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Vesicular GABA release delays the onset of the Purkinje cell terminal depolarization without affecting tissue swelling in cerebellar slices during simulated ischemia

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Abstract

Neurosteroids that can enhance GABA_A receptor sensitivity protect cerebellar Purkinje cells against transient episodes of global brain ischemia, but little is known about how ischemia affects GABAergic transmission onto Purkinje cells. Here we use patch-clamp recording from Purkinje cells in acutely prepared slices of rat cerebellum to determine how ischemia affects GABAergic signaling to Purkinje cells. In voltage-clamped Purkinje cells, exposing slices to solutions designed to simulate brain ischemia caused an early, partial suppression of the frequency of spontaneous inhibitory post synaptic currents (sIPSCs), but after 5-8 minutes GABA accumulated in the extracellular space around Purkinje cells, generating a large (~17 nS), sustained GABA_A receptor-mediated conductance. The sustained GABA_A conductance occurred in parallel with an even larger (~117 nS) glutamate receptor-mediated conductance, but blocking GABA_A receptors did not affect the timing or magnitude of the glutamate conductance, and blocking glutamate receptors did not affect the timing or magnitude of the GABA_A conductance. Despite the lack of interaction between GABA and glutamate, blocking GABA_A receptors significantly accelerated the onset of the Purkinje cell “ischemic” depolarization (ID), as assessed with current-clamp recordings from Purkinje cells or field potential recordings in the dendritic field of the Purkinje cells. The Purkinje cell ID occurred ~2 minutes prior to the sustained glutamate release under control conditions and a further 1-2 minutes earlier when GABA_A receptors were blocked. Tissue swelling, as assessed by monitoring light transmittance through the slice, peaked just after the ID, prior to the sustained glutamate release, but was not affected by blocking GABA_A receptors. These data indicate that ischemia induces the Purkinje cell ID and tissue swelling prior to glutamate release, and that blocking GABA_A receptors accelerates the onset of the ID without affecting tissue swelling. Taken together these data may explain why Purkinje cells are one of the

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most ischemia sensitive neurons in the brain despite lacking NMDA receptors, and why neurosteroids that enhance GABA_A receptor function protect Purkinje cells against transient episodes of global brain ischemia.

Introduction

A variety of compounds that enhance GABA_A receptor sensitivity, such as diazepam and neurosteroids, protect brain tissue from damage induced by episodes of brain ischemia (Galeffi *et al.*, 2000;Kelley *et al.*, 2008;Nelson *et al.*, 2001;Schwartz-Bloom *et al.*, 1998), but the mechanisms mediating this protection are not fully understood (Sarnowska *et al.*, 2009). Besides the complication added by non-GABA_A receptor actions of these compounds, even actions mediated by increasing GABA_A receptor sensitivity are complicated by the diversity of GABAergic processes both across different brain regions and during different phases of ischemia and reperfusion.

During forebrain ischemia, release of GABA (γ -Aminobutyric acid), like other neurotransmitters, increases in two phases: an early increase in vesicular release of GABA, followed by a second, larger phase of release, mediated primarily by GABA transporter reversal (Allen *et al.*, 2004;Allen & Attwell, 2004;Phillis & O'Regan, 2003;Saransaari & Oja, 2008;Saransaari & Oja, 2005). The early phase of elevated GABA could protect ischemic brain tissue by dampening excitability, thereby conserving ATP and delaying the onset of multiple damage pathways triggered by ATP depletion (Kass & Lipton, 1989;Abramowicz *et al.*, 1991;Abel & McCandless, 1992). The second phase of GABA release, which occurs in parallel with excitotoxic glutamate release could, in principle, by keeping cells hyperpolarized counteract excitotoxic glutamate release, and possibly opening of other channels implicated in ischemic brain damage, such as TRP, ASIC and gap junction hemi-channels (Rossi *et al.*, 2007). However, if GABA_A receptor activation does not prevent these processes, then it could increase the Cl⁻ entry which occurs when cells depolarize (Hansen, 1985;Inglefield & Schwartz-Bloom, 1998), and thus increase water entry and cell swelling (Allen *et al.*, 2004). Cell swelling can be damaging, so excessive GABA_A receptor activation could be harmful. Consistent with this, in retinal and cerebellar cells, cell death evoked by activation of glutamate receptors can be reduced by blocking GABA_A receptors (Chen *et al.*, 1999). Thus, it is not clear whether increasing GABA_A receptor sensitivity during the acute phase of brain ischemia will be beneficial or harmful, and many studies indicate that the protection provided by GABA_A receptor enhancement occurs during the post-ischemic period (Galeffi *et al.*, 2000;Sarnowska *et al.*, 2009).

In vitro studies of brain slices of forebrain from post ischemic animals or after washout of ischemia simulation solutions indicate that the GABAergic system is impaired by episodes of ischemia, including a down regulation of GABA_A receptors (Alicke & Schwartz-Bloom, 1995;Allen *et al.*, 2004;Zhan *et al.*, 2006), a loss of the chloride gradient (Galeffi *et al.*, 2004), and impaired functioning of GABAergic interneurons (Zhan *et al.*, 2007). Thus, protection provided by enhancing GABA_A receptor sensitivity in the post ischemic period may reflect a normalization of the GABAergic system rather than an enhancement compared to pre-ischemic baseline conditions.

The specific details of the preceding text primarily concerns studies of the hippocampus, and may not apply to other brain structures or cell types with differing properties and responses to ischemia. An important example is the cerebellar Purkinje cell (PC), which is one of the most ischemia sensitive neurons in the brain (Pulsinelli, 1985), but has a very unusual configuration of many of the key molecules implicated in ischemia-induced damage in the hippocampus. Of particular relevance to the role of GABA_A receptors in modulating ischemic damage, mature PCs do not express functional NMDA receptors (Hausser & Roth, 1997; Llano *et al.*, 1991), which in the hippocampus are a primary cause of excitotoxic Ca²⁺ entry (Zhang & Lipton, 1999), and have several important interactions with GABA_A receptors during ischemia. First, since Ca²⁺ entry through NMDA receptor-gated channels is reduced by membrane hyperpolarization (Mayer *et al.*, 1984), activation of GABA_A receptors will reduce Ca²⁺ influx through NMDA channels. Second, since glutamate release during brain ischemia is driven by membrane depolarization (Rossi *et al.*, 2000), activation of GABA_A receptors should reduce the glutamate release that activates NMDA receptors. Finally, Ca²⁺ influx through NMDA channels during ischemia causes a long-term inactivation of GABA_A receptors which contributes to neuronal damage in the post-ischemic period (Alicke & Schwartz-Bloom, 1995; Allen *et al.*, 2004; Zhan *et al.*, 2006; Schwartz *et al.*, 1994). Thus, the lack of NMDA receptors on PCs makes it difficult to extrapolate the role of PC GABA_A receptors from what has been observed for hippocampal cells. Nonetheless, recent studies have demonstrated that 2 days after recovery from cardiac arrest, PC GABA_A receptor expression is suppressed, and in cultured PCs, GABA_A synaptic currents are reduced after 1 hour of recovery from oxygen glucose deprivation (Ardeshiri *et al.*, 2006; Kelley *et al.*, 2008). Intriguingly, allopregnenolone, a neurosteroid that potentiates GABA_A receptors, protects PCs against ischemic damage *in vivo*, and against oxygen glucose deprivation in culture, and at least for the cultured PCs the mechanism of protection requires GABA_A receptor activity (Ardeshiri *et al.*, 2006; Kelley *et al.*, 2008). However, it is not known whether the protection provided by allopregnenolone is mediated during ischemia or during reperfusion/recovery, and the potential for Cl⁻ influx through GABA_A receptors to exacerbate tissue swelling during ischemia is especially important for cerebellum due to its proximity to the brain stem, compression of which makes cerebellar stroke particularly lethal (Kelly *et al.*, 2001; Amarenco, 1991). Importantly, although Kelley *et al.* (2008) have determined that the function of PC GABA_A receptors is impaired in the early reperfusion stage following a lethal dose of ischemia, there is currently no information available about the response or function of the PC GABA_A system while ischemia is actually occurring. Here we use patch-clamp recording of PCs in cerebellar slices to determine how conditions simulating ischemia affect GABAergic transmission onto PCs during ischemia, and how GABA_A activity during ischemia affects acute PC responses to ischemia.

Experimental Procedures

Preparation of brain slices

Seventy Sprague–Dawley rats contributed to the present study. The animals were housed with *ad libitum* access to food and water in a room air-conditioned at 22–23°C with a standard 12 h light–dark cycle. All procedures conform to the regulations detailed in the

National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Animal Care and Use Committee of the Oregon Health and Science University. Cerebellar slices were prepared acutely on each day of experimentation (Rossi & Slater, 1993; Rossi & Hamann, 1998; Rossi *et al.*, 2000). Rats (18-21 days old) were anaesthetized with Isoflurane and killed by decapitation. The whole brain was rapidly isolated and immersed in ice cold (0-2°C) artificial cerebrospinal fluid (ACSF) containing (in mM): 124 NaCl, 26 NaHCO₃, 1 NaH₂PO₄, 2.5 KCl, 2.5 CaCl₂, 2 MgCl₂, 10 D-glucose, and bubbled with 95% O₂/5% CO₂ (pH 7.4). The cerebellum was dissected out of the brain and mounted, parallel to the sagittal plane, in a slicing chamber filled with ice cold (0-2°C) ACSF. Parasagittal slices (225 µm) were made with a vibrating tissue slicer (Vibratome). Slices were incubated in warmed ACSF (33±1°C) for one hour after dissection and then held at 22-23°C until used. Kynurenic acid (1 mM) was included in the dissection, incubation and holding solution (to block glutamate receptors to reduce potential excitotoxic damage) but was omitted from the experimental solutions. All experiments were conducted on slices within 8 hours after dissection, most within 5-6 hours of dissection. No parameter that we have analyzed has shown any significant difference across time after dissection.

Electrophysiology

Slices were placed in a submersion chamber on an upright microscope, and viewed with an Olympus 60X (0.9 numerical aperture) water immersion objective with differential interference contrast and infrared optics. Slices were perfused with heated (32-34°C) ACSF at a rate of ~7 ml/min. Drugs were dissolved in ACSF and applied by bath perfusion. Whole-cell recordings were made from the somata of visually identified Purkinje cells. Patch pipettes were constructed from thick-walled borosilicate glass capillaries and filled with an internal solution containing (in mM) for voltage-clamp experiments with E_{Cl}⁻ = -68 mV: Cesium gluconate 130, NaCl 4, CaCl₂ 0.5, HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) 10, EGTA (Ethylene glycol-bis(2-aminoethyl-ether)-N,N', N'-tetraacetic acid) 5, MgATP 4, Na₂GTP 0.5, QX-314 (Lidocaine N-ethyl chloride) 5 (to suppress voltage-gated sodium currents), for voltage-clamp experiments with E_{Cl}⁻ = 8 mV: CsCl 130, NaCl 4, CaCl₂ 0.5, HEPES 10, EGTA 5, MgATP 4, Na₂GTP 0.5, QX-314 5, and for current-clamp recording: Potassium gluconate 132.3, KCl 7.7, NaCl 4, CaCl₂ 0.5, HEPES 10, EGTA 5, MgATP 4, Na₂GTP 0.5. Voltage-clamp and current-clamp solutions were pH adjusted to 7.2 with CsOH or KOH respectively. Electrode resistance was 1.5 to 2.5 MΩ. Cells were rejected if access resistance was greater than 5 MΩ. Cells were also rejected if the access resistance, monitored with -5 mV voltage steps, changed by more than 20% during the course of an experiment. Spontaneous synaptic currents are acquired at 20 KHz after being filtered at 10 KHz. For analysis, spontaneous synaptic currents are digitally filtered at 2-5 KHz and analyzed with synaptosoft software (Synaptosoft Inc., GA). Spontaneous synaptic currents are defined as current deflections which have an amplitude (measured from the mean current) two times greater than the peak to peak amplitude of the current noise, and which have at least a 3 fold slower decay than rise time (to avoid inclusion of channel openings or momentary seal loss). In experiments examining sIPSCs, GABA_A receptor mediated sIPSCs are distinguished from glutamatergic sEPSCs by clamping the PCs at the reversal potential for glutamatergic sEPSCs. Under such conditions all PC spontaneous synaptic currents are blocked by the GABA_A receptor antagonists

GABAzine (10 μ M) or Bicuculline (25 μ M), confirming their identity as GABA_A receptor mediated sIPSCs. In experiments examining sEPSCs, glutamatergic sEPSCs are isolated by blocking GABA_A receptors with GABAzine (10 μ M). Under such conditions, all PC spontaneous synaptic currents are blocked by the AMPA/KA receptor antagonist NBQX (25 μ M), confirming their identity as AMPA receptor mediated sEPSCs. There was no consistent change in access or input resistance during the time frame used for analyzing spontaneous synaptic currents, and analysis of individual cells was terminated if either attribute changed by more than 20%. For field potential recording, a glass capillary electrode (tip diameter = 3–4 μ m), filled with ACSF, was placed into the molecular layer, 40–70 μ m above the Purkinje cell layer.

Simulating ischemia in brain slices

We simulated severe brain ischemia by exposing slices to a modified ACSF in which glucose and oxygen were replaced with sucrose and nitrogen (oxygen glucose deprivation, OGD), or OGD solution supplemented with iodoacetic acid (IAA, 2 mM) to block glycolysis. As will be seen in the results section, the only significant difference that we observed between these two methods of simulating ischemia was a more rapidly developing response when IAA was included. Previously we also supplemented our ischemia simulation solution with cyanide (1 mM) to block oxidative phosphorylation (Hamann *et al.*, 2005; Rossi *et al.*, 2000). Because cyanide may have chemical interactions with either glutamate receptors or glutamate transporters independent of cellular responses to energy deprivation, in this project we did not include cyanide. Similar to OGD and OGD+IAA, adding cyanide further increased the rate of progression the response, without affecting the magnitude (Data not shown).

Tissue swelling assay

Light transmittance was used to assay tissue swelling. transmitted white light images were collected at 1 Hz using a 20x water immersion lens. The spatially averaged intensity of light transmitted through the entire field of view was used for analysis, and the field of view always contained similar proportions of the molecular layer, the Purkinje cell layer, the granule cell layer, and some of the interior white matter. The percent change in light transmittance was calculated using the following equation: $((T_t - T_0)/T_0) * 100$, where T_t is the intensity at time t, and T_0 is the intensity just prior to any treatment. Low osmolarity ACSF was made by diluting the normal ACSF with distilled water.

Statistics

All data are expressed as the mean $\pm$ the standard error of the mean. Statistical comparisons of mean values were made with unpaired t-tests, with $p < 0.05$ as the threshold for significance. In the figures, * signifies $p < 0.05$, ** signifies $p < 0.01$, and *** signifies $p < 0.001$.

Reagents

All reagents were from Sigma Chemicals (St. Louis, MO) except D-AP5(D-(-)-2-Amino-5phosphonopentanoic acid), bicuculline, GABAzine (SR 95531 hydrobromide or 6-

Imino-3-(4-methoxyphenyl)-1(6H)-pyridazinebutanoic acid hydrobromide), kynurenic acid, NBQX (2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium salt) (all from Ascent scientific, UK), and iodoacetic acid (from Acros/Fisher).

Results

Ischemia causes a concurrent but mutually independent release of GABA and glutamate

To monitor the ischemia-induced GABA release that is experienced by Purkinje cells (PCs), we made voltage-clamp recordings of GABA_A receptor-mediated currents generated by PCs in acutely prepared rat (18-21 days old) cerebellar slices, and exposed them to solutions designed to mimic severe brain ischemia (OGD, supplemented with IAA, see methods for details). In the ischemic hippocampus, GABA and glutamate are released in parallel, but Ca²⁺ influx through glutamate gated channels inactivates GABA_A receptors, which may contribute to cellular damage, but also complicates the quantification of GABA release using GABA_A receptor currents. Accordingly, here we used several approaches, which combined enable us to quantify both GABA and glutamate release, and to determine if the two receptor systems interact during cerebellar ischemia. First, to isolate currents mediated by GABA_A receptors without actually blocking glutamate receptors, we used a low Cl⁻ solution in the patch pipette ($E_{Cl^-} = -68$ mV), and set the membrane potential of the PC to the reversal potential of glutamate gated currents ($E_{glu} = \sim 0$ mV, adjusted empirically for each cell), thereby preventing current flow through glutamate receptors in the PC being recorded. Figure 1A shows a representative response of a PC under these recording conditions. In contrast to other cell types, such as CA1 pyramidal cells (Allen *et al.*, 2004), simulated ischemia caused an early suppression of the amplitude and frequency of GABA_A receptor-mediated sIPSCs (spontaneous inhibitory postsynaptic currents) (Fig. 1A&B). Despite the apparent suppression of sIPSCs, after about 5 minutes, a large sustained GABA_A mediated current developed (Fig. 1A), which we quantified by measuring the current blocked by the GABA_A receptor antagonist bicuculline (40 μ M, Fig A&D, $I_{GABA} = 1.02 \pm 0.14$ nA, $n=10$). To determine if activation of glutamate receptors during ischemia affected the GABA_A receptor-mediated response, we repeated the experiment in the continuous presence of glutamate receptor antagonists (NBQX 25 μ M + AP5 50 μ M, Fig 1C). In contrast to what we found previously for hippocampal cells (Allen *et al.*, 2004), blocking glutamate receptors did not affect the magnitude of the ischemia-induced GABA_A current in PCs (Fig. 1C&D, $I_{GABA} = 1.32 \pm 0.36$, $n=9$, $p>0.05$). To check for the possibility that net current flow through PC glutamate receptors is required to affect PC GABA_A receptors, we repeated the experiment with the PC clamped at -60 mV (to enable current flow through glutamate gated channels), and used a high Cl⁻ intracellular solution ($E_{Cl^-} = +8$ mV, thereby maintaining the magnitude of the driving force for Cl⁻ but reversing the polarity). However, because the magnitude of the GABA_A current is small relative to the glutamate receptor current (Fig. 1C bottom), it was not possible to confidently distinguish the bicuculline blocked current from random fluctuations in the glutamate current. Therefore, we recorded for several minutes after the glutamate current developed, then applied glutamate receptor antagonists, followed, after the current stabilized, by bicuculline (Fig. 1C, bottom). With this protocol we were able to confidently quantify the magnitude of the GABA_A current, which was not significantly different in magnitude to the current

recorded in the other two recording configurations (Fig. 1D, $I_{\text{GABA}}=1.23\pm0.21$, $n=10$). Thus, the large PC glutamate current does not affect PC GABA_A receptors during ischemia.

We routinely supplement our ischemia simulation solutions with IAA (OGD + IAA) to inhibit glycolysis (Rossi *et al.*, 2000; Hamann *et al.*, 2002; Hamann *et al.*, 2005; Allen *et al.*, 2004). We reason that inhibiting glycolysis reduces variability in the response to ischemia across cells and slices that might be introduced by differences in cellular stores of glucose or other substrates such as glycogen, and generally speeds up responses without affecting their quality or magnitude. However, since many of the PC responses to ischemia appear to be distinct from those in other brain regions, before exploring further the role of GABAergic transmission in PC ischemia, we decided to confirm that the addition of IAA simply speeds up the response to ischemia, relative to OGD alone, without fundamentally influencing the quality and magnitude of the response. Figures 2A&B show representative responses of PCs to simulating ischemia with OGD alone, either at -60 mV (with E_{Cl^-} set to 0 mV, Fig. 2A) or at 0 mV (with E_{Cl^-} set to -60 mV, Fig. 2B). As we predicted, although the response to OGD alone is slower to develop than with OGD + IAA (Fig. 2C&D; time to peak at 0 mV = 4.67 ± 0.44 min. and 8.46 ± 0.74 min. for OGD+IAA and OGD alone respectively ($p<0.001$, $n=8$ for each), time to peak at -60 mV = 4.55 ± 0.36 min. and 9.9 ± 0.91 min. for OGD+IAA and OGD alone respectively ($p<0.001$, $n=7$ for each)), the amplitude of the response at 0 or -60 mV, and the magnitude of the glutamate and GABA currents are essentially the same (Fig. 2C&D; $I_{\text{glu}}=6.8\pm0.61$ nA and 6.81 ± 1.2 nA ($p>0.05$, $n=12\&7$), $I_{\text{GABA}}=1.02\pm0.14$ nA and 1.14 ± 0.14 nA ($p>0.05$, $n=8\&8$) for IAA+OGD or OGD alone respectively). In the process of these experiments, we also used an alternative GABA_A receptor antagonist, GABAzine (10 μ M), and found no difference in the magnitude of current blocked by bicuculline and GABAzine (compare Fig. 1D and 2C), further confirming that the current is mediated by GABA_A receptors.

To determine if the GABA that is released during ischemia affects the timing or magnitude of excitotoxic glutamate that is released onto PCs, we monitored the glutamate mediated current response to ischemia either alone, or in the continued presence of GABAzine (Fig. 3). Blocking GABA_A receptors did not affect any aspect of the glutamate receptor-mediated current induced by either OGD alone or OGD + IAA (Fig. 3B).

Blocking GABA_A receptors quickens the onset of the PC ischemic depolarization

Since blocking GABA_A receptors did not affect ischemic glutamate release onto PCs, we wanted to determine if blocking GABA_A receptors could nonetheless affect the PC response to ischemia under more physiological conditions. Toward that end we made current-clamp recordings from PCs, and used a “physiological” internal solution with low $[\text{Cl}^-]$, and without the channel blockers we use to optimize voltage-clamp recording (see methods for details). Under these conditions, simulating ischemia with either OGD alone or OGD + IAA causes, after several minutes, the PC to depolarize to about -5 mV (the “ischemic” depolarization (ID), Fig. 4A; time to ID = 5.22 ± 0.42 min. for IAA+OGD ($n=12$) and 8.04 ± 0.33 min. for OGD alone ($n=12$)). As was the case for the timing of glutamate and GABA release (Fig. 2), the ID occurred more slowly with OGD alone compared to OGD + IAA (Fig. 4B, $p<0.001$). With either method of simulating ischemia, blocking GABA_A

receptors with GABAzine (10 μ M) significantly decreased the time of onset of the ID (Fig. 4B; time to ID = 4.52 ± 0.18 min. ($p < 0.01$, $n=8$) for OGD+IAA+ GABAzine and 6.75 ± 0.39 min. ($p < 0.01$, $n=8$) for OGD+GABAzine), and slightly but significantly increased the magnitude of the ID (Fig. 4B; ID amplitude = 62.8 ± 1.54 mV for IAA+OGD+GABAzine ($p < 0.01$ versus 57.4 ± 0.75 mV without GABAzine, $n=8 \& 12$) and 58.9 ± 0.68 min. for OGD +GABAzine ($p < 0.01$ versus 53.5 ± 1.05 without GABAzine, $n=8 \& 12$)). As a further confirmation of the influence of blocking GABA_A receptors, we also used field potential recording to measure the ID without interfering with the content and integrity of the PCs (Fig. 4C). The timing of the ID recorded using field potential recording was the same as that recorded with current clamp (Fig. 4E; time to ID = 7.82 ± 0.25 min. ($n=15$) for OGD alone, $p > 0.05$ compared to current clamp recording), and similarly, blocking GABA_A receptors significantly decreased the time of onset of the ID (Fig. 4D; time to ID = 6.57 ± 0.53 , $p < 0.01$, $n=8$). Thus, although blocking GABA_A receptors does not affect ischemic glutamate release, it does hasten the onset of the ID by 1-2 min. depending on the severity of the ischemic insult, and the ID precedes the massive glutamate release by 2 min. for OGD alone and by 3-4 minutes for OGD with GABAzine (Fig. 4E, $p < 0.01$).

The fact that the ID precedes the massive release of GABA and Glutamate, and blocking GABA_A receptors hastens the onset of the ID, indicates that despite the suppression of sIPSC frequency and amplitude (Fig. 1A&B), the persistent IPSCs are nonetheless counteracting some drive toward depolarization that occurs before the large release of glutamate (Fig. 4E). In many cell types ischemia causes an early increase in spontaneous excitatory post synaptic currents (sEPSCs) prior to the large release of glutamate by transporter reversal. Accordingly we examined sEPSCs in PCs during simulated ischemia. Although delayed relative to the suppression of sIPSCs, ischemia did cause a dramatic increase in PC sEPSC frequency, starting at about 3 minutes in ischemia (sEPSC frequency = $978 \pm 402\%$ of control, $p < 0.01$, $n=9$), and steadily increasing over a two minute period leading up to the time point when the ID occurs with GABA_A receptors blocked (Fig. 4F&G). The timing of the increase in sEPSC frequency, combined with our previous finding that blocking glutamate receptors delays the PC ID by at least 20 minutes (Hamann *et al.*, 2005), indicates that the ischemia-induced increase in sEPSCs drives the earlier onset ID when GABA_A receptors are blocked.

Blocking GABA_A receptors does not affect ischemia-induced swelling

Our finding that blocking GABA_A receptors accelerates the onset of the ID suggests that activation of GABA_A receptors is protective. However, the large GABA_A current that is generated in parallel with the large glutamate current (Fig. 2), could exacerbate cell swelling, which could counteract any benefit gained by the delay in ID onset. To test for this possibility, we used a well established assay for cellular swelling, light transmittance, which increases as cells swell (Allen *et al.*, 2004; Anderson *et al.*, 2005; Jarvis *et al.*, 2001; Joshi & Andrew, 2001). Figure 5A shows a representative trace of light transmittance through a slice of cerebellum under control conditions and upon exposure to hypoosmotic solution (240 mosm). As has been reported for other brain regions, exposing the slice to hypoosmotic solution causes a reproducible and reversible increase in light transmittance (Fig. 5A&B; light transmittance increased by $11.5 \pm 1.5\%$, $p < 0.01$ compared to control, $n=3$). Exposing

the slice to simulated ischemia caused a biphasic increase in light transmittance, starting off gradually with a secondary, faster rise to peak (Fig. 5C; time to peak = 8.67 ± 0.36 min., peak increase = $10.2 \pm 1.0\%$, $n=18$). Blocking GABA_A receptors did not affect the timing or magnitude of the change in light transmittance (Fig. 5C&D; time to peak = 8.72 ± 0.54 min., peak increase = $11.96 \pm 1.05\%$, $p>0.05$, $n=16$). Thus, neither the early vesicular release of GABA nor the sustained GABA_A current contribute to cerebellar swelling during simulated ischemia.

Discussion

In this study we observed a biphasic response of the PC GABA_A system to simulated ischemia: an early, but incomplete suppression of sIPSCs, followed after several minutes by a sustained release of GABA that generates a large tonic current in the PC. The early suppression of sIPSCs is the opposite of what occurs in hippocampal cells, where ischemia leads to a 30 fold increase in the frequency of sIPSCs (Allen & Attwell, 2004). The subsequent sustained release of GABA parallels a sustained release of glutamate, which is typical of other brain regions, and is likely mediated by GABA transporter reversal, consequent to a rundown of ionic gradients. In contrast to the hippocampus (Redecker *et al.*, 2002; Aliche & Schwartz-Bloom, 1995; Allen *et al.*, 2004; Zhan *et al.*, 2006), activation of glutamate receptors during ischemia did not affect PC GABA_A receptors or the release of GABA (Fig. 1D). Similarly, blocking GABA_A receptors did not affect glutamate release induced by ischemia (Fig. 3).

GABA_A receptor activity delays the onset of the ID

Blocking GABA_A receptors during simulated ischemia accelerated the onset of the ID by about 2 minutes (Fig. 4). The earlier onset ID was not mediated by an accelerated or larger magnitude of glutamate release, because the ID occurs 2-3 minutes before the ischemia-induced sustained glutamate current occurs (Fig. 4E), and blocking GABA_A receptors did not affect the timing or magnitude of the glutamate current (Fig. 3). Similarly, since the sustained GABA_A current occurred in parallel with the sustained glutamate current, both delayed relative to the ID by 2 minutes, the speeding of the ID by blocking GABA_A receptors cannot result from the loss of the sustained GABA_A current. Rather, the speeding of the ID must be due to blocking sIPSCs, even though they are partially suppressed by ischemia. This suggests that a depolarizing force develops early during ischemia which the persistent sIPSCs are able to counteract for 1-2 minutes (the difference in the time of ID with and without GABA_A receptors blocked, Fig. 4B). With voltage-clamp recordings, ischemia induced a dramatic increase in sEPSC frequency that escalated progressively over the two minutes leading up to the time the ID occurs in unclamped cells (Fig. 4F&G). This temporal correlation, combined with our previous study showing that the ID is prevented by blocking glutamate receptors (Hamann *et al.*, 2005), suggests that it is the increase in sEPSCs that drives the PC ID.

Blocking GABA_A receptors does not affect ischemia-induced cerebellar swelling

Previous brain slice studies of different brain regions have determined that significant tissue swelling occurs in parallel with the ID (Allen *et al.*, 2004; Anderson *et al.*, 2005; Jarvis *et al.*,

2001; Joshi & Andrew, 2001). Here we observe a similar phenomenon in the cerebellum, where swelling abruptly increases during the ID, both occurring before the sustained glutamate release (Fig. 4E & 5D). In the hippocampus, the ischemia-induced swelling is exacerbated by ischemic GABA release and the resultant Cl^- influx through GABA_A channels (Allen *et al.*, 2004). In contrast, we found that blocking GABA_A receptors did not affect cerebellar swelling (Fig. 5). This difference may stem from the fact that in the cerebellum, both the ID and peak swelling are triggered before the large release of glutamate and GABA occurs, whereas in the hippocampus, both the ID and peak swelling occur during the sustained glutamate and GABA release (Allen *et al.*, 2004). Alternatively, the influence of GABA_A receptors on ischemia induced swelling may depend on the relative magnitude of the GABA to glutamate current, which is much smaller in PCs (the magnitude of the GABA_A current is ~14 % of the glutamate current, Fig. 2C) than in hippocampal pyramidal cells (the magnitude of the GABA_A current is ~70 % of the glutamate current (Allen *et al.*, 2004)). Either the smaller proportional contribution to total cellular conductance of GABA_A receptors or the much larger absolute magnitude of glutamate currents in PCs compared to pyramidal cells could preclude the GABA_A current contribution to cellular swelling in cerebellum compared to hippocampus.

Possible roles of GABAergic transmission in modulating ischemia-induced PC damage

If the PC ID persists for more than ~2 minutes, PCs are irrevocably damaged (Hamann *et al.*, 2005; Mohr *et al.*, 2010). In contrast to the hippocampus and other forebrain regions (Joshi & Andrew, 2001; Jarvis *et al.*, 2001; Anderson *et al.*, 2005), our data suggests that the PC ID is triggered by an imbalance in excitatory and inhibitory synaptic transmission, with sIPSCs decreasing and sEPSCs increasing early in ischemia (Fig 1A&4F&G). Accordingly, blocking glutamate receptors delays the onset of the PC ID by at least 20 minutes (Hamann *et al.*, 2005), and blocking GABA_A receptors hastens the onset of the PC ID by ~2 minutes (Fig. 4B). Given this scenario, we hypothesize that preventing the ischemia-induced suppression of sIPSCs, or enhancing sIPSC frequency or amplitude could further delay the onset of the PC ID, and thus reduce or prevent PC damage. However, we would place an upper limit on such a delay of two minutes, at which point the sustained glutamate release will undoubtedly depolarize and kill PCs (Hamann *et al.*, 2005; Mohr *et al.*, 2010). While two minutes may not be particularly meaningful in the core of an ischemic stroke, it is a fairly good portion of the time window for survival during cardiac arrest (5-10 minutes), and could therefore have a significant impact on delayed neuronal death in survivors of heart attack. In support of this contention, treating rodents with allopregnanolone, which, amongst other things, can potentiate GABA_A receptor responses, protects PCs against damage induced by a transient, global ischemia model of heart attack (Kelley *et al.*, 2008). Furthermore, in the penumbral region of focal ischemia, where energy deprivation is less severe (Lipton, 1999; Rossi *et al.*, 2007; Hata *et al.*, 2000; Obrenovitch, 1995), the time frame for affecting the balance between sIPSCs and sEPSCs may be prolonged, similar to the slowing of responses we observed in comparing OGD to OGD + IAA (Fig. 2C).

Conclusions

During simulated ischemia, PCs in cerebellar slices generate a terminal ischemic depolarization and rapid tissue swelling 2 minutes prior to the sustained release of neurotransmitters glutamate and GABA. The early onset of these two damage responses, prior to the sustained release of glutamate, may explain why PCs are one of the most sensitive cells to transient episodes of global brain ischemia despite lacking NMDA receptors. The ischemia induced PC ID appears to be triggered by a shift in balance of the excitatory and inhibitory tone mediated by sEPSCs and sIPSCs. Thus, drugs that can influence this balance in favor of inhibition could delay the triggering of PC damage during episodes of cerebellar ischemia.

Reference List

- Abel MS, McCandless DW. Elevated gamma-aminobutyric acid levels attenuate the metabolic response to bilateral ischemia. *J.Neurochem.* 1992; 58:740–744. [PubMed: 1729415]
- Abramowicz AE, Kass IS, Chambers G, Cottrell JE. Midazolam improves electrophysiologic recovery after anoxia and reduces the changes in ATP levels and calcium influx during anoxia in the rat hippocampal slice. *Anesthesiology.* 1991; 74:1121–1128. [PubMed: 2042764]
- Alicke B, Schwartz-Bloom RD. Rapid down-regulation of GABA_A receptors in the gerbil hippocampus following transient cerebral ischemia. *J.Neurochem.* 1995; 65:2808–2811. [PubMed: 7595583]
- Allen NJ, Attwell D. The effect of simulated ischaemia on spontaneous GABA release in area CA1 of the juvenile rat hippocampus. *J.Physiol.* 2004; 561:485–498. [PubMed: 15459240]
- Allen NJ, Rossi DJ, Attwell D. Sequential release of GABA by exocytosis and reversed uptake leads to neuronal swelling in simulated ischemia of hippocampal slices. *J.Neurosci.* 2004; 24:3837–3849. [PubMed: 15084665]
- Amarenco P. The spectrum of cerebellar infarctions. *Neurology.* 1991; 41:973–979. [PubMed: 2067660]
- Anderson TR, Jarvis CR, Biedermann AJ, Molnar C, Andrew RD. Blocking the anoxic depolarization protects without functional compromise following simulated stroke in cortical brain slices. *J.Neurophysiol.* 2005; 93:963–979. [PubMed: 15456803]
- Ardeshiri A, Kelley MH, Korner IP, Hurn PD, Herson PS. Mechanism of progesterone neuroprotection of rat cerebellar Purkinje cells following oxygen-glucose deprivation. *Eur.J.Neurosci.* 2006; 24:2567–2574. [PubMed: 17100844]
- Chen Q, Moulder K, Tenkova T, Hardy K, Olney JW, Romano C. Excitotoxic cell death dependent on inhibitory receptor activation. *Exp.Neurol.* 1999; 160:215–225. [PubMed: 10630206]
- Galeffi F, Sah R, Pond BB, George A, Schwartz-Bloom RD. Changes in intracellular chloride after oxygen-glucose deprivation of the adult hippocampal slice: effect of diazepam. *J.Neurosci.* 2004; 24:4478–4488. [PubMed: 15128862]
- 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]
- Hamann M, Rossi DJ, Marie H, Attwell D. Knocking out the glial glutamate transporter GLT-1 reduces glutamate uptake but does not affect hippocampal glutamate dynamics in early simulated ischaemia. *Eur.J.Neurosci.* 2002; 15:308–314. [PubMed: 11849297]
- Hamann M, Rossi DJ, Mohr C, Andrade AL, Attwell D. The electrical response of cerebellar Purkinje neurons to simulated ischaemia. *Brain.* 2005; 128:2408–2420. [PubMed: 16123143]
- Hansen AJ. Effect of anoxia on ion distribution in the brain. *Physiol. Rev.* 1985; 65:101–148. [PubMed: 3880896]

- Hata R, Maeda K, Hermann D, Mies G, Hossmann KA. Dynamics of regional brain metabolism and gene expression after middle cerebral artery occlusion in mice. *J.Cereb.Blood Flow Metab.* 2000; 20:306–315. [PubMed: 10698068]
- Hausser M, Roth A. Dendritic and somatic glutamate receptor channels in rat cerebellar Purkinje cells. *J.Physiol.* 1997; 501(Pt 1):77–95. [PubMed: 9174996]
- Inglefield JR, Schwartz-Bloom RD. Optical imaging of hippocampal neurons with a chloride-sensitive dye: early effects of in vitro ischemia. *J.Neurochem.* 1998; 70:2500–2509. [PubMed: 9603215]
- Jarvis CR, Anderson TR, Andrew RD. Anoxic depolarization mediates acute damage independent of glutamate in neocortical brain slices. *Cereb.Cortex.* 2001; 11:249–259. [PubMed: 11230096]
- Joshi I, Andrew RD. Imaging anoxic depolarization during ischemia-like conditions in the mouse hemi-brain slice. *J.Neurophysiol.* 2001; 85:414–424. [PubMed: 11152742]
- Kass IS, Lipton P. Protection of hippocampal slices from young rats against anoxic transmission damage is due to better maintenance of ATP. *J.Physiol.* 1989; 413:1–11. [PubMed: 2557434]
- Kelley MH, Taguchi N, Ardeshiri A, Kuroiwa M, Hurn PD, Traystman RJ, Herson PS. Ischemic insult to cerebellar Purkinje cells causes diminished GABA_A receptor function and allopregnanolone neuroprotection is associated with GABA_A receptor stabilization. *J.Neurochem.* 2008; 107:668–678. [PubMed: 18699862]
- Kelly PJ, Stein J, Shafqat S, Eskey C, Doherty D, Chang Y, Kurina A, Furie KL. Functional recovery after rehabilitation for cerebellar stroke. *Stroke.* 2001; 32:530–534. [PubMed: 11157193]
- Lipton P. Ischemic cell death in brain neurons. *Physiol Rev.* 1999; 79:1431–1568. [PubMed: 10508238]
- Llano I, Marty A, Armstrong CM, Konnerth A. Synaptic- and agonist-induced excitatory currents of Purkinje cells in rat cerebellar slices. *J.Physiol.* 1991; 434:183–213. [PubMed: 1673717]
- Mayer ML, Westbrook GL, Guthrie PB. Voltage-dependent block by Mg²⁺ of NMDA responses in spinal cord neurones. *Nature.* 1984; 309:261–263. [PubMed: 6325946]
- Mohr C, Brady JD, Rossi DJ. Young age and low temperature, but not female gender delay ATP loss and glutamate release, and protect Purkinje cells during simulated ischemia in cerebellar slices. *Neuropharmacology.* 2010; 58:392–403. [PubMed: 19825379]
- Nelson RM, Hainsworth AH, Lambert DG, Jones JA, Murray TK, Richards DA, Gabrielsson J, Cross AJ, Green AR. Neuroprotective efficacy of AR-A008055, a clomethiazole analogue, in a global model of acute ischaemic stroke and its effect on ischaemia-induced glutamate and GABA efflux in vitro. *Neuropharmacology.* 2001; 41:159–166. [PubMed: 11489452]
- Obrenovitch TP. The ischaemic penumbra: twenty years on. *Cerebrovasc.Brain Metab. Rev.* 1995; 7:297–323. [PubMed: 8703672]
- Phillis JW, O'Regan MH. Characterization of modes of release of amino acids in the ischemic/reperfused rat cerebral cortex. *Neurochem.Int.* 2003; 43:461–467. [PubMed: 12742092]
- Pulsinelli WA. Selective neuronal vulnerability: morphological and molecular characteristics. *Prog.Brain Res.* 1985; 63:29–37. [PubMed: 2872695]
- Redecker C, Wang W, Fritschy JM, Witte OW. Widespread and long-lasting alterations in GABA(A)-receptor subtypes after focal cortical infarcts in rats: mediation by NMDA-dependent processes. *J.Cereb.Blood Flow Metab.* 2002; 22:1463–1475. [PubMed: 12468891]
- Rossi DJ, Brady JD, Mohr C. Astrocyte metabolism and signaling during brain ischemia. *Nat.Neurosci.* 2007; 10:1377–1386. [PubMed: 17965658]
- Rossi DJ, Hamann M. Spillover-mediated transmission at inhibitory synapses promoted by high affinity alpha6 subunit GABA(A) receptors and glomerular geometry. *Neuron.* 1998; 20:783–795. [PubMed: 9581769]
- Rossi DJ, Oshima T, Attwell D. Glutamate release in severe brain ischaemia is mainly by reversed uptake. *Nature.* 2000; 403:316–321. [PubMed: 10659851]
- Rossi DJ, Slater NT. The developmental onset of NMDA receptor-channel activity during neuronal migration. *Neuropharmacology.* 1993; 32:1239–1248. [PubMed: 7509049]
- Saransaari P, Oja SS. Characteristics of GABA release in mouse brain stem slices under normal and ischemic conditions. *Neurochem.Res.* 2005; 30:1549–1556. [PubMed: 16362774]

- Saransaari P, Oja SS. GABA release under normal and ischemic conditions. *Neurochem.Res.* 2008; 33:962–969. [PubMed: 17940883]
- Sarnowska A, Beresewicz M, Zablocka B, Domanska-Janik K. Diazepam neuroprotection in excitotoxic and oxidative stress involves a mitochondrial mechanism additional to the GABA_AR and hypothermic effects. *Neurochem.Int.* 2009; 55:164–173. [PubMed: 19428822]
- Schwartz RD, Wagner JP, Yu X, Martin D. Bidirectional modulation of GABA-gated chloride channels by divalent cations: inhibition by Ca²⁺ and enhancement by Mg²⁺. *J.Neurochem.* 1994; 62:916–922. [PubMed: 8113813]
- Schwartz-Bloom RD, McDonough KJ, Chase PJ, Chadwick LE, Inglefield JR, Levin ED. Long-term neuroprotection by benzodiazepine full versus partial agonists after transient cerebral ischemia in the gerbil [corrected]. *J.Cereb.Blood Flow Metab.* 1998; 18:548–558. [PubMed: 9591847]
- Zhan RZ, Nadler JV, Schwartz-Bloom RD. Depressed responses to applied and synaptically-released GABA in CA1 pyramidal cells, but not in CA1 interneurons, after transient forebrain ischemia. *J.Cereb.Blood Flow Metab.* 2006; 26:112–124. [PubMed: 15959457]
- Zhan RZ, Nadler JV, Schwartz-Bloom RD. Impaired firing and sodium channel function in CA1 hippocampal interneurons after transient cerebral ischemia. *J.Cereb.Blood Flow Metab.* 2007; 27:1444–1452. [PubMed: 17228331]
- Zhang Y, Lipton P. Cytosolic Ca²⁺ changes during in vitro ischemia in rat hippocampal slices: major roles for glutamate and Na⁺-dependent Ca²⁺ release from mitochondria. *J.Neurosci.* 1999; 19:3307–3315. [PubMed: 10212290]

Figure 1. Simulated ischemia suppresses PC sIPSCs and generates a large, sustained GABA_A current.

A. Representative voltage-clamp recording ($V_h = 0$ mV, $E_{Cl} = -68$ mV) from a PC in a cerebellar slice during simulated ischemia. Insets are representative segments of PC current displayed at higher gain and time scale showing sIPSCs in control and early during simulated ischemia. In this and all figures, the horizontal black bars above or below the current trace indicate the timing of exposure to the conditions described immediately above or below the black bar. **B.** Graph displays the mean frequency and amplitude of PC sIPSCs at various times during simulated ischemia. **C.** PC response to simulated ischemia (same recording configuration as in A) in the continuous presence of glutamate receptor antagonists, AP5 (50 μ M) and NBQX (25 μ M). **D.** Representative voltage-clamp recording ($V_h = -60$ mV, $E_{Cl} = +8$ mV) of a PC response to simulated ischemia and sequential block of glutamate receptors and GABA_A receptors. **E.** Bar chart displays the mean amplitude of the current blocked by the GABA_A receptor antagonist, bicuculline (10 μ M, as in A-C) for the 3 conditions depicted in A-C.

Figure 2. Including a glycolytic inhibitor with oxygen and glucose deprivation accelerates PC responses to simulated ischemia without affecting the quality or magnitude

A. Representative voltage-clamp recording ($V_h = -60$ mV, $E_{\text{Cl}} = +8$ mV, as in Fig. 1D) of a PC response to simulating ischemia by depleting oxygen and glucose (OGD) without the glycolytic inhibitor, IAA. **B.** Representative voltage-clamp recording ($V_h = 0$ mV, $E_{\text{Cl}} = -68$ mV) of a PC response to OGD. **C&D.** Bar charts summarize the timing and amplitude of responses to OGD or OGD + IAA for glutamate currents (**C**) or GABA_A currents (**D**), as in A&B respectively. I_{glu} and I_{GABA} are the magnitude of current blocked by AP5 (50 μM) + NBQX (25 μM) or GABAzine (10 μM) respectively.

Figure 3. Blocking GABA_A receptors does not affect ischemia-induced glutamate release

A. PC response to simulated ischemia (OGD only) in the continuous presence of the GABA_A antagonist GABA_Azine (10 μ M), and during subsequent block of glutamate receptors with AP5 (50 μ M) + NBQX (25 μ M). **B.** Bar charts summarize the timing and magnitude of ischemia-induced glutamate currents (for both methods of simulating ischemia) with and without GABA_A receptors blocked. I_{glu} is the magnitude of current blocked by AP5 (50 μ M) + NBQX (25 μ M).

Figure 4. Blocking GABA_A receptors reduces the time to onset of ischemic depolarization

A. Representative current clamp recordings of Purkinje cell responses to simulated ischemia alone (top) or in the presence of GABAzine (10 μ M, bottom). **B.** Bar charts summarize current clamp recording of time to ID (right) and ID amplitude (left) for OGD and OGD+IAA, with and without GABAzine (10 μ M). **C.** Representative field potential recordings of the ID for OGD alone (top) or in the presence of GABAzine (10 μ M, bottom). **D.** Bar charts summarize field potential recording of time to ID (right) and ID amplitude (left) for OGD, with and without GABAzine (10 μ M). **E.** Bar chart compares the time to peak for the large glutamate current (as measured in voltage-clamp recording, from fig. 3C) and time to ID recorded with current-clamp (from fig. 4B) and field potentials (from fig. 4D), and the effect of blocking GABA_A receptors for each measurement. All values are for simulating ischemia with OGD alone. **F.** Representative traces displaying voltage clamp recordings of sEPSCs under control conditions and after ~4 minutes in ischemia (OGD+IAA) and GABAzine. **G.** Graph plots the mean frequency of sEPSCs (black, from voltage clamp recordings as in F) and sIPSCs (gray, from voltage clamp recordings as in fig. 1B). Dashed line indicates the mean time to ID in ischemia (OGD+IAA) in the presence of GABAzine, from current clamp recordings as in A, bottom panel. Asterisks indicate time points in ischemia in which the sEPSC or sIPSC frequency is significantly different from the control frequency (* = $p < 0.05$ and ** = $p < 0.01$).

Figure 5. Blocking GABA_A receptors does not affect ischemia induced cell swelling

A. Representative trace depicting total light transmittance through a slice of cerebellum during and after exposure to hypotonic solution. **B.** Bar chart summarizes the mean light transmittance before, during and after exposure to hypotonic solution. **C.** Specimen traces showing changes in light transmittance during exposure to simulated ischemia, with (grey) and without (black) GABA_A receptors blocked with GABAzine (10 μ M). **D.** Bar charts summarize the timing and magnitude of ischemia-induced (OGD) changes in light transmittance, with and without GABA_A receptors blocked.