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Alterations in Purkinje cell GABA_A receptor pharmacology following oxygen and glucose deprivation and cerebral ischemia reveal novel contribution of β_1 -subunit-containing receptors

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Abstract

Cerebellar Purkinje cells (PCs) are particularly sensitive to cerebral ischemia, and decreased GABA_A receptor function following injury is thought to contribute to PC sensitivity to ischemia-induced excitotoxicity. Here we examined the functional properties of the GABA_A receptors that are spared following ischemia in cultured Purkinje cells from rat and *in vivo* ischemia in mouse. Using subunit-specific positive modulators of GABA_A receptors, we observed that oxygen and glucose deprivation (OGD) and cardiac arrest-induced cerebral ischemia cause a decrease in sensitivity to the α_2/β_3 -subunit-preferring compound, etomidate. However, sensitivity to propofol, a β_1 -subunit-acting compound that modulates α_1/β_3 -subunits, was not affected by OGD. The α_1/β_1 -subunit-acting compounds, diazepam and zolpidem, were also unaffected by OGD. We performed single-cell reverse transcription–polymerase chain reaction on isolated PCs from acutely dissociated cerebellar tissue and observed that PCs expressed the β_1 -subunit, contrary to previous reports examining GABA_A receptor subunit expression in PCs. GABA_A receptor β_1 -subunit protein was also detected in cultured PCs by western blot and by immunohistochemistry in the adult mouse cerebellum and levels remained unaffected by ischemia. High concentrations of loreclezole (30 μ M) inhibited PC GABA-mediated currents, as previously demonstrated with β_1 -subunit-containing GABA_A receptors expressed in heterologous systems. From our data we conclude that PCs express the β_1 -subunit and that there is a greater contribution of β_1 -subunit-containing GABA_A receptors following OGD.

Keywords

electrophysiology; GABA_A receptor; mouse; oxygen–glucose deprivation; Purkinje cells; rat

Introduction

Cerebellar Purkinje cells (PCs) are the output neurons of the cerebellar cortex and are particularly susceptible to ischemic injury. The large number of excitatory glutamatergic

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synapses onto PCs contributes to their vulnerability to excitotoxic damage after ischemia (Horn & Schlote, 1992; Brasko *et al.*, 1995; Fonnum & Lock, 2000; Ardeshiri *et al.*, 2006; Kelley *et al.*, 2008). Many neuroprotective strategies have been explored using PCs as a model system, with variable success. One approach is to increase the inhibitory tone by enhancing GABA_A receptor activity, thus hyperpolarising cells and leaving them less vulnerable to excitotoxic damage (Schwartz-Bloom & Sah, 2001). One consideration in targeting GABA receptors is that, following cerebral ischemia, as experienced during a stroke or cardiac arrest (CA), GABA_A receptors are down-regulated in various brain regions (Li *et al.*, 1993; Schiene *et al.*, 1996; Qu *et al.*, 1998; Kelley *et al.*, 2008; Arancibia-Carcamo *et al.*, 2009), resulting in decreased inhibitory potential and increased susceptibility to excitotoxic damage. We also observed that oxygen and glucose deprivation (OGD) causes a rapid and sustained reduction in GABA-mediated current and GABA_A receptor $\alpha 1$ -subunit protein (Kelley *et al.*, 2008). Therefore, preserving GABA_A receptor function may play an important role in neuroprotection from ischemia. We have recently demonstrated that the neurosteroid allopregnanolone protects PCs from ischemic damage both *in vitro* and *in vivo* via a GABA_A receptor-dependent mechanism (Ardeshiri *et al.*, 2006; Kelley *et al.*, 2008, 2011). Although it is clear that GABA receptor function is altered by ischemia, there is currently limited information regarding GABA_A receptor structural changes and trafficking in pathophysiological states, making the full nature and consequences of these changes to GABA_A receptors unknown. This apparent disease-induced plasticity could contribute to the difficulty in finding GABA_A receptor-modulating agents that are effective neuroprotectants. Pharmacotherapies targeting GABA_A receptors may be effective if we improve our understanding of receptor subunit composition in neurons that have undergone ischemia.

The GABA_A receptors are pentameric proteins thought to be composed of two α -subunits, two β -subunits, and one γ - or δ -subunit (or less commonly an ϵ , θ , or ζ -subunit). Sixteen subtypes of GABA_A receptor subunits have been cloned (Olsen & Sieghart, 2009), allowing for heterogeneous expression profiles across brain regions with distinct channel properties. The subunit composition dictates the specific properties of the receptor, including ligand binding, channel kinetics, and response to allosteric modulators. Additionally, GABA_A receptor phosphorylation and association with trafficking proteins are subunit dependent. Cerebellar PCs are thought to express a homogeneous population of GABA_A receptors, consisting of $\alpha 1 \beta 2 \gamma 2$ (Wisden *et al.*, 1992). Following ischemia, PC GABA_A receptor function and protein levels are significantly reduced (Kelley *et al.*, 2008). In the current set of experiments, we examined the function of PC GABA_A receptors that remain following ischemia using subunit-specific allosteric modulators. Single-cell polymerase chain reaction (PCR) from isolated PCs and western blot were also used to study GABA_A receptor subunit expression. Our findings suggest that PCs express a more heterogeneous population of GABA_A receptors than previously believed and that the relative subunit contribution is altered by ischemia.

Materials and methods

Primary cerebellar culture

All experiments were performed in accordance with National Institute of Health guidelines and experimental protocols approved by the institutional animal care and use committee (IACUC). Cerebellar neurons were cultured from embryonic day 18 Sprague-Dawley rats as previously described (Kelley *et al.*, 2008). In brief, time-pregnant Sprague-Dawley rats (Charles River, Wilmington, MA, USA) were killed with CO₂ and embryos removed by caesarean section. Embryonic brains were isolated and placed in ice-cold dissection solution composed of Hank's balanced salt solution (Gibco/Invitrogen, Carlsbad, CA, USA) supplemented with 0.03% bovine serum albumin and 10 mM MgSO₄ (Sigma-Aldrich, St Louis, MO, USA). Cerebella were dissected, meninges removed, and tissue digested with

0.2% trypsin in dissection solution at 37 °C. Digestion was halted by washing with cerebellar culture medium composed of Dulbecco's modified Eagle's medium/F-12 (1 : 1) mix supplemented with 1.4 mM L-glutamine, 50 units*µg/mL penicillin–streptomycin, b-27 supplement (1 ×), and 10% fetal bovine serum (Hyclone, Logan, UT, USA). Tissue was triturated, filtered through a 70 µm cell sorting nylon mesh, and the suspension centrifuged at 1000 g for 10 min to isolate cells. The cell pellet was resuspended in cerebellar culture medium without fetal bovine serum. Cells were plated at a density of 2.8×10^5 cells per round 12 mm glass coverslip and grown at 37 °C in a humidified incubator containing 5% CO₂. Cultured neurons were grown for 10–14 days *in vitro* to allow maturation and formation of synapses.

Oxygen and glucose deprivation

Cells were transferred to a glucose-free saline solution composed of (in mM): 140 NaCl, 5 KCl, 0.8 MgCl₂, 1 CaCl₂, 10 HEPES, pH 7.35, with NaOH, and placed in an anaerobic incubator (at 37 °C) containing an oxygen-reacting catalyst and 5% CO₂, 95% N₂ (Coy Laboratory Products, Grass Lake, MI, USA) for 2 h. This duration of OGD was previously determined to cause significant levels of cell death in our cultures following 24 h of reperfusion (Ardeshiri *et al.*, 2006). Reoxygenation was initiated by transferring cells to glucose-containing saline for electrophysiological recordings (see below).

Electrophysiology

Cells were transferred to a recording chamber mounted on an inverted microscope (DM IRB; Leica, Houston, TX, USA) containing normal saline composed of (in mM): 140 NaCl, 5 KCl, 0.8 MgCl₂, 1 CaCl₂, 10 HEPES, 10 glucose, pH 7.35, with NaOH, and a gravity-fed bath flow rate of 2–5 mL/min. Whole-cell voltage-clamp recordings were made from the somas of PCs using an Axopatch 200B amplifier (Axon Instruments, Union City, CA, USA) interfaced to a computer (Dell, Round Rock, TX, USA). PCs were selected by their large soma, low input resistance, and extensive dendritic arbor. Data were excluded from cells in which the series resistance changed more than 20% throughout the course of the experiment. Electrodes were pulled from borosilicate glass capillaries with inner filaments using a Flaming Brown electrode puller (Sutter Instrument Co., Novato, CA, USA) and had resistances of 2–3 MΩ when filled with internal pipette solution composed of (in mM): 140 CsCl, 1 EGTA, 10 HEPES, 1 MgCl₂, 5 MgATP, pH 7.3 with CsOH. Data were collected at a sample frequency of 20 kHz and using pCLAMP9 (Molecular Devices, Sunnyvale, CA, USA). Whole-cell capacitance and series resistance were electronically compensated to 60–70%. Adequate whole cell access ($R_a < 20 \text{ M}\Omega$) was achieved prior to measuring responses to compounds and verified at the end of recording. A pressurised microperfusion system with a wide barrel manifold (250 µm) was used for rapid solution exchange around the entire cell body and dendritic processes (ALA Scientific Instruments, Westbury, NY, USA) to measure PC response to GABA and subunit-specific modulators. The amplitude of currents, in response to GABA and test compounds, was measured using Clampfit analysis software (Axon Instruments). For concentration–response analysis, only cells where a complete concentration–response was obtained were included. Data from each cell were plotted and fit with the following equation to determine EC₅₀ using Igor Pro software (WaveMetrics, Lake Oswego, OR, USA): $f(x) = (I_{\text{max}} - I_0) / (1 + (EC_{50}/x)^n) + I_0$, where x equals the concentration of drug and n equals the Hill coefficient. The EC₅₀ is presented as the mean ± SEM for individual cells. Data were expressed as the percent potentiation of 2 or 5 µM GABA [the concentration of GABA required to activate 20% of total GABA_A receptors (GABA₂₀)]. The maximum GABA-mediated current, achieved by applying a saturating concentration of GABA (1 mM, 1 s), was measured at the end of each recording to ensure that the effect of drugs was submaximal and to verify that the concentration of GABA being used would activate 20% of the total GABA_A receptors. The concentrations of drugs used

for dose–response analysis were based on previous pharmacological studies using heterologous expression systems (Mihic *et al.*, 1994; Sanna *et al.*, 1995; Hill-Venning *et al.*, 1997; Neelands *et al.*, 1998). For loreclezole inhibition experiments, 1 s pulses of 20 μM GABA were applied every 30 s to establish a stable baseline response, 30 μM loreclezole was then preapplied by local perfusion for 30 s and remained present through three subsequent pulses of 20 μM GABA every 30 s. Washout of drug was achieved by local perfusion of saline in addition to bath perfusion.

Cardiac arrest and slice electrophysiology

Briefly, adult male mice (7–9 weeks) were anesthetised with isoflurane (1–3%) via face mask. Temperature probes were placed into the left temporalis muscle and rectum. The rectal temperature was controlled at 37 °C during surgery. For drug administration, the right jugular vein was cannulated with a PE-10 catheter. The electrocardiogram was monitored throughout the experimental procedures. Mice were endotracheally intubated with a 22G intravenous catheter and mechanically ventilated (Minivent; Hugo Sachs Elektronik, March-Hugstetten, Germany). CA was induced by intravenous injection of 50 μL of 0.5 M KCl and was confirmed by the appearance of asystole on the electrocardiography monitor and no spontaneous breathing. Anesthesia was then stopped and the endotracheal tube was disconnected from the ventilator. During CA, the pericranial temperature was maintained at 37.5 ± 0.2 °C and the body temperature at 36 °C. Cardiopulmonary resuscitation (CPR) was initiated at 8 min after the induction of CA by slow injection of 0.5 mL of epinephrine (8 μg), chest compressions (approximately 300/min), and ventilation with 100% oxygen. As soon as return of spontaneous circulation was achieved, defined as electrocardiographic activity with visible cardiac contractions, chest compression was stopped. If return of spontaneous circulation could not be achieved within 2.5 min of CPR, resuscitation was stopped and the animal was excluded from the study. At 25 min after return of spontaneous circulation, temperature probes were removed, skin incisions were closed, and animals were weaned from the ventilator. Animals were extubated after confirmation of spontaneous ventilation/breathing and mice were returned to their housing cage.

At 3 h after CA/CPR, mice were anesthetised and decapitated, and brains were removed. Parasagittal cerebellar sections (300 μm) were prepared using a vibratome. Slices were prepared and maintained in artificial cerebrospinal fluid containing (in mM): 126 NaCl, 2.5 KCl, 2.5 CaCl_2 , 1.2 MgCl_2 , 1.2 NaH_2PO_4 , 26 NaHCO_3 , and 10 D-glucose. Slices were transferred to a recording chamber with perfused artificial cerebrospinal fluid (1.5 mL/min) and Purkinje neurons were identified by their large soma and location in the PC layer. Whole-cell recordings were obtained using a 2–3 M pipette containing (in mM): 135 K-gluconate, 8 NaCl, 10 HEPES, 0.05 EGTA, 2 MgCl_2 , 4 MgATP, and 0.3 NaGTP.

The series resistance was < 20 M Ω and did not change more than 20% during the experiment. Cells were voltage clamped at –60 mV and a stimulating electrode placed in the inner molecular layer was used to stimulate GABA release. Stimulation resulted in an outward inhibitory postsynaptic current (IPSC). GABA IPSCs were recorded in the presence of 10 μM NBQX (2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide) to block glutamatergic transmission.

Solutions and drugs

Etomidate and zolpidem were dissolved in EtOH at a stock concentration of 10 mM. Loreclezole was dissolved in dimethylsulfoxide at a stock concentration of 5 mM. Diazepam was dissolved in dimethylsulfoxide at a stock concentration of 10 mM. Propofol was dissolved in EtOH at a stock concentration of 20 mM. GABA was dissolved in water at a stock concentration of 500 mM. Working solutions were prepared by careful serial dilution of

stock compounds in saline. GABA currents were unaffected by vehicle controls containing EtOH or dimethylsulfoxide (data not shown). Components of the internal and external solutions, propofol and diazepam were obtained from Sigma-Aldrich. Etomidate, loreclezole, GABA and zolpidem were obtained from Tocris (Ellisville, MO, USA).

Immunohistochemistry

Following CA/CPR (or sham control) and 24 h recovery, mice were perfused through the left ventricle with saline followed by 4% paraformaldehyde, then removed and postfixed for an additional 8–12 h. Cerebelli were dehydrated and embedded in paraffin. Coronal sections (6 μ m) were cut and three sections, 100 μ m apart (beginning at approximately –5.6 from Bregma), were mounted for immunohistochemistry. Briefly, sections underwent deparaffinisation and antigen retrieval using citrate buffer (10 mM, pH 6.0) at 95 °C for 30 min. Sections were incubated in blocking solution (10% normal goat serum and 0.5% triton x-100) for 2 h at room temperature (22 °C). GABA_A beta 1 antibody and beta 2 antibody (Novus Biologicals, Littleton, CO, USA) were diluted 1 : 100 in blocking solution and incubated for 8–12 h at 4 °C. The secondary antibody goat anti-rabbit Alexa-488 (Molecular Probes, Eugene, OR, USA) was diluted 1 : 500 in blocking solution to visualise GABA_A receptors and incubated for 2 h at room temperature. A total of nine images were captured per animal (three per level) and analysed by a separate investigator. For analysis, the mean intensity of the soma was measured and background was subtracted. Soma intensities from all nine images were averaged to generate a mean intensity (arbitrary units) per animal ($n = 4$ in control and CA/CPR).

Acute cerebellar dissociation and Purkinje cell collection

Cerebellar dissociation for the isolation of PCs was performed according to Raman and Bean (Raman & Bean, 1997; Benton & Raman, 2009) with the modification of the use of RNase-free H₂O for all solutions. Postnatal day 14–20 C57BL/6 mice (Charles River) were anaesthetised with isoflurane, decapitated, and superficial layers of the vermis of the cerebellum were dissected for dissociation. Tissue was minced in ice-cold, oxygenated dissociation solution composed of (in mM): 82 Na₂SO₄, 30 K₂SO₄, 5 MgCl₂, 10 HEPES, 10 glucose, and 0.001% phenol red (buffered to pH 7.4 with NaOH). Tissue was then incubated in 10 mL oxygenated dissociation solution containing 3 mg/mL protease XXIII (Sigma-Aldrich), pH 7.4 with NaOH, at 37 °C for 7 min. Enzymatic digestion was halted by washing tissue in warmed, oxygenated dissociation solution containing 1 mg/mL bovine serum albumin and 1 mg/mL trypsin inhibitor, tissue was microdissected to roughly 1 mm pieces, and transferred to oxygenated Tyrode's solution composed of (in mM): 150 NaCl, 4 KCl, 2 CaCl₂, 2 MgCl₂, 10 HEPES, and 10 glucose, pH 7.4 with NaOH, at room temperature. To disperse cells, tissue was triturated with fire-polished RNase-free Pasteur pipettes. Dissociated cells were plated onto 35 mm Petri dishes and allowed to settle for 20–30 min. PCs were visualised using an inverted microscope (DM IRB; Leica) and identified by their large size, teardrop shape, and proximal dendritic stump. Pipettes (2–3 M Ω) were pulled from RNase-free borosilicate glass, filled with RNase-free Tyrode's solution and manipulated with an oocyte injector. Pipettes were positioned near the cell and the tip was broken, by lowering the pipette into the Petri dish, to create a large enough opening to capture PCs with applied negative pressure. Immediately following isolation, four to six cells were expelled into each siliconised microcentrifuge tube containing 1.6 μ L of 5 \times Superscript buffer (Invitrogen, Carlsbad, CA, USA), 18 U of RNAsin, 0.8 μ L of 100 mM dithiothreitol, and diethylpyrocarbonate-treated water to 8 μ L. The negative control consisted of Tyrode's solution collected from the vicinity of PCs. Samples were frozen at –70 °C until used for reverse transcription–PCR.

Single-cell polymerase chain reaction

Each sample, containing four to six cells, was thawed on ice and 0.2 μM dNTPs, 100 ng of random hexamers, and 400 ng OligodT were added. Samples were heat denatured for 5 min at 65 °C and then cooled on ice for 5 min. Cellular RNA was reverse transcribed by adding 100 U of Superscript III reverse transcriptase, 3.4 μL of 5 \times Superscript buffer, 15.2 U of RNAsin, 1.2 μL of 100 mM dithiothreitol, and diethylpyrocarbonate-treated water to 14 μL , together with cells for a final volume of 25 μL . Reverse transcription reactions were incubated at 25 °C for 5 min, transcribed at 50 °C for 60 min, denatured at 70 °C for 15 min, and cooled on ice.

Nested PCR was used for all samples, using degenerate outer and inner primers for α - and β -subunits, with the exception of the reaction for the β_{1b} (from the Allen Mouse Brain Atlas; www.brain-map.org) primer set, for which conventional PCR was used. PCR primers were designed using DNA Star software (Madison, WI, USA). For α degenerate primers, sequences corresponded to the α_1 -subunit [accession number NM_010250, forward primer 347–365 nucleotides (nts), reverse primer 1000–1020 nt] and α_6 -subunit (accession number NM_008068, forward primer 290–308 nt, reverse primer 853–872 nt). For β degenerate primers, sequences corresponded to the β_1 -subunit (accession number NM_008069, forward primer 201–223 nt, reverse primer 907–929 nt), β_2 -subunit (accession number NM_008070, forward primer 206–227 nt, reverse primer 911–933 nt), and β_3 -subunit (accession number NM_008071, forward primer 305–326 nt, reverse primer 1010–1032 nt). All other primers were as follows: α_1 (273 nt product, accession number NM_010250, forward primer 413–434 nt, reverse primer 674–686 nt), α_6 (287 nt product, accession number NM_008068, forward primer 395–417 nt, reverse primer 661–682 nt), β_1 (206 nt product, accession number NM_008069, forward primer 464–483 nt, reverse primer 651–670 nt), β_{1b} (669 nt product, accession number NM_008069, forward primer 1145–1166 nt, reverse primer 1792–1814 nt), β_2 (271 nt product, accession number NM_008070, forward primer 428–445 nt, reverse primer 679–699 nt), and β_3 (260 nt product, accession number NM_008071, forward primer 519–535 nt, reverse primer 760–779 nt). PCR was performed using 2 μL of cDNA template from each reverse transcription reaction in a 25 μL PCR volume containing 5 μL 5 \times Mg-free buffer, 2 μL 25 mM MgCl_2 , 0.5 μL 10 mM dNTPs, 0.4 μL GoTaq Flexi DNA polymerase (Promega), and 0.2 μM each of the forward and reverse primers. Thirty-five cycles of amplification were performed using a Gene Amp PCR system 9700 thermal cycler (Applied Biosystems) in 0.2 mL thin-walled PCR tubes with the following protocol: 94 °C for 2 min; 35 cycles of 96 °C for 30 s, 54–57 °C for 0.5–1 min, and 72 °C for 1 min; with a final 72 °C extension for 5 min. PCR products were visualised with ethidium bromide on a 2% agarose gel. Reverse transcription–PCR experiments revealed the presence of all α -subunits; however, not every reaction on PCs yielded a signal, with four out of six PC reactions exhibiting α_2 , three out of six showing α_3 and two out of six showing α_1 .

Western blot

Total protein was collected from cultures by lysing cells with Cel-Lytic-M lysis buffer (Sigma-Aldrich), centrifuging at 10 000 g for 5 min at 4 °C and discarding the pellet to remove nuclei and debris. The total protein content of the supernatant was measured using a BCA (bicinchoninic acid) protein assay (Pierce/Thermo Scientific, Rockford, IL, USA) and 30 μg of total protein from each sample was run on a precast 4–12% stacking polyacrylamide gel (Invitrogen) for 1 h at 200 V. Protein was transferred to a polyvinylidene difluoride membrane (Invitrogen) for 1 h at 30 V. Blots were incubated in primary antibody (1 : 1000 dilution of rabbit anti-GABA_A receptor α_1 -subunit) (Novus Biologicals) overnight at 4 °C, followed by secondary antibody (1 : 1000 donkey anti-rabbit IgG ECL-HRP; enhanced chemiluminescence-horseradish peroxidase) (GE Healthcare, Piscataway, NJ, USA) for 1 h at room temperature. Blots were then stripped and reprobed with primary

antibody (1 : 7000 mouse anti- β -actin) (Sigma-Aldrich) for 1 h at room temperature, followed by secondary antibody (1 : 1000 goat anti-mouse IgG ECL HRP-linked) (GE Healthcare) for 1 h at room temperature. The SuperSignal West Dura chemiluminescent detection system (Thermo Scientific) was used to visualise protein and blots were imaged and analysed using a FluorChem FC2 gel imager (Alpha Innotech, San Leandro, CA, USA). GABA_A receptor α 1-subunit protein levels were normalised to β -actin and expressed as arbitrary units.

Statistical analysis

All data are presented as mean \pm SEM. Each n represents an individual cell for electrophysiology experiments and an individual culture for western blot experiments. For pharmacological studies, EC₅₀ was calculated for each individual cell and mean EC₅₀s were compared. Statistical significance was determined using Student's t -test (unpaired, two-tailed, if $P < 0.05$).

Results

Purkinje cells have decreased sensitivity to etomidate following oxygen and glucose deprivation

Whole-cell voltage-clamp recordings were made from cultured cerebellar PCs under control conditions or within 1 h of reoxygenation following 2 h OGD, a time point following ischemic insult that reflects data from neurons that are undergoing excitotoxicity and a duration of OGD that produces PC injury with a high probability of cell death (Ardeshiri *et al.*, 2006). We tested the effect of ischemia on GABA_A receptor sensitivity to etomidate by measuring peak current amplitudes in response to 1 s pulses of GABA₂₀ (2–5 μ M) plus increasing concentrations of etomidate (0.3–10 μ M) before and after OGD (Fig. 1A; (Table 1)). Etomidate is a positive modulator of GABA_A receptor activity that preferentially binds to α 2/3-containing receptors. Concentration–response relationships were plotted and the EC₅₀ for each individual cell was determined. For control cells, the average EC₅₀ was $2.9 \pm 0.3 \mu$ M ($n = 8$). Two hours of OGD caused a significant increase in the average EC₅₀ to $3.8 \pm 0.2 \mu$ M ($P < 0.05$; $n = 6$; Fig. 1B).

Oxygen and glucose deprivation does not alter Purkinje cell GABA_A receptor sensitivity to propofol

The shift in etomidate sensitivity following ischemia led us to hypothesise that the α 1-subunit of GABA_A receptors is altered following ischemia. We tested propofol, another GABA_A receptor modulator that binds to the α 1-subunit, enhancing the activity of all α 1-subunit-containing receptors (α 1– α 3) equally. Peak current amplitudes in response to 1 s pulses of GABA₂₀ plus increasing concentrations of propofol (1–15 μ M) were measured before and after OGD (Fig. 2A). The sensitivity and efficacy of propofol potentiation of GABA_A receptors remained unchanged by ischemia (control EC₅₀, $5.6 \pm 0.5 \mu$ M, $n = 9$; OGD EC₅₀, $5.8 \pm 0.5 \mu$ M, $n = 12$; Fig. 2B).

Oxygen and glucose deprivation does not alter GABA_A receptor sensitivity to zolpidem and diazepam

We tested the effect of ischemia on the α 1- and α 2-subunits by measuring responses to diazepam and zolpidem, respectively. These compounds bind the α 1-subunit and potentiate GABA_A receptor response to GABA by increasing the current amplitude (Sigel & Buhr, 1997). Zolpidem is more selective for α 1-containing receptors and requires the α 2-subunit for potentiation (Buhr & Sigel, 1997). Therefore, we utilised diazepam as an indicator of α 1-subunit function and zolpidem to more specifically assess α 1 \times α 2-subunit function.

Responses to 1 s pulses of GABA₂₀ plus increasing concentrations of diazepam (0.1–10 μ M) or zolpidem (50–3000 nM) were measured before and after OGD. OGD did not significantly alter the efficacy or affinity for diazepam (control EC₅₀, 2.27 \pm 0.4 μ M, n = 7; OGD EC₅₀, 2.81 \pm 0.4 μ M, n = 6; Fig. 3A) or zolpidem (control EC₅₀, 465.7 \pm 75.9 nM, n = 9; OGD EC₅₀, 482.6 \pm 62.4 nM, n = 8; Fig. 3B).

Loss of $\beta_{2/3}$ -containing receptors following in vivo global ischemia

Global ischemia was induced with 8 min of CA followed by CPR. At 3 h after resuscitation, brains were removed and the cerebellum was sectioned for whole-cell electrophysiology experiments. Stimulation in the inner molecular layer resulted in an outward GABAergic IPSC in Purkinje neurons that was blocked by picrotoxin (data not shown). The decay of the IPSC was fit with a single exponential that was not different between sham and mice that had undergone CA/CPR (Fig. 4) (19.8 \pm 4.8 vs. 21.1 \pm 1.3 ms). To confirm the loss of $\beta_{2/3}$ -containing receptors after ischemia, etomidate was applied to slices (3 μ M, 10 min) and decay constants were measured. Etomidate prolonged the decay kinetics of the IPSC to 837 \pm 128 ms in slices from control sham-operated mice. In contrast, etomidate produced a significantly smaller prolongation after CA/CPR (534.5 \pm 42.3 ms) compared with sham (P < 0.05), suggesting a loss of $\beta_{2/3}$ -containing receptors after *in vivo* global ischemia.

To confirm the loss of $\beta_{2/3}$ protein, immunohistochemistry was performed in sections from control mice and after CA with an antibody recognising the β_2 -subunit. GABA_A β_2 -subunit was clearly detectable in PCs from control and there was a significant reduction after CA/CPR (Fig. 5A). In contrast, GABA_A β_1 -subunit was detected at similar levels in sections from control and CA/CPR (Fig. 5B).

GABA_A receptor β_1 -subunit is expressed in Purkinje cells

Purkinje cells are thought to express a homogeneous population of GABA_A receptors composed of β_1 -, $\beta_{2/3}$ -, and β_2 -subunits (Wisden *et al.*, 1992; McKernan & Whiting, 1996). However, the pharmacological shift in sensitivity that we observed with etomidate, but not propofol, is consistent with the expression of the β_1 -subunit. We used single-cell PCR (four to six pooled isolated PCs) to test for the presence of β_1 mRNA in PCs isolated from acutely dissociated mouse cerebellum (Mintz *et al.*, 1992; Raman & Bean, 1997). The presence of PCs and purity of samples were confirmed by examining β_6 -subunit mRNA expression. In order to verify that we did not contaminate our PC samples with granule cells, the most abundant cell type in the cerebellum, we tested for the presence of β_6 -subunits. The presence of β_1 and absence of β_6 are indicative of PCs, whereas the presence of β_6 indicates granule cells (Fig. 6A). PC pure samples (lacking β_6) were then tested for β_1 -subunit expression. Our PCR results indicate the presence of all three β -subunits (β_{1-3}) (Fig. 6A). Western blot analysis of protein collected from cultured cerebellar neurons confirmed the expression of β_1 -subunit protein (Fig. 6B). Unlike the β_1 -subunit, which is degraded following simulated ischemia (Kelley *et al.*, 2008), β_1 -subunit protein remained unaffected by OGD (Fig. 6C).

To confirm functional expression of the β_1 -subunit, we tested the effect of loreclezole, a GABA_A receptor modulator with both agonist and antagonistic properties. Loreclezole, at low doses, can act as a positive modulator of GABA_A receptors containing the β_{2-3} -subunits (Wafford *et al.*, 1994; Wingrove *et al.*, 1994). Conversely, at high concentrations (> 6 μ M), loreclezole inhibits GABA_A receptors containing the β_1 -subunit by decreasing channel open time and increasing the occurrence of a closed channel state (Fisher *et al.*, 2000). Consistent with PC expression of β_1 -subunits, we observed that pre-application of loreclezole (30 μ M, 30 s) caused an initial 30% reduction in GABA-mediated current that maximally decreased

with subsequent pulses of GABA by $65.5 \pm 6.8\%$ ($n = 4$), and almost completely reversed following 60–120 s washout ($92 \pm 5.2\%$ recovery) (Fig. 6D).

Discussion

Summary of findings

Ischemia significantly decreased PC GABA_A receptor sensitivity to etomidate with no change in propofol sensitivity, thus revealing the presence of α_1 -containing receptors. We verified α -subunit expression in PCs by single-cell PCR analysis and detected α_1 -subunit transcripts. Western blot analysis also confirmed that α_1 -subunit protein was expressed in cultured cerebellar neurons and we observed that loreclezole inhibited PC GABA-mediated current. This is further supported by our immunohistochemical data in adult mouse cerebellum. Taken together, our data indicate the presence of α_1 -containing GABA_A receptors in Purkinje neurons, which challenges current dogma that only α_2/α_3 -subunits are expressed in this cell type (Wisden *et al.*, 1992; McKernan & Whiting, 1996). The shift in etomidate sensitivity observed following OGD in PCs suggested a reduced contribution of α_2/α_3 -subunit-containing GABA_A receptors. The sensitivity to other GABA_A receptor modulators, propofol, diazepam, and zolpidem, remained unaffected by ischemia.

Etomidate sensitivity was altered by ischemia but propofol was not

Etomidate acts on the α -subunit of GABA_A receptors with a higher affinity for the α_2 - and α_3 -subunits compared with α_1 (Belelli *et al.*, 1997). Using heterologous expression systems, the EC₅₀ for etomidate has been reported to range between 0.6 and 1.2 μM for GABA_A receptors composed of $\alpha_1\alpha_2\alpha_2$ (Hill-Venning *et al.*, 1997), and 4 μM for receptors composed of $\alpha_1\alpha_3\alpha_2$ (Belelli *et al.*, 2003). The presence of α_1 increases the EC₅₀ to a range between 6 and 11 μM (Hill-Venning *et al.*, 1997). Our data are consistent with several previous reports indicating that PCs predominantly express α_2/α_3 -containing receptors, exhibiting an EC₅₀ for etomidate of 2.9 μM under control conditions. Following OGD, the EC₅₀ for etomidate significantly increased to 3.8 μM with no change in propofol sensitivity. Similarly, following CA/CPR, the efficacy of etomidate is reduced in synaptic recordings of PC GABAergic IPSCs. The simplest explanation is that ischemia preferentially decreases expression of α_2/α_3 -containing receptors, sparing α_1 -containing receptors. Indeed, OGD had no effect on α_1 -subunit protein levels, suggesting that this subunit is spared from ischemia-induced downregulation, in contrast to our previous observation of OGD-induced degradation of α_1 -subunits (Kelley *et al.*, 2008). Our immunohistochemistry data also support our finding that there is a preferential loss of α_2 -subunit with no detectable change in α_1 -subunit expression following *in vivo* ischemia. Interestingly, ischemia did not alter the sensitivity of GABA_A receptors to compounds known to interact with α - and β -subunits. In total, these data lead to the conclusion that ischemia causes decreased total GABA_A receptor levels (Kelley *et al.*, 2008) and that $\alpha_1\alpha_2\alpha_3$ are preferentially silenced/degraded, leaving a larger relative complement of $\alpha_1\alpha_1\alpha_2$ receptors.

Single-cell polymerase chain reaction demonstrated β_1 expression

Contrary to our findings, the literature indicates that cerebellar PCs do not express α_1 -subunits (Wisden *et al.*, 1992; Sergeeva *et al.*, 2010). The α_1 -subunit has not been detected in PCs by single-cell PCR (Ruano *et al.*, 1997; Sim *et al.*, 2000) or *in situ* hybridisation (Laurie *et al.*, 1992; Wisden *et al.*, 1992). However, there are reports describing α_1 mRNA labeling a distinct band at the interface of the granule cell layer and molecular layer, where PCs reside (Zhang *et al.*, 1991; Zdilar *et al.*, 1992; Lein *et al.*, 2007). One explanation for this discrepancy is that PCs express the α_1 -subunit transcript at low levels. Indeed, in our hands, PC α -subunit transcripts were more difficult to detect compared with α_1 . Additional experimental measures were employed to accurately detect low-copy genes by single-cell

PCR. First, we tested multiple sets of primers to find those optimised for low-copy transcripts, including utilising a primer sequence for the α_1 -subunit from the Allen Mouse Brain Atlas (www.brain-map.org) that positively labeled PCs by *in situ* hybridisation (Lein *et al.*, 2007). We utilised the nested PCR technique to enhance our amplicon levels from dilute cDNA and avoid false negatives from non-specific amplification. Consistent with our single-cell PCR data, the actions and immunohistochemistry data of lorecelezole suggest that cerebellar PCs express GABA_A receptors containing functional α_1 -subunits.

The molecular mechanism of the selective loss of α_2/α_3 observed after ischemia remains unclear. α -subunits are substrates for several protein kinases, including protein kinase A and C and Ca²⁺/calmodulin-dependent protein kinase II, and phosphorylation of the various α -subunits has been shown to modulate receptor function and trafficking. It was recently demonstrated that etomidate sensitivity is not altered by acute phosphorylation (Drexler *et al.*, 2009), indicating that altered trafficking is a more likely explanation for the ischemia-induced decrease in etomidate sensitivity. *In vitro* ischemia in hippocampal neurons results in a loss of surface expression of GABA_A receptors that is dependent on an interaction of the α -subunit with the clathrin adaptor protein 2 (AP2) (Smith *et al.*, 2012) and previous work has demonstrated that the interaction of AP2 with GABA_A receptors is modulated by phosphorylation (Kittler *et al.*, 2005). Thus, further work is needed to explore the possibility that ischemia simulates differential phosphorylation of α_1 - compared with α_2/α_3 -containing receptors, and consequent changes in trafficking and GABA_A receptor subunit composition.

Conclusion

From our data we conclude that PCs express the α_1 -subunit, contrary to previous reports. The relative contribution of α_1 -containing GABA_A receptors appears to increase following ischemia, thereby decreasing sensitivity to etomidate. Although α_1 transcript levels may be lower than α_2/α_3 levels, α_1 -subunit protein is easily detectable by western blot and protein levels remain unaffected by ischemia. α_1 -subunit expression in PCs is novel and controversial, but is important for understanding inhibition in the cerebellum and GABA_A receptor expression and trafficking in ischemia. We provide molecular and functional data indicating that PCs express α_1 -subunits and the re-evaluation of previous findings may be warranted. Recently, a new GABA_A receptor α_1 -subunit antagonist was found to reduce ataxia in mice (Gee *et al.*, 2010). Motor coordination deficits and ataxia are commonly experienced following cerebral ischemia (Lim *et al.*, 2004; Venkatesan & Frucht, 2006; Lu-Emerson & Khot, 2010), likely due to PC dysfunction and cell death. Therefore, the increased propensity for ataxia following ischemia may be due to the increased relative contribution of α_1 -containing GABA_A receptors in PCs. The physiological and pathophysiological significance of α_1 -subunit expression in PCs remains to be determined.

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Abbreviations

| | |
|-----|-------------------------------|
| CA | cardiac arrest |
| CPR | cardiopulmonary resuscitation |

| | |
|-------------|---------------------------------|
| IPSC | inhibitory postsynaptic current |
| nt | nucleotide |
| OGD | oxygen and glucose deprivation |
| PC | Purkinje cell |
| PCR | polymerase chain reaction |

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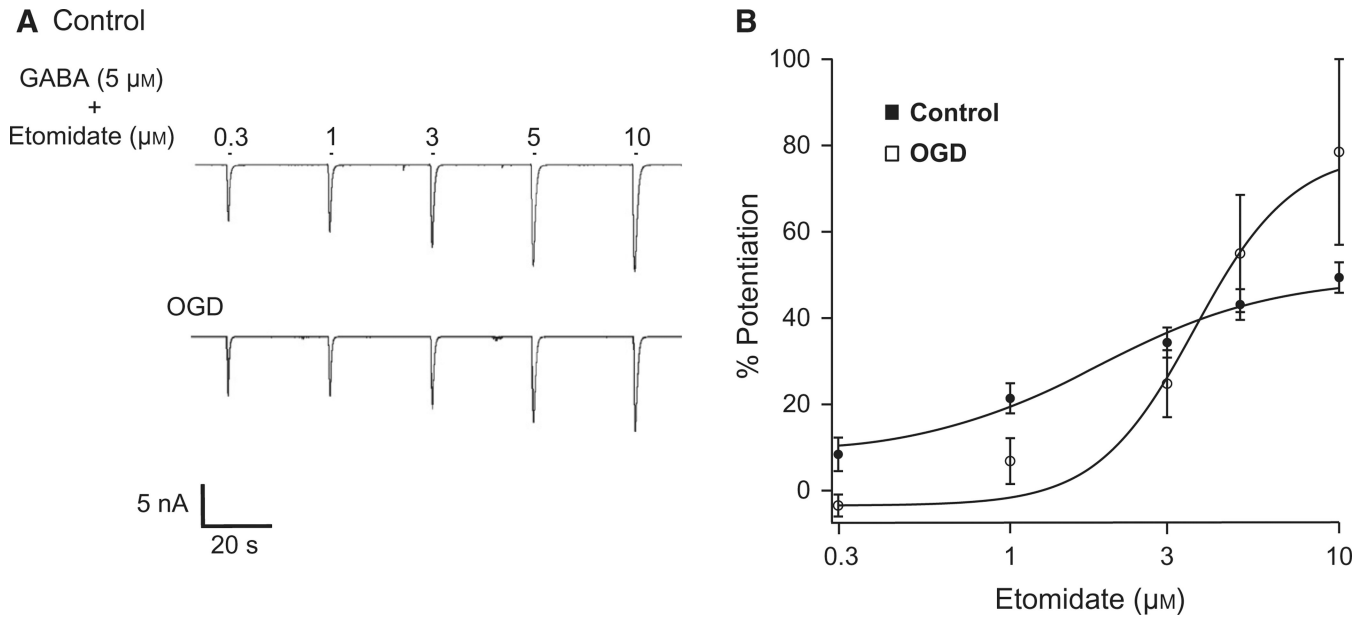


Fig. 1. OGD decreases PC GABA_A receptor sensitivity to etomidate. (A) Representative recordings from cultured PCs illustrating responses to 1 s applications of 5 μM GABA plus 0.3, 1, 3, 5, or 10 μM etomidate (bars above trace indicate application). (B) Average concentration-response relationships for etomidate from control and OGD PCs. The average EC₅₀ for control cells was $2.9 \pm 0.3 \mu\text{M}$. The OGD cell average EC₅₀ was $3.8 \pm 0.2 \mu\text{M}$ ($P < 0.05$). Data presented are the average of eight control cells and six OGD cells.

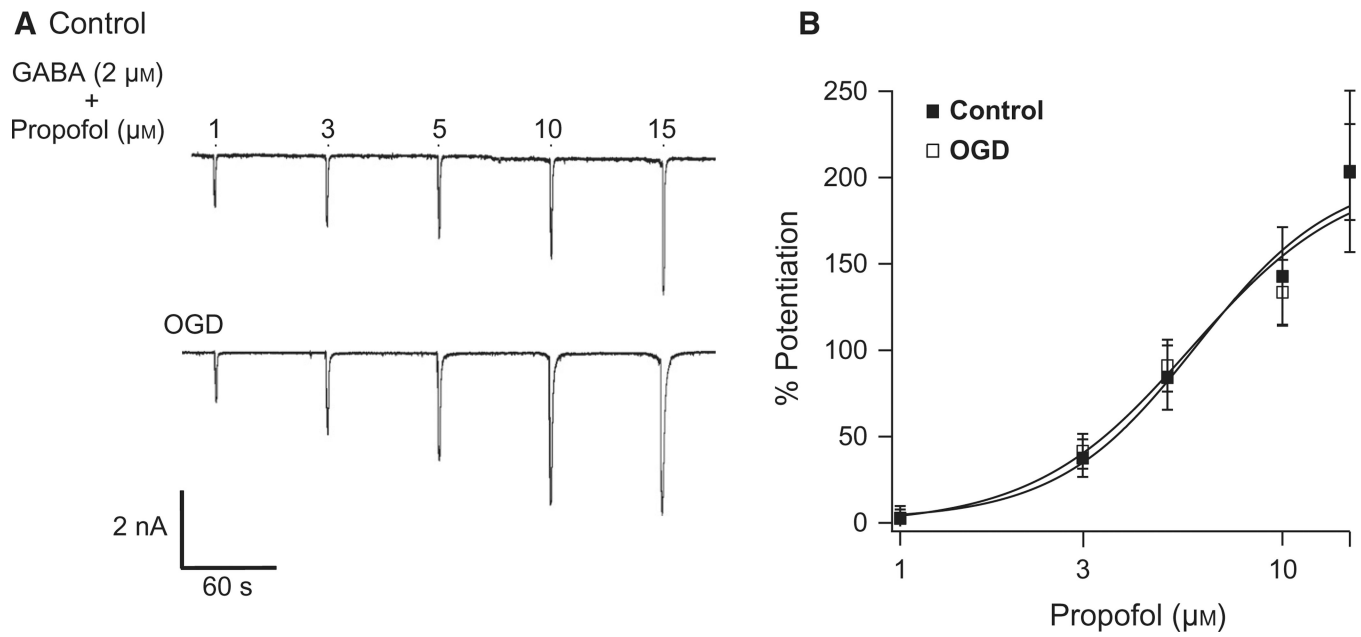


Fig. 2. OGD does not affect PC GABA_A receptor sensitivity to propofol. (A) Representative recordings from cultured PCs illustrating responses to 1 s applications of 2 μM GABA plus 1, 3, 5, 10, or 15 μM propofol (bars above trace indicate application). (B) Average concentration-response relationships for propofol from control and OGD PCs. The average EC₅₀ for control cells was $5.6 \pm 0.5 \mu\text{M}$ ($n = 9$). The OGD cell EC₅₀ was $5.8 \pm 0.5 \mu\text{M}$ ($n = 12$).

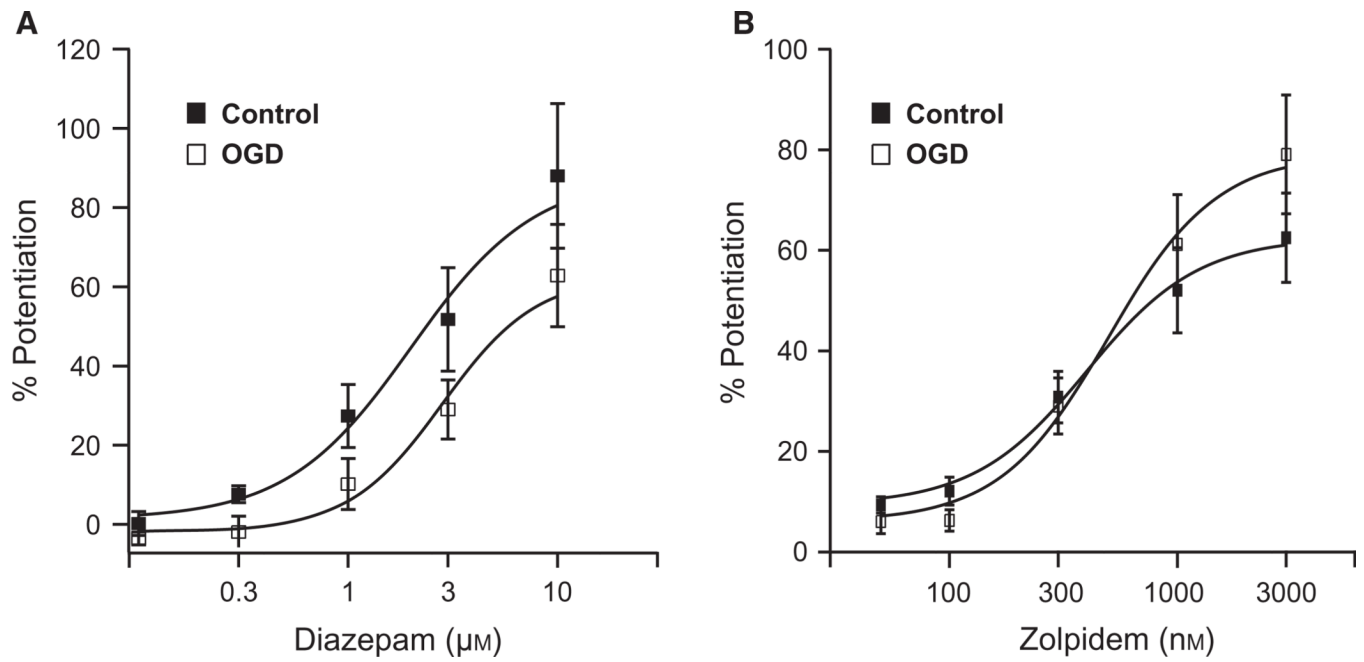


Fig. 3. OGD does not affect PC GABA_A receptor sensitivity to diazepam or zolpidem. (A) Average concentration-response relationships for diazepam from control and OGD PCs. The average EC₅₀ for control PCs was $2.27 \pm 0.4 \mu\text{M}$ ($n = 7$). For OGD cells the average EC₅₀ was $2.81 \pm 0.4 \mu\text{M}$ ($n = 6$). (B) Average concentration-response relationships for zolpidem from control and OGD PCs. The average EC₅₀ for control cells was $465.7 \pm 75.9 \text{ nM}$ ($n = 9$). The average OGD cell EC₅₀ was $482.6 \pm 62.4 \text{ nM}$ ($n = 8$).

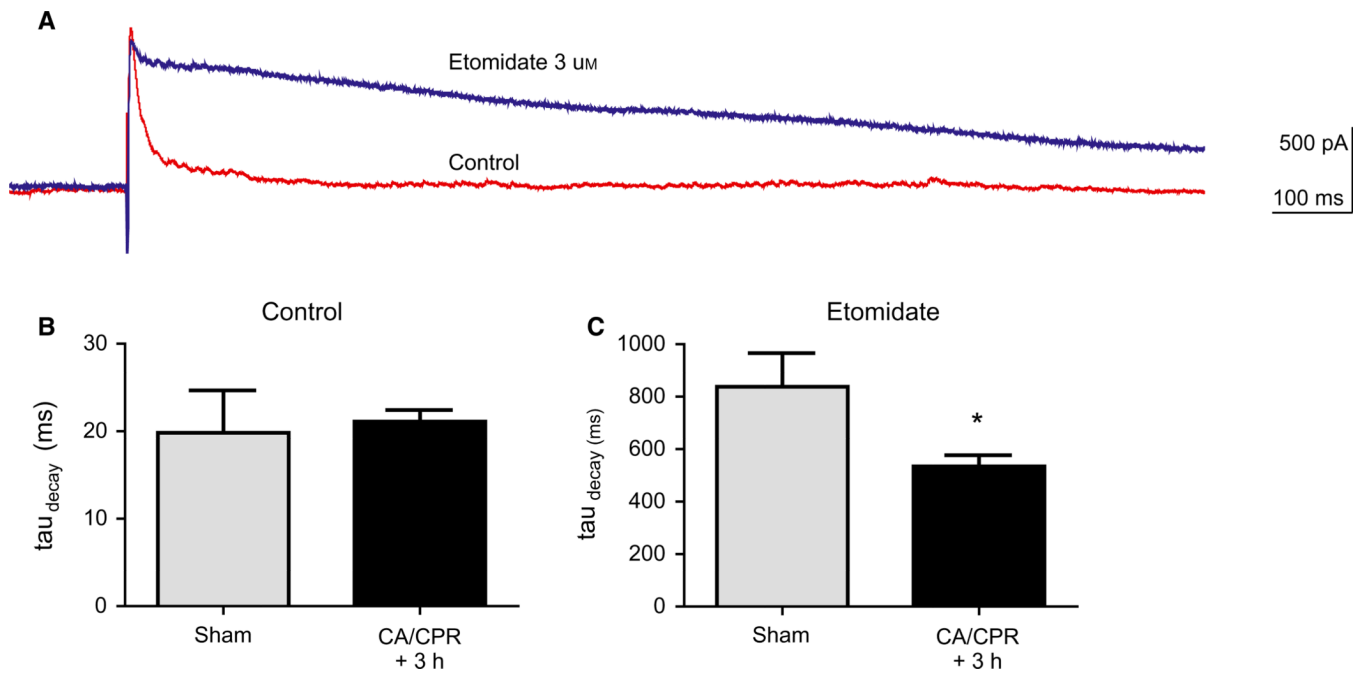


Fig. 4. *In vivo* global ischemia results in reduced potentiation of IPSC decay kinetics. (A) Representative recording showing prolongation of the decay kinetics of the IPSC before (control) and after application of etomidate (3 μM). (B) Summary of IPSC decay time constant (ms) in control conditions in sham and CA/CPR. (C) Summary of IPSC decay time constant in the presence of etomidate. Mean \pm SEM; * $P < 0.05$.

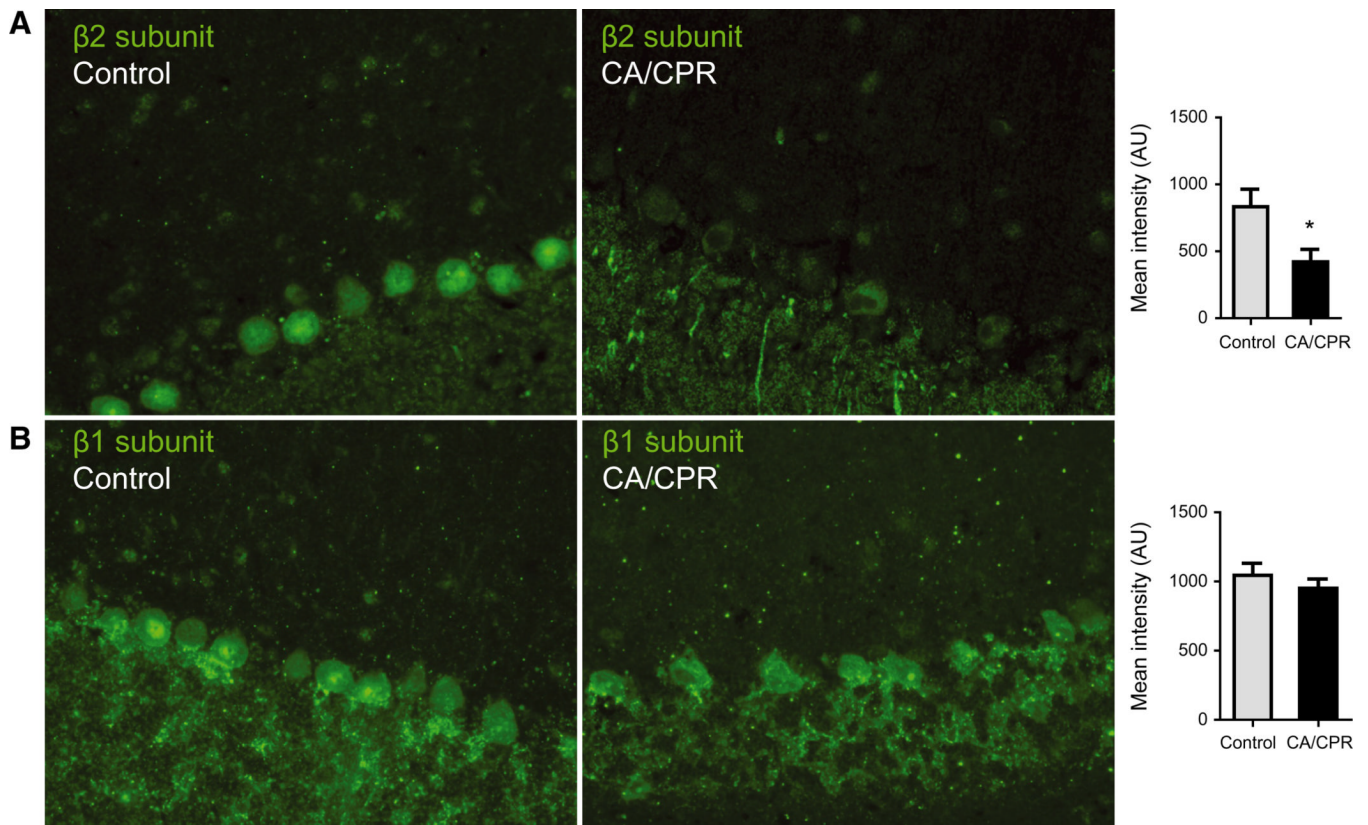


Fig. 5. Reduced β_2 - but not β_1 -subunit expression following *in vivo* global ischemia. (A) Immunohistochemistry using GABA_A receptor β_2 -subunit antibody on paraffin-embedded sections from control (left) or after CA (middle). Mean intensity of fluorescence [arbitrary units (AUs)] in the soma was significantly reduced (* indicates $P < 0.05$, $n = 4$) 24 h after CA (right). (B) Immunohistochemistry using GABA_A receptor β_1 -subunit antibody on sections from control (left) or after CA (middle). Mean intensity of fluorescence was not different between control and CA/CPR.

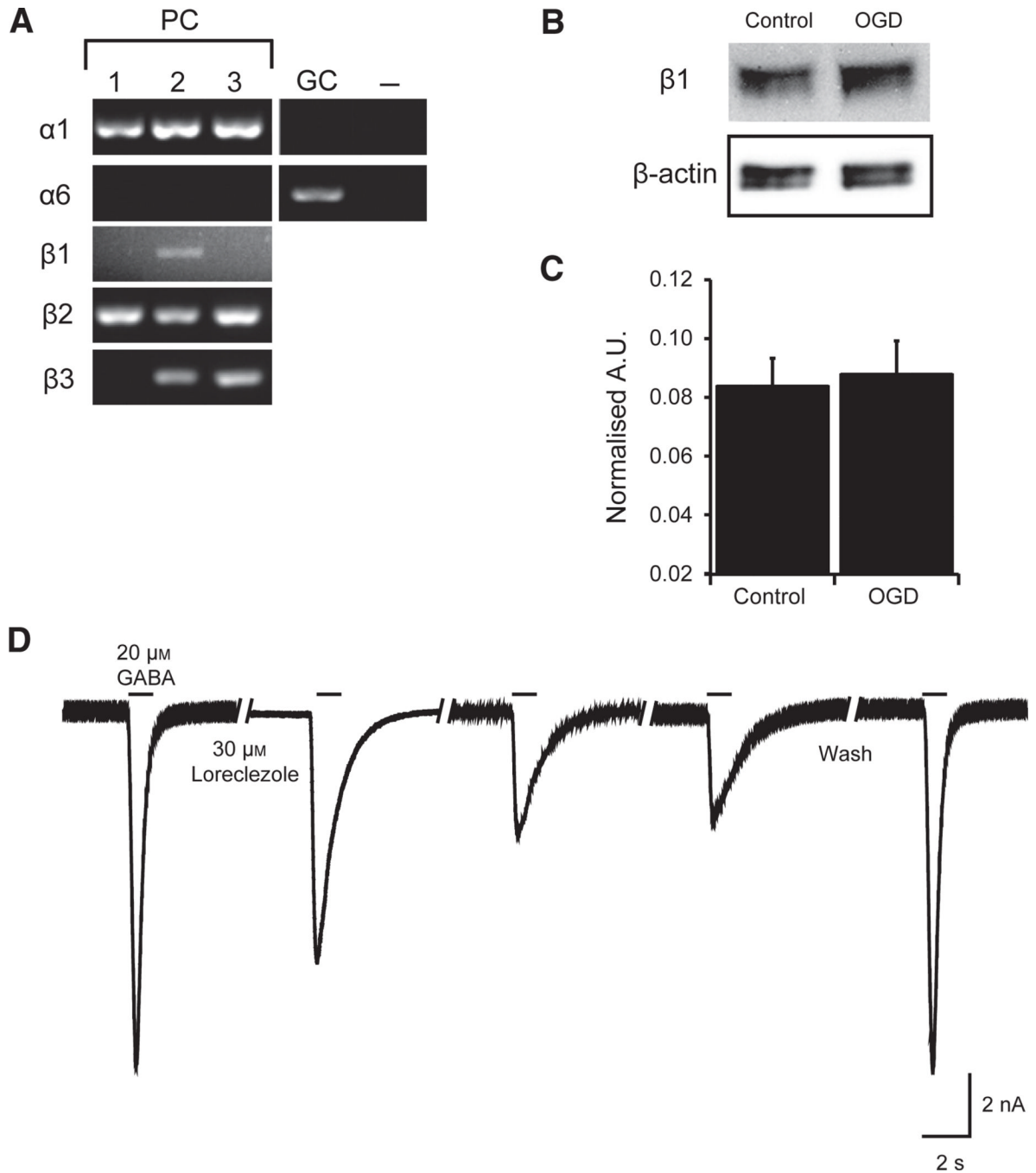


Fig. 6. PCs express the GABA_A receptor $\beta 1$ -subunit and protein expression of $\beta 1$ is not affected by OGD. (A) Representative gels illustrating the mRNA expression of $\alpha 1$, $\alpha 6$, $\beta 1$, $\beta 2$, and $\beta 3$ -subunits in isolated PCs by single-cell reverse transcription-PCR. Each lane (1–3) represents one sample containing four to six PCs. Purity of PC samples was confirmed by the absence of $\alpha 6$. Granule cells (GCs) were the positive control for $\alpha 6$. Negative control (–) was Tyrode’s solution collected from the vicinity of PCs. (B) Representative western blot of cerebellar GABA_A receptor $\beta 1$ -subunit protein from control cultured PCs and cells that received 2 h OGD and 1 h reoxygenation. β -actin loading control shown in lower panel. (C) Quantification of GABA_A receptor $\beta 1$ -subunit protein, normalised to β -actin loading control

and expressed as optical density units ($n = 5$ for each group). Data are mean \pm SEM. (D) Representative recording depicting loreclezole inhibition of GABA_A receptor-mediated currents. Each bar represents application of 20 μM GABA. After stable peak amplitude response to GABA was achieved, 30 μM loreclezole was preapplied by local perfusion onto the cell for 30 s and loreclezole remained present through three subsequent GABA pulses applied in 30 s intervals. Following 60 s wash out of loreclezole, peak amplitude of GABA response was restored. A.U., arbitrary unit.

Table 1PC GABA_A receptor sensitivity to subunit-specific positive modulators

| Drug | Subunit | Control EC₅₀ | OGD EC₅₀ | Control vs. OGD P < 0.05 |
|-------------|----------------|--------------------------------|----------------------------|--|
| Etomidate | 2-3 | 2.9 ± 0.3 μM (8) | 3.8 ± 0.2 μM (6) | * |
| Propofol | 1-3 | 5.6 ± 0.5 μM (9) | 5.8 ± 0.5 μM (12) | – |
| Zolpidem | 1, 2 | 466.1 ± 75.8 nM (9) | 491.5 ± 55.6 nM (8) | – |
| Diazepam | 1,2,3,5, | 2.3 ± 0.4 μM (7) | 2.8 ± 0.3 μM (7) | – |

Note significant change in sensitivity to etomidate. Numbers inside parentheses indicate number of cells/condition. Summary of PC sensitivities to various drugs before and after OGD. Value are given as the mean ± SEM.

Significant difference.