induced reduction in GABA_{B2}.

2. Methods

Animal care and all experimental procedures were conducted in accordance with British animal protection legislation and the experimental protocols were approved by the British National Committee for Ethics in Animal Research.

2.1. Organotypic slice cultures of rat hippocampus

Organotypic hippocampal slice cultures were prepared using the method of Stoppini and colleagues (Stoppini et al., 1991). Transverse hippocampal slices (400 µm) from 7-day-old male Wistar rats were cut using a McIlwain tissue chopper and transferred to Hank's balanced salt solution (HBSS). Six slices were placed on each Millicell culture insert in 6-well culture plates together with 1 ml of culture medium per well. Culture medium (pH 7.3) consisted of minimum essential medium 50%, horse serum 25% and HBSS 25% supplemented with glucose 36 mM, HEPES 25 mM, NaHCO₃ 4 mM, and penicillin/streptomycin 1%. The cultures were incubated at 37 °C in an atmosphere of 5% CO₂ for 14 days *in vitro* (DIV) prior to use. Medium was changed every 3 days.

2.2. OGD and NMDA treatment

On DIV 14, the cultures were exposed to OGD (Cimarosti et al., 2001). Each insert was washed twice with OGD medium (pH 7.2) composed of CaCl₂ 1.26 mM, KCl 5.36 mM, NaCl 136.89 mM, KH₂ PO₄ 0.44 mM, Na₂HPO₄ 0.34 mM, MgCl₂ 0.49 mM, MgSO₄ 0.44 mM, HEPES 25 mM, NaHCO₃ 4 mM and penicillin/streptomycin 1%. To deplete glucose from intracellular stores and extracellular space, the inserts were incubated in 1 ml of OGD medium for 10 min. Following this period, the medium was exchanged for OGD medium

previously bubbled with N₂/CO₂ (95%/5%) for 10 min. The slice cultures were then transferred to an anaerobic chamber at 37 °C with N₂-enriched atmosphere where they were maintained for 45 min. After OGD, the slices were removed from the chamber, washed twice with HBSS, returned to culture medium, and incubated for a further 24 h. Where appropriate baclofen or AP5 was incorporated in culture medium and in OGD medium during the periods indicated.

For assessments of NMDA-induced cell death, on DIV 14 the cultures were exposed to NMDA 50 µM for 45 min (in the same culture medium; 37 °C atmosphere air with 5% CO₂). After NMDA exposure, the inserts were washed twice with HBSS and returned to culture medium for 24 h before being assessed for cell death. Baclofen or AP5 was incorporated in culture medium with or without NMDA during the periods indicated.

Slices in the control groups were treated in parallel to slices in the OGD or NMDA groups, and incubated for 45 min, but were washed and maintained in culture medium and exposed to 37 °C atmosphere air with 5% CO₂.

2.3. Assessment of cell death due to OGD or NMDA excitotoxicity

Cellular damage was assessed by fluorescent image analysis of propidium iodide (PI) uptake (Cimarosti et al., 2001). PI is excluded from healthy cells, but following loss of membrane integrity it enters cells, binds to DNA and becomes highly fluorescent. Twenty-four hours after OGD or NMDA-induced excitotoxicity, PI 7.5 µM was added to cultures and incubated for 1 h. Cultures were observed with an inverted microscope (Nikon Eclipse TE 300) fitted with a standard rhodamine filter set. Images were captured and analysed using Scion Image software. The intensity of PI fluorescence in the selected region of interest, (CA1, CA2 + CA3 or DG (dentate gyrus)), was used as an index of cell death. The pixel intensity and area in which PI fluorescence was detectable above background level was determined using the 'density slice' function of the software. The mean percentage values of fluorescence in the slices treated with test compounds (baclofen or AP5) were calculated and compared to standard damage (Cimarosti et al., 2001). Standard damage was obtained as the mean of the intensity of PI fluorescence in the organotypic slices subjected to OGD or NMDA with no added drug.

2.4. Western blots and densitometry

For Western blot analysis, slices were added to Tris-HCl (pH 7.4) 50 mM, NaCl 150 mM, EDTA 1 mM, SDS 0.1%, Triton X-100 1% and mammalian protease inhibitor 1%, and homogenized on ice. The homogenates were sonicated for 10–15 s at 4 °C and the protein concentration determined. Samples were heated for 5 min at 37 °C with β-mercaptoethanol 5%, and subjected to SDS-PAGE (8%) loaded at 15 µg protein/lane. Proteins were blotted onto Immobilon-P membrane (Millipore Corporation, Bedford, MA, USA) and probed with appropriate primary antibodies after blocking with non-fat dry milk. The primary antibodies used were: rabbit polyclonal anti-GABA_{B1a,b} antibody (1 µg/ml, Santa Cruz Biotechnology), guinea pig polyclonal anti-GABA_{B2} (1:1000, Chemicon), and mouse monoclonal anti-β-actin (1:5000, Sigma Chemicals). The membrane was incubated with horseradish peroxidase-conjugated secondary antibodies (1:10 000, Sigma Chemicals) for 1 h followed by substrate incubation with BM Chemiluminescence Blotting Substrate (POD, Roche Molecular Biochemicals) or SuperSignal West Femto (Pierce). The chemiluminescence signal was detected on Hyperfilm HP (Amersham Biosciences). The band intensity was quantified by densitometry using ImageJ (NIH). OGD and NMDA groups were compared to control groups, which were designated as 100%, on the same Western blot to avoid any differences in signal intensity due to exposure times. All the blots

were then re-probed with β -actin antibody as an internal control to ensure equal protein loading in all lanes.

2.5. Statistical analysis

Data are presented as mean $\pm$ SEM (standard error of the mean) of the indicated number of independent experiments. One-way analysis of variance (ANOVA), followed by Duncan's multiple-range method, was applied to the means to determine significant differences between experimental groups. p Values < 0.05 were considered statistically significant.

3. Results

3.1. Dose–response effect of baclofen present during oxygen–glucose deprivation (OGD)

Organotypic hippocampal slice cultures were subjected to OGD for 45 min in the absence or presence of baclofen. In the CA1 region of OGD slices, baclofen at concentrations of 5, 50, 100 and 200 μ M decreased cell death assessed by the incorporation of propidium iodide (PI) by $37 \pm 7\%$, $36 \pm 10\%$, $34 \pm 7\%$ and $49 \pm 10\%$, respectively, compared to the CA1 region of untreated OGD slices (Fig. 1B). Similar neuroprotection was observed in the CA2 + CA3 region, where baclofen at the concentrations of 5, 50 and 100 μ M decreased PI uptake by $30 \pm 10\%$, $27 \pm 10\%$ and $38 \pm 8\%$, respectively whereas the concentration of 200 μ M conferred higher levels of neuroprotection ($71 \pm 11\%$, Fig. 1C). In the DG baclofen decreased the PI uptake by $32 \pm 7\%$ at 100 μ M and by $33 \pm 15\%$ at 200 μ M (Fig. 1D). To validate the OGD protocol as a model to assess neuroprotection, we tested the NMDA receptor antagonist AP5, an established neuroprotective agent (Fatokun et al., 2008). AP5 (50 μ M) added during exposure to OGD decreased PI incorporation by $\sim 95\%$ in all regions analysed (see Fig. 3) indicating that nearly all OGD-invoked cell death is mediated by NMDA receptor activation.

3.2. GABA_B receptors levels after OGD are not affected by baclofen treatment

We next investigated the effects of OGD on GABA_B receptor levels in control and baclofen-treated slices. Slices analysed 24 h after OGD showed dramatically decreased levels of GABA_{B2}. Levels of GABA_{B1}, however, remained unchanged by OGD (Fig. 2A and B). Interestingly, inclusion of baclofen (100 μ M) during the 45 min OGD did not alter the profiles of GABA_B receptor subunits (Fig. 2A and B, see Fig. 4). To determine whether the OGD effect on GABA_B receptor levels was an immediate or delayed response to cellular stress, we analysed slices collected immediately after (0 min), 60 and 120 min after OGD. The levels of GABA_{B2} were decreased at 0 min and decreased further at 60 min and 120 min time points (Fig. 2C and E). In contrast, GABA_{B1} did not decrease rather there was a trend towards increased levels following OGD, and the changes were statistically significant at 120 min (Fig. 2C and D).

We next tested the time window for neuroprotection by baclofen. Baclofen (100 μ M) was added either 45 min before, during or immediately after OGD (Fig. 3). While not as effective as AP5, significant levels of protection were observed with baclofen in all the analysed regions. In the CA1 region the PI incorporation was reduced by $28 \pm 7\%$, $42 \pm 11\%$ and $56 \pm 9\%$, when baclofen was added before, during or after OGD, respectively (Fig. 3B). Similar neuroprotection was conferred in the CA2 + CA3 region ($38 \pm 11\%$, $63 \pm 9\%$ and $50 \pm 10\%$, respectively, Fig. 3C) and in the DG ($40 \pm 11\%$, $48 \pm 12\%$ and $50 \pm 9\%$, respectively, Fig. 3D).

Baclofen either before or during OGD did not affect the OGD-induced changes in the levels of GABA_{B2} (Fig. 4). However, when baclofen was added after OGD, a slight but significant increase in the levels of GABA_{B2} compared to baclofen-untreated OGD slices was observed

(Fig. 4), indicating that baclofen stimulation may provide some level of protection against OGD-induced GABA_{B2} degradation.

3.3. Effects of baclofen on NMDA-induced excitotoxicity

In parallel experiments we investigated the fate of GABA_B receptor subunits and the effects of baclofen on slice cultures exposed to NMDA (50 μ M) for 45 min. Baclofen (100 μ M) was added for 45 min before, during or after NMDA-induced excitotoxicity. When added before or during NMDA exposure, baclofen decreased the incorporation of PI by ~50% in the CA1 region, ~40% in the CA2 + CA3 region and ~35% in the DG (Fig. 5). However, when baclofen was added after NMDA treatment, it did not exert a significant neuroprotective effect (Fig. 5). Surprisingly, the presence of baclofen before, during or after NMDA treatment caused a significant increase in the levels of GABA_{B1} compared to control exposed slices whereas GABA_{B2} levels were decreased in all NMDA-treated slices both with and without baclofen (Fig. 6).

4. Discussion

We used rat organotypic hippocampal slice cultures to investigate the effects of *in vitro* models of ischaemia on the total levels of GABA_B receptor subunits and to assess the neuroprotective effect of baclofen. Unlike dispersed neuronal cell cultures, organotypic slice cultures retain some structure and neuronal pathways (Cimarosti and Henley, 2008). This makes them well suited to study the balance between excitatory and inhibitory neurotransmission regulated by neuronal network connections involving distinct types of GABAergic interneurons and glial cells (DeFazio et al., 2009; Maccaferri et al., 2000).

The roles of GABA in brain damage after energy deprivation have been investigated by using several experimental approaches (Green et al., 2000; Schwartz-Bloom and Sah, 2001) however there is some discrepancy in the literature. Enhanced GABAergic transmission has been reported to be neuroprotective (Calabresi et al., 2003; Chen Xu et al., 2000; Galeffi et al., 2000; Schwartz-Bloom et al., 2000). Conversely, GABA receptor agonists have been reported to exacerbate cerebral damage caused by energy deprivation (Erdo et al., 1991; Muir et al., 1996; Stokes et al., 2001). In our *in vitro* ischaemic models baclofen was strongly neuroprotective but this effect did not appear to be related to changes in the total levels of GABA_B receptor subunits. Ischaemic preconditioning (IPC) *in vivo* in rats promotes a robust release of GABA after lethal ischaemia compared with control rats and IPC also increases the activity of glutamate decarboxylase, the major GABA synthesis pathway in the brain (Dave et al., 2005). In rat organotypic hippocampal slices, IPC and preconditioning by activation of the epsilon-isoform of protein kinase C confer neuroprotection that depends on functional modifications of GABA synapses (DeFazio et al., 2009). In addition, baclofen also provided a significant decrease in cellular death in organotypic hippocampal slices exposed to OGD suggesting that activation of GABA_B contributes significantly to neuroprotection against ischaemia (Dave et al., 2005). Similarly, baclofen administration *in vivo* (50 mg/kg) prevented both the loss of hippocampal CA1 pyramidal cells and the reduction in hippocampal CaM kinase immunoreactivity observed in control animals following ischaemic insult although it did not prevent ischaemia-induced working memory deficits in behavioral tests (Babcock et al., 2002).

A major factor on neuronal death following OGD is cytotoxic calcium entry through NMDA receptors (Papadia and Hardingham, 2007). Consistent with this, we show that inclusion of AP5, an NMDA receptor antagonist, during OGD resulted in very high levels of neuroprotection. Nonetheless, OGD is an extreme insult that likely also affects other signalling pathways. We therefore propose that the increase in GABA_{B1} following NMDA-induced excitotoxicity but not OGD may be due to subtle differences in the signalling

cascades activated. Baclofen was most neuroprotective in CA1, moderately neuroprotective in CA3 regions of hippocampal slices but least effective in DG. These results are consistent with previously published expression studies of distributions of the GABA_{B1} subunit which is strongly expressed in the CA1, less strongly expressed in CA3 regions and weakly expressed in DG (López-Bendito et al., 2004). Similarly NMDA receptors are weakly expressed in the CA3 region and highly expressed in DG (Coultrap et al., 2005). Indeed, consistent with this we found that in NMDA-induced excitotoxicity, AP5 was more neuroprotective in DG compared to other regions.

We have shown previously that ischaemia *in vivo* results in marked decreases in both AMPAR and KAR levels (Cimarosti et al., 2008). Further, ischaemia has been shown to dramatically down-regulate expression of the AMPA receptor subunit GluR2 and thereby increase the Ca²⁺ permeability of AMPARs (reviewed by Liu and Zukin, 2007; Soundarapandian et al., 2005). Here we demonstrate a decrease in GABA_{B2} protein levels 24 h after OGD or NMDA-induced excitotoxicity in organotypic hippocampal slice cultures, both in the absence or presence of baclofen. Interestingly, there was large reduction of GABA_{B2} protein levels immediately following OGD suggesting that these subunits were degraded during OGD protocol. Although unexpected, similar rapid rates of receptor turnover (~50% loss within 45 min) have been reported previously. For example we, and others, have shown similar extents and rates of degradation for EGF receptor (Kantamneni et al., 2009; Lu et al., 2003). In contrast, the levels of the GABA_{B1} subunit were largely unchanged following our *in vitro* ischaemia models. This observation of differential GABA_B subunit regulation argues against a general loss of surface receptor proteins in response to excitotoxic stress, and supports a model whereby GABA_{B2} is being specifically degraded.

The data presented here are broadly consistent with analysis of GABA_B subunit mRNAs using *in situ* hybridization following transient global ischaemia in the gerbil hippocampus. Both GABA_{B1} and GABA_{B2} mRNAs decreased and then disappeared in the CA1 region of the hippocampus in conjunction with neuronal death (Vollenweider et al., 2006). GABA_{B2}, which is less abundant, was more affected than the GABA_{B1}. Two days after ischaemia there was a pronounced decrease in GABA_{B2} while GABA_{B1} was only slightly altered. However, four days after ischaemia when there was widespread cell death, expression of both subunits was nearly absent in CA1. In organotypic hippocampal slice cultures, ischaemic models induce neuronal death within 24 h in the CA1 region, and the damage extends to the CA3 region during the following 72 h (Cho et al., 2004). The analysis at different time points after ischaemia could explain the different findings between our *in vitro* study in organotypic cultures and the previous *in vivo* study in gerbils (Vollenweider et al., 2006). Furthermore, it is difficult to directly correlate mRNA levels with protein levels. This may be especially true in the case of GABA_B – levels of GABA_{B2} in GABA_{B1}^{-/-} mice are almost undetectable, despite GABA_{B2} mRNA levels and distribution in the brain being unchanged (Schuler et al., 2001).

The reasons and mechanisms for the differential regulation of GABA_B receptor subunits following ischaemic insult observed here are unclear. Although it has been published previously that plasma membrane expression of GABA_B receptors is stable with little net endocytosis even in the presence of baclofen (Fairfax et al., 2004), there is general theme emerging in the field that GABA_B receptors undergo constitutive cycling. This constitutive cycling is increased upon baclofen application (Wilkins et al., 2008; Laffray et al., 2007). It is possible this may be a way of keeping the receptors at the surface active during prolonged stimulation by exchanging desensitized surface receptors for newly exocytosed non-desensitized receptors. This would provide an explanation, at least in part, for the apparent lack of GABA_B desensitization in some systems (Cruz et al., 2004; Wetherington and Lambert, 2002).

Despite the ~50% loss of GABA_{B2} during the 45 min OGD baclofen is still neuroprotective. It therefore remains possible that during excitotoxic/ischaemic conditions there is a compensatory increase in GABA_B receptor signalling sensitivity. Interestingly, there is some evidence to support this. GABA_B receptors are regulated by 5' AMP-dependent protein kinase (AMPK) (Kuramoto et al., 2007). AMPK regulates cellular energy levels by stimulating catabolic metabolism and simultaneously inhibiting anabolic pathways and is rapidly activated following ischaemia (Kahn et al., 2005; Ramamurthy and Ronnett, 2006). AMPK binds GABA_{B1} and directly phosphorylates S783 in GABA_{B2} to enhance GABA_B receptor activation of GIRKs. Phosphorylation of S783 was dramatically increased following ischaemic injury leading to the suggestion that it may play a role in neuroprotection after ischaemia by increasing GABA_B receptor function and reducing excitotoxicity (Kuramoto et al., 2007). In that study, no change in GABA_{B2} staining was detected following middle cerebral artery occlusion (MCAO) *in vivo* or 80 min after transient anoxic insult (10 mM deoxyglucose and 10 mM azide) in dispersed neuronal cultures. However, because of the different model systems, ischaemia paradigms and sampling times used it is difficult to compare directly the reduced levels of GABA_{B2} after ischaemic insult here and not in the AMPK study.

It is feasible that GABA_{B1} is constitutively synthesized and that GABA_{B2} is the determining, and therefore most stringently regulated, factor in the number of functional GABA_B receptors. In that case, down-regulation of GABA_{B2} will result in decreased inhibitory neurotransmission and remove a counter balance of excitatory glutamate release and enhance the neurotoxicity. While further research work is required to resolve these questions, GABA_B receptor agonists that can access the brain could provide a new potential avenue for the development of neuroprotective therapies in the treatment of ischaemia.

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References

- Babcock AM, Everingham A, Paden CM, Kimura M. Baclofen is neuroprotective and prevents loss of calcium/calmodulin-dependent protein kinase II immunoreactivity in the ischemic gerbil hippocampus. *J. Neurosci. Res.* 2002; 67:804–811. [PubMed: 11891795]
- Bettler B, Tiao JY. Molecular diversity, trafficking and subcellular localization of GABA_B receptors. *Pharmacol. Ther.* 2006; 110:533–543. [PubMed: 16644017]
- Bowery NG. GABA_B receptor: a site of therapeutic benefit. *Curr. Opin. Pharmacol.* 2006; 6:37–43. [PubMed: 16361115]
- Calabresi P, Cupini LM, Centonze D, Pisani F, Bernardi G. Antiepileptic drugs as a possible neuroprotective strategy in brain ischemia. *Ann. Neurol.* 2003; 53:693–702. [PubMed: 12783414]
- Chen Xu W, Yi Y, Qiu L, Shuaib A. Neuroprotective activity of tiagabine in a focal embolic model of cerebral ischemia. *Brain Res.* 2000; 874:75–77. [PubMed: 10936225]
- Cho S, Liu D, Fairman D, Li P, Jenkins L, McGonigle P, Wood A. Spatiotemporal evidence of apoptosis-mediated ischemic injury in organotypic hippocampal slice cultures. *Neurochem. Int.* 2004; 45:117–127. [PubMed: 15082229]
- Choi DW. Excitotoxic cell death. *J. Neurobiol.* 1992; 23:1261–1276. [PubMed: 1361523]
- Cimarosti H, Rodnight R, Tavares A, Paiva R, Valentim L, Rocha E, Salbego C. An investigation of the neuroprotective effect of lithium in organotypic slice cultures of rat hippocampus exposed to oxygen and glucose deprivation. *Neurosci. Lett.* 2001; 315:33–36. [PubMed: 11711208]
- Cimarosti H, Lindberg C, Bomholt SF, Ronn LC, Henley JM. Increased protein SUMOylation following focal cerebral ischemia. *Neuropharmacology.* 2008; 54:280–289. [PubMed: 17991493]

- Cimarosti H, Henley JM. Investigating the mechanisms underlying neuronal death in ischemia using in vitro oxygen-glucose deprivation: potential involvement of protein SUMOylation. *Neuroscientist*. 2008; 14:626–636. [PubMed: 19029060]
- Coultrap SJ, Nixon KM, Alvestad RM, Valenzuela CF, Browning MD. Differential expression of NMDA receptor subunits and splice variants among the CA1, CA3 and dentate gyrus of the adult rat. *Brain Res. Mol. Brain Res*. 2005; 135:104–111. [PubMed: 15857673]
- Cruz HG, Ivanova T, Lunn ML, Stoffel M, Slesinger PA, Lüscher C. Bi-directional effects of GABA(B) receptor agonists on the mesolimbic dopamine system. *Nat. Neurosci*. 2004; 7:153–159. [PubMed: 14745451]
- Dave KR, Lange-Asschenfeldt C, Raval AP, Prado R, Busto R, Saul I, Perez-Pinzon MA. Ischemic preconditioning ameliorates excitotoxicity by shifting glutamate/gamma-aminobutyric acid release and biosynthesis. *J. Neurosci. Res*. 2005; 82:665–673. [PubMed: 16247804]
- DeFazio RA, Raval AP, Lin HW, Dave KR, Della-Morte D, Perez-Pinzon MA. GABA synapses mediate neuroprotection after ischemic and epsilonPKC preconditioning in rat hippocampal slice cultures. *J. Cereb. Blood Flow Metab*. 2009; 29:375–384. [PubMed: 18957990]
- Erdo S, Michler A, Wolff JR. GABA accelerates excitotoxic cell death in cortical cultures: protection by blockers of GABA-gated chloride channels. *Brain Res*. 1991; 542:254–258. [PubMed: 1851456]
- Fairfax BP, Pitcher JA, Scott MG, Calver AR, Pangalos MN, Moss SJ, Couve A. Phosphorylation and chronic agonist treatment atypically modulate GABAB receptor cell surface stability. *J. Biol. Chem*. 2004; 279:12565–12573. [PubMed: 14707142]
- Fatokun AA, Stone TW, Smith RA. Adenosine receptor ligands protect against a combination of apoptotic and necrotic cell death in cerebellar granule neurons. *Exp. Brain Res*. 2008; 186:151–160. [PubMed: 18040669]
- Galeffi F, Sinnar S, Schwartz-Bloom RD. Diazepam promotes ATP recovery and prevents cytochrome c release in hippocampal slices after in vitro ischemia. *J. Neurochem*. 2000; 75:1242–1249. [PubMed: 10936207]
- Green AR, Hainsworth AH, Jackson DM. GABA potentiation: a logical pharmacological approach for the treatment of acute ischaemic stroke. *Neuropharmacology*. 2000; 39:1483–1494. [PubMed: 10854894]
- Huston E, Cullen GP, Burley JR, Dolphin AC. The involvement of multiple calcium channel sub-types in glutamate release from cerebellar granule cells and its modulation by GABAB receptor activation. *Neuroscience*. 1995; 68:465–478. [PubMed: 7477957]
- Jackson-Friedman C, Lyden PD, Nunez S, Jin A, Zweifler R. High dose baclofen is neuroprotective but also causes intracerebral hemorrhage: a quantal bioassay study using the intraluminal suture occlusion method. *Exp. Neurol*. 1997; 147:346–352. [PubMed: 9344559]
- Kahn BB, Alquier T, Carling D, Hardie DG. AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metab*. 2005; 1:15–25. [PubMed: 16054041]
- Kantamneni S, Holman D, Wilkinson KA, Nishimune A, Henley JM. GISP increases neurotransmitter receptor stability by down-regulating ESCRT-mediated lysosomal degradation. *Neurosci. Lett*. 2009; 452:106–110. [PubMed: 19154774]
- Kuramoto N, Wilkins ME, Fairfax BP, Revilla-Sanchez R, Terunuma M, Tamaki K, Iemata M, Warren N, Couve A, Calver A, Horvath Z, Freeman K, Carling D, Huang L, Gonzales C, Cooper E, Smart TG, Pangalos MN, Moss SJ. Phospho-dependent functional modulation of GABA(B) receptors by the metabolic sensor AMP-dependent protein kinase. *Neuron*. 2007; 53:233–247. [PubMed: 17224405]
- Laffray S, Tan K, Dulluc J, Bouali-Benazzouz R, Calver AR, Nagy F, Landry M. Dissociation and trafficking of rat GABAB receptor heterodimer upon chronic capsaicin stimulation. *Eur. J. Neurosci*. 2007; 25:1402–1416. [PubMed: 17425567]
- Lal S, Shuaib A, Ijaz S. Baclofen is cytoprotective to cerebral ischemia in gerbils. *Neurochem. Res*. 1995; 20:115–119. [PubMed: 7783835]
- Liu SJ, Zukin RS. Ca²⁺-permeable AMPA receptors in synaptic plasticity and neuronal death. *Trends Neurosci*. 2007; 30:126–134. [PubMed: 17275103]

- López-Bendito G, Shigemoto R, Kulik A, Vida I, Fairén A, Luján R. Distribution of metabotropic GABA receptor subunits GABAB1a/b and GABAB2 in the rat hippocampus during prenatal and postnatal development. *Hippocampus*. 2004; 14:836–848. [PubMed: 15382254]
- Lu Q, Hope LW, Brasch M, Reinhard C, Cohen SN. TSG101 interaction with HRS mediates endosomal trafficking and receptor down-regulation. *Proc. Natl. Acad. Sci. U.S.A.* 2003; 100:7626–7631. [PubMed: 12802020]
- Maccaferri G, Roberts JD, Szucs P, Cottingham CA, Somogyi P. Cell surface domain specific postsynaptic currents evoked by identified GABAergic neurones in rat hippocampus in vitro. *J. Physiol.* 2000; 524(Pt 1):91–116. [PubMed: 10747186]
- Muir JK, Lobner D, Monyer H, Choi DW. GABAA receptor activation attenuates excitotoxicity but exacerbates oxygen–glucose deprivation-induced neuronal injury in vitro. *J. Cereb. Blood Flow Metab.* 1996; 16:1211–1218. [PubMed: 8898693]
- Ouyang C, Guo L, Lu Q, Xu X, Wang H. Enhanced activity of GABA receptors inhibits glutamate release induced by focal cerebral ischemia in rat striatum. *Neurosci. Lett.* 2007; 420:174–178. [PubMed: 17531382]
- Papadia S, Hardingham GE. The dichotomy of NMDA receptor signaling. *Neuroscientist*. 2007; 13:572–579. [PubMed: 18000068]
- Ramamurthy S, Ronnett GV. Developing a head for energy sensing: AMP-activated protein kinase as a multifunctional metabolic sensor in the brain. *J. Physiol.* 2006; 574:85–93. [PubMed: 16690704]
- Schuler V, Luscher C, Blanchet C, Klix N, Sansig G, Klebs K, Schmutz M, Heid J, Gentry C, Urban L, Fox A, Spooren W, Jatón AL, Vigouret J, Pozza M, Kelly PH, Mosbacher J, Froestl W, Kaslin E, Korn R, Bischoff S, Kaupmann K, van der Putten H, Bettler B. Epilepsy, hyperalgesia, impaired memory, and loss of pre- and postsynaptic GABA(B) responses in mice lacking GABA(B1). *Neuron*. 2001; 31:47–58. [PubMed: 11498050]
- Schwartz-Bloom RD, Sah R. Gamma-aminobutyric acid(A) neurotransmission and cerebral ischemia. *J. Neurochem.* 2001; 77:353–371. [PubMed: 11299298]
- Schwartz-Bloom RD, Miller KA, Evenson DA, Crain BJ, Nadler JV. Benzodiazepines protect hippocampal neurons from degeneration after transient cerebral ischemia: an ultrastructural study. *Neuroscience*. 2000; 98:471–484. [PubMed: 10869841]
- Soundarapandian MM, Tu WH, Peng PL, Zervos AS, Lu Y. AMPA receptor subunit GluR2 gates injurious signals in ischemic stroke. *Mol. Neurobiol.* 2005; 32:145–155. [PubMed: 16215279]
- Stokes AH, Bernard LP, Nicklas WJ, Zeevalk GD. Attenuation of malonate toxicity in primary mesencephalic cultures using the GABA transport blocker, NO-711. *J. Neurosci. Res.* 2001; 64:43–52. [PubMed: 11276050]
- Stoppini L, Buchs PA, Muller D. A simple method for organotypic cultures of nervous tissue. *J. Neurosci. Methods*. 1991; 37:173–182. [PubMed: 1715499]
- Vollenweider F, Bendfeldt K, Maetzler W, Otten U, Nitsch C. GABA(B) receptor expression and cellular localization in gerbil hippocampus after transient global ischemia. *Neurosci. Lett.* 2006; 395:118–123. [PubMed: 16298486]
- Wetherington JP, Lambert NA. GABA(B) receptor activation desensitizes postsynaptic GABA(B) and A(1) adenosine responses in rat hippocampal neurones. *J. Physiol.* 2002; 544:459–467. [PubMed: 12381818]
- Wilkins ME, Li X, Smart TG. Tracking cell surface GABAB receptors using an alpha-bungarotoxin tag. *J. Biol. Chem.* 2008; 283:34745–34752. [PubMed: 18812318]
- Zhou C, Li C, Yu HM, Zhang F, Han D, Zhang GY. Neuroprotection of gamma-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.* 2008; 86:2973–2983. [PubMed: 18512761]

Fig. 1.

Effects of different doses of baclofen on cellular damage induced by oxygen–glucose deprivation (OGD) for 45 min in organotypic slice cultures of rat hippocampus. (A) Representative images of cultures showing propidium iodide (PI) uptake 24 h after OGD. (B)–(D) Quantitative analysis of incorporation of PI in CA1, CA2 + CA3 and DG of control and baclofen-treated OGD slices compared with the corresponding region of untreated OGD slices (standard damage). The results are presented as percentage of standard damage $\pm$ SEM ($n = 9$). (*) indicates significant difference from standard damage and (#) indicates significant difference from all groups, $p < 0.05$ (one-way ANOVA and Duncan's test).

Fig. 2.

Effects of oxygen–glucose deprivation (OGD) for 45 min on the protein levels of GABA_B receptors in organotypic slice cultures of rat hippocampus. (A) and (B) Untreated and baclofen-treated (100 μ M) organotypic slice cultures of rat hippocampus were subjected to 45 min OGD and analysed 24 h after the lesion induction. Baclofen was present during the 45 min of OGD. (A) Representative pattern of GABA_{B1} and GABA_{B2} immunoreactivity detected using Santa Cruz anti-GABA_{B1} antibody and Chemicon anti-GABA_{B2} antibody. (B) Cumulative GABA_{B1} and GABA_{B2} results showing quantified data from separate immunoblots using slices from three different experiments. (C)–(E) Protein levels of GABA_B receptors in organotypic slice cultures of rat hippocampus exposed to 45 min OGD and analysed immediately (0 min), 60 and 120 min after OGD. (C) Representative blots showing the pattern of GABA_{B1} and GABA_{B2} immunoreactivity detected using Santa Cruz anti-GABA_{B1} antibody and Chemicon anti-GABA_{B2} antibody. (D) and (E) Cumulative GABA_{B1} and GABA_{B2} results showing quantified data from separate immunoblots using slices from five different experiments. The results are presented as percentage of control $\pm$ SEM. (*) indicates significant difference $p < 0.05$ (one-way ANOVA and Duncan's test).

Fig. 3.

Effects of baclofen (100 μ M) added for 45 min before, during or after oxygen–glucose deprivation (OGD) on cellular damage induced by OGD for 45 min in organotypic slice cultures of rat hippocampus. (A) Representative images of cultures showing propidium iodide (PI) uptake 24 h after OGD. (B)–(D) Quantitative analysis of incorporation of PI in CA1, CA2 + CA3 and DG of baclofen-treated OGD slices compared with the corresponding region of untreated OGD slices (standard damage). The results are presented as percentage of standard damage $\pm$ SEM ($n = 9$). (*) indicates significant difference from standard damage and (#) indicates significant difference from all groups, $p < 0.05$ (one-way ANOVA and Duncan's test).

Fig. 4.

Effects of oxygen–glucose deprivation (OGD) for 45 min on the protein levels of GABA_B receptors in untreated and baclofen-treated (100 μM) organotypic slice cultures of rat hippocampus. Baclofen was added for 45 min before, during or after OGD. (A) Representative pattern of GABA_{B1} and GABA_{B2} immunoreactivity detected using Santa Cruz anti-GABA_{B1} antibody and Chemicon anti-GABA_{B2} antibody. (B) and (C) Cumulative GABA_{B1} and GABA_{B2} results showing quantified data from separate immunoblots using slices from three different experiments. The results are presented as percentage of control ± SEM. (*) indicates significant difference $p < 0.05$ (one-way ANOVA and Duncan's test).

Fig. 5.

Effects of baclofen (100 μ M) added for 45 min before, during or after NMDA exposure on cellular damage induced by NMDA (50 μ M) added for 45 min in organotypic slice cultures of rat hippocampus. (A) Representative images of cultures showing propidium iodide (PI) uptake 24 h after OGD. (B)–(D) Quantitative analysis of incorporation of PI in CA1, CA2 + CA3 and DG of baclofen-treated NMDA-exposed slices compared with the corresponding region of untreated NMDA-exposed slices (standard damage). The results are presented as percentage of standard damage $\pm$ SEM ($n = 6$). (*) indicates significant difference from standard damage and (#) indicates significant difference from all groups, $p < 0.05$ (one-way ANOVA and Duncan's test).

Fig. 6.

Effects of NMDA (50 μ M) exposure for 45 min on the protein levels of GABA_B receptors in untreated and baclofen-treated (100 μ M) organotypic slice cultures of rat hippocampus. Baclofen was added for 45 min before, during or after NMDA exposure. (A) Representative pattern of GABA_{B1} and GABA_{B2} immunoreactivity detected using Santa Cruz anti-GABA_{B1} antibody and Chemicon anti-GABA_{B2} antibody. (B) and (C) Cumulative GABA_{B1} and GABA_{B2} results showing quantified data from separate immunoblots using slices from three different experiments. The results are presented as percentage of control $\pm$ SEM. (*) indicates significant difference $p < 0.05$ (one-way ANOVA and Duncan's test).