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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

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Abstract

Excessive activation of glutamate receptors contributes to Purkinje cell (PC) damage during brain ischemia, but the mechanisms of glutamate release are contentious. Age, gender and temperature all strongly influence ischemic brain damage, but the mechanisms underlying their influence are not fully understood. We determined how age, gender and temperature influence ATP loss, glutamate release, glutamate receptor activation and PC damage during cerebellar ischemia. We used voltage-clamped PCs to monitor glutamate release during simulated ischemia in slices of cerebellum of different ages and genders, and at different temperatures. While gender did not affect ischemic glutamate release, both young age and low temperature dramatically delayed the onset of glutamate release without affecting its magnitude. Glutamate receptor and transporter density were similar around young and old PCs, but the rate of ATP decline during ischemia was dramatically slowed in young animals and by lowered temperature. Bypassing the ischemia-induced loss of ATP, and disrupting ionic gradients directly by pharmacologically inhibiting the Na⁺/K⁺-ATPase, reduced the difference in timing of glutamate release in newborn and mature cerebellum. Ischemic damage in newborn and mature cerebellum paralleled ATP loss and glutamate release, but blocking glutamate receptors did not prevent ischemic damage. Thus, protection against brain ischemia provided by young age or lowered temperature is due to slower consumption and hence delayed loss of ATP, with a corresponding delay in glutamate release and other undetermined damage mechanisms. The protection afforded by female gender must occur downstream of ATP decline, glutamate release, and activation of glutamate receptors on PCs.

Introduction

Ischemic brain damage, as occurs during stroke and cardiac arrest is a leading cause of death and disability. A primary cause of ischemic brain damage is excessive activation of glutamate receptors, and the consequent influx of Ca²⁺ (Choi et al., 1987; Choi and Rothman, 1990; Lipton, 1999; Perez Velazquez et al., 1997; Zhang and Lipton, 1999). In the forebrain, a major source of ischemic glutamate release is via plasma membrane glutamate transporters (named EAATs) running backwards due to the ischemia-induced rundown of ionic gradients

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that normally power glutamate uptake on these transporters (Phillis et al., 2000; Rossi et al., 2000).

Approximately 3% of strokes involve the cerebellum, and cerebellar Purkinje cells (PCs) are amongst the first brain cells to die during global ischemic events (Kelly et al., 2001; Pulsinelli, 1985; Welsh et al., 2002; Amarenco, 1991; Mercuri et al., 1997; Northington et al., 2001). Despite their extreme sensitivity and frequent involvement in ischemic brain damage, relatively little is known about how PCs are damaged by ischemia. This lack of information is problematic because many of the ischemic processes that have been characterized in forebrain utilize cellular components that are not expressed by PCs or have an atypical configuration, thereby limiting extrapolation. First, PCs do not express significant levels of functional NMDA receptors (Hausser and Roth, 1997; Llano et al., 1991; Rosenmund et al., 1992), which, because of their high permeability to Ca^{2+} , are a primary source of damaging Ca^{2+} entry. Second, PCs have a uniquely high density of postsynaptic EAATs (Danbolt, 2001) which should strongly influence glutamate accumulation around PCs. Finally, unlike other ischemia-sensitive principle neurons, PCs are GABAergic, and so have low intracellular concentrations of glutamate, which will lower the driving force for glutamate release by EAATs.

Despite the atypical properties of PC glutamatergic components, simulating ischemia in cerebellar slices induces glutamate release, and a consequent large PC current that is mediated by desensitization-resistant AMPA receptors (Hamann et al., 2005). The ischemia-induced AMPA current causes PCs to depolarize to $\sim 0\text{mV}$, presumably leading to toxic Ca^{2+} influx through voltage-activated Ca^{2+} channels. Similar to forebrain, pharmacologically blocking EAATs dramatically ($\sim 90\%$) reduces the glutamate release onto PCs (Hamann et al., 2005). However, in contrast to forebrain, removing extracellular Ca^{2+} also dramatically ($\sim 70\%$) reduces ischemic glutamate release (Hamann et al., 2005). The super additive reduction of glutamate release by blocking EAATs and removing external Ca^{2+} is surprising since Ca^{2+} is not known to influence glutamate transport by EAATs. Further, *in vivo* studies suggest that both neuronal and glial EAATs are protective against ischemic damage of PCs (Welsh et al., 2002; Yamashita et al., 2006). Thus, despite theoretical and pharmacological support, the role of glutamate transporter reversal in ischemic glutamate release remains equivocal (Rossi et al., 2007).

We sought an alternative to pharmacological or genetic manipulations to investigate mechanisms of ischemic damage of Purkinje cells. We examined PC responses to ischemia under naturally occurring conditions that influence ischemic damage: age and gender (Towfighi et al., 1997; Vannucci and Hagberg, 2004; Murphy et al., 2004; Alkayed et al., 1998; Payan and Conrad, 1977), and during lowered temperature (Zhao et al., 2007). We hypothesized that if the resistance to ischemic damage afforded by young age, female gender, or lowered temperature manifested as altered glutamatergic responses, identifying the underlying mechanisms should both clarify critical glutamatergic processes and identify potential therapeutic targets.

Methods

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 $22\text{--}23^{\circ}\text{C}$ 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 and Slater, 1993; Rossi and Hamann, 1998; Rossi et al., 2000). Rats (from 6–21 days old depending on experiment) 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.

Visualized patch-clamp recording from cells in brain slices

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 (from 22–34°C depending on experiment) ACSF at a rate of ~4ml/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 (mM) Cs gluconate 130, NaCl 4, CaCl₂ 0.5, HEPES 10, EGTA 5, MgATP 4, Na₂GTP 0.5, QX-314 5 (to suppress voltage-gated sodium currents), pH adjusted to 7.2 with CsOH. 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 –5mV voltage steps, changed by more than 20% during the course of an experiment.

Although it is impossible to entirely account for the less than perfect voltage-clamp in adult Purkinje cells, we have in the past attempted to deal with series resistance errors created during the passage of large currents generated by hippocampal CA1 cells and Purkinje cells during ischemia, but have generally found that doing so has not affected our conclusions (Hamann et al., 2002; Hamann et al., 2005). However, when comparing adult cells to young, more “clampable” cells, such voltage-clamp errors could have a more significant impact on our conclusions. For this reason, and since it is impossible to completely and accurately remove all voltage-clamp errors electrically or mathematically, we have tried to normalize all of our measured currents to other measured currents in the same cell before comparing across cells of different age and hence electrical properties. With this approach we intend for voltage-clamp errors to be “cancelled out”, before we compare across cells with differing electrical properties. Specifically, in Figure 4C, we have scaled the amplitude of the ischemia-induced current to the AMPA evoked current in the same cell, and then compared the scaled current between young and old cells. Similarly, in Figure 5B&C, we have scaled glutamate-evoked currents to AMPA-evoked currents (Fig. 5B) or TBOA-evoked currents to glutamate evoked currents (Fig. 5C) in the same cell, before comparing across cell types (right panels).

Although in principle, differences in the quality of voltage clamp can affect the timing of currents, we do not believe that the differences in cell morphology and corresponding electrical properties is a significant issue in the current context. In particular, in the absence of current flow, there are no voltage errors, and so cannot explain the difference in timing of the onset of ischemia evoked currents (although it probably does affect the rise time of the current, the rise time of the ischemia-induced current in adult cells is on the order of seconds, whereas the difference in time of occurrence between young and adult cells is nearly 45 minutes, Fig. 2). That voltage-clamp errors are not dramatically affecting the timing of events is supported by comparing voltage-clamp recording to field potential recording (we have shown previously that the ischemic depolarization is generated by the large current recorded during voltage-

clamp recording (Hamann et al., 2005)). Specifically, the ischemic depolarization recorded with field potential recordings occurs at the same time as the large glutamate-mediated current (compare Fig. 3F middle bar to Fig. 2C), indicating that the timing is not significantly affected by voltage-clamp errors, and certainly cannot explain the dramatic difference in timing between young and old slices.

Thus, although the quality of voltage-clamp in adult cells is not perfect, we believe that the errors created do not fundamentally alter the conclusions we have drawn about the differences in the response of young and old Purkinje cells that we have observed.

Simulating ischemia in brain slices

We simulated severe brain ischemia by exposing slices to solution in which glucose and oxygen were replaced with sucrose and nitrogen, and supplemented with iodoacetic acid (2mM) to block glycolysis. Previously we also supplemented our ischemia simulation solution with cyanide (1mM) 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. The only significant difference that we observed between these two methods of simulating ischemia was a slightly more rapidly developing response when cyanide was included (Data not shown).

Immunocytochemistry

Slices were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 17 hours. Slices were then washed and incubated for 40 minutes in blocking solution (PBS, 0.5% Triton X-100, and bovine serum albumin (0.5mg/ml)). Next, they were incubated for 1–2 days with primary antibody in PBS and Triton. Slices were washed 3 times (10 minutes each) in PBS, then incubated for 45 minutes with an Alexa-conjugated secondary antibody. Slices were mounted in Citifluor and imaged with confocal microscopy. See reagents (below) for source and dilution of antibodies used.

Confocal microscopy

Images were acquired with a Zeiss confocal LSM510 laser scanning Microscope, using accompanying Zeiss software for acquisition, processing and subsequent analysis. Stacks of image planes were acquired from the PC layers in different lobes and then projected into a single stacked image (28 μ m thick for mature, 24 μ m thick for young slices). A laser line falling within 20nm of the peak absorbance was used for each of the various fluorophores, with appropriate excitation, dichroic and emission filters. Pinhole diameter and slice step thickness were optimized for the objective used.

Cell death assay

After the post surgery incubation period, slices were equilibrated in a chamber containing heated (33–34°C) control ACSF for 40 minutes (for slices that were going to be exposed to ischemia plus glutamate receptor blockers, this solution was supplemented with glutamate receptor antagonists to ensure adequate equilibration prior to the actual ischemic episode). Slices were then transferred to a heated chamber containing either control ACSF, ischemia simulation solution, or ischemia simulation solution with glutamate receptor antagonists (NBQX 25 μ M + AP5 50 μ M) for either 10 or 60 minutes. Subsequently all slices were allowed to recover/equilibrate for 2 hours in room temperature ACSF with kynurenic acid (1mM). All slices were then placed in ACSF supplemented with propidium iodide (6 μ M) for 20 minutes. Finally, all slices were fixed and processed for subsequent immunocytochemistry and confocal microscopy (as described above). 3-D projections of confocally acquired stacks were used to

analyze calbindin density in PCs. A roughly rectangular region of interest was placed over the 3-D projection, extending from the base of the PC layer to 110 μm (for mature slices) or 45 μm (for young slices) into the molecular layer (occupied by the PCs dendrites), and across the length of the field of view. Since ischemia typically damaged PC dendrites beyond clear identity, the dimensions of the analyzed region were set as the average dimensions of the PC and molecular layer of control slices for a given age, and were identical for all slices and conditions of a given age group. The mean density of the calbindin fluorescence was measured for the same three lobes in each slice and in each condition. Propidium iodide (PI) staining was also examined for all conditions, but since there was no PI staining in control slices, and virtually no PI staining of PCs in ischemia damaged slices (due to the severity of the damage), the PI signal was not analyzed. However, the PI signal in granule cells was used to help identify the base of the Purkinje cell layer in ischemic conditions, and is included in the figures for visualization purposes. Statistical comparisons of mean density of calbindin staining were made with unpaired t-tests.

ATP assay

Following 1 hour at 33–34°C in oxygenated ACSF containing 1 mM kynurenic acid, slices were incubated in standard oxygenated ACSF at either 33–34°C or 22–23° for 30 minutes. At the end of this period, 2 slices were immediately removed for analysis of the initial ATP content before further treatments. The mean initial ATP content was $221 \pm 18 \text{ nM/mg}$ for young animals at 33–34°C, $245 \pm 21 \text{ nM/mg}$ for young animals at 22–23°, 185 ± 30 for old animals at 33–34°C, and 165 nM/mg for old animals at 22–23°. Control slices then remained in standard oxygenated ACSF while ischemia slices were transferred to a chamber containing ischemia solution (see above). Incubation times and temperatures are detailed in the figure legends. 1–2 slices were removed for analysis at each time point. For ATP and protein determination, slices were homogenized in 0.3 M perchloric acid, neutralized to pH 6.8 with an appropriate volume of 1.0 M K_2HPO_4 , and stored at –20°C for up to 1 week. Samples were centrifuged at 13K rpm at 4°C for 5 minutes before ATP and protein measurements. For ATP determination, the supernatants were diluted 100-fold and assayed using a luciferase-based reaction kit (Molecular Probes, Eugene, OR), and luminescence was measured on a Turner Biosystems luminometer. For each assay, a standard curve was run using known ATP concentrations, and all measurements were within the linear range of the assay. Protein content of the undiluted supernatants was assayed using Bradford reagent (Sigma, St Louis, MO).

Statistics

All values are expressed as the mean $\pm$ S.E.M with statistical significance defined as $p < 0.05$ using a student's t-test. In all figures, p-values are displayed for all comparisons that are significantly different, and comparisons that are not significantly different ($p > 0.05$) are unlabelled.

Reagents

Glutamate, Ouabain (Sigma, St. Louis, MO), AMPA, TBOA (Tocris, Ellisville, MO), NMDA, TTX, NBQX (Ascent scientific, Great Britain), Propidium Iodide (Molecular Probes, Eugene, OR). Antibodies: Rabbit anti Calbindin D-28K, affinity purified antibody (Chemicon, Temecula CA) used at 1:2000 dilution. Guinea pig anti-EAAT1, antiserum (Chemicon, Temecula CA) used at 1:1000 dilution. Rabbit anti-EAAT4, affinity pure antibody (Alpha diagnostics, San Antonio, TX) used at 1:100 dilution. Alexa fluor conjugated secondary antibodies (Molecular Probes, Eugene, OR) used at 1:500 dilution.

Results

Gender does not affect ischemic glutamate release

To monitor the ischemia-induced glutamate release that is experienced by Purkinje cells (PCs), we made voltage-clamp recordings from PCs in acutely prepared mature (18–21 days old) rat cerebellar slices exposed to solutions designed to mimic severe brain ischemia (see methods for details). Figure 1A shows a representative response of a PC in a cerebellar slice from a female rat. Similar to other brain regions, and in agreement to what we have previously reported, simulated ischemia leads to two sequential phases of enhanced glutamate release. The first phase is typified by a gradual but ultimately profound increase in the frequency of spontaneous excitatory postsynaptic currents (sEPSCs, Fig. 1A), which culminates in the second phase, a rapidly developing, large, sustained glutamate receptor generated current (Peak= 11.0 ± 0.9 nA, $n=11$, Fig. 1A&B), that we have previously shown is mediated predominantly by AMPA receptors (I_{glu}= 6.7 ± 0.8 nA, Fig. 1A&B). This ischemia-induced glutamate gated current generates the ischemic depolarization (ID) that occurs in unclamped cells (Hamann et al., 2005), so we will refer to it as the ID current. The timing and magnitude of these responses in PCs from males and females were indistinguishable ($p > 0.05$, $n=5$ and 11 respectively, Fig. 1B&C). This suggests that the influence of gender on ischemic brain damage is not mediated by the timing or magnitude of glutamate release, nor by the density of glutamate receptors.

Ischemic glutamate release is dramatically delayed in newborn cerebellum

To determine the influence of age on the PC response to ischemia, we recorded responses to simulated ischemia by PCs in slices from animals ranging in age from 6 to 21 days old (Fig. 2). Recordings from 12–14 day old Purkinje cells showed qualitatively similar responses to recordings from the mature (18–21 day old) PCs shown in Figure 1, but the timing of both the increase in sEPSCs, and the ID current is delayed by several minutes (Time to peak current = 23.5 ± 2.3 min. compared to 4.5 ± 0.2 min. in mature PCs, $n=27$, $p < 0.001$, not shown), and the amplitude of the ID current was significantly smaller than those observed in older animals (peak= 4.0 ± 0.5 nA, $p < 0.001$, not shown). Recording from Purkinje cells in slices from animals considered to be developmentally analogous to newborn humans (6–7 day olds) (Vannucci et al., 1999; Vannucci and Vannucci, 2005) also showed qualitatively similar responses (Fig. 2B), but the time to onset of ID current is delayed by nearly 40 minutes (Time to peak = 42.9 ± 4.3 , $n=9$, $p < 0.001$ compared to mature PCs, Fig. 2B&C), and is only about 1/4th the magnitude of mature PCs (Peak= 2.8 ± 1.7 nA, I_{glu}= 2.5 ± 0.8 nA, $p < 0.001$, Fig 2B&D). These results suggest that the relative resistance of newborn brain tissue to ischemic damage stems in part from a dramatic delay in the time for excitotoxic glutamate to accumulate extracellularly. Another notable difference between newborn and mature PCs is that while glutamate receptor antagonists block all of the ischemia-induced current in newborn PCs (Fig. 2B), a portion of the ischemia-induced current (~1–2 nA) in mature PCs is not blocked by glutamate receptor antagonists (Fig. 2A). The residual current in mature PCs is not due to inadequate inhibition of glutamate receptors, as doubling the antagonist concentration did not produce any additional suppression ($n=4$, not shown).

Reduced temperature delays the onset of ischemic glutamate release

Cooling of the brain is a well documented protectant against ischemic brain damage particularly in newborns, but the mechanisms underlying this protection are not clear (Zhao et al., 2007). Reducing the temperature of the bathing solution from our standard recording temperature ($33 \pm 1^\circ\text{C}$) to $\sim 22^\circ\text{C}$ significantly delayed the onset of the ID current for both newborn (Time to peak= 86.9 ± 14.7 min., $n=8$, $p < 0.01$, Fig. 3B&C) and mature cerebellum (Time to peak= 11.6 ± 0.6 min., $n=20$, $p < 0.01$, Fig. 3A&C), but the delay was much greater for Purkinje cells in newborn cerebellum (Fig. 3A–C). In fact, 50% of newborn PCs did not generate detectable

glutamate current by 2 hours of ischemia at 22°C, at which point experiments were terminated due to diminished recording quality. Despite significant delays in the onset of glutamate accumulation, lowering the temperature did not affect the magnitude of the ID current (Fig. 3D). While cooling to 22°C is feasible in our brain slice studies, clinical cooling is currently more modest. To check that more modest changes in temperature also affect the timing of glutamate release we conducted field potential recordings (Fig. 3E), for which each recording is already the average of numerous cells, and thus makes it easier to detect small differences in timing. With this approach we determined the time to the “ischemic” terminal depolarization (which we have shown previously is driven by the large glutamate current, Hamann et al. 2005) for three different temperature groups: 29–31°C, 32–34°C, and 35–37°C, with corresponding times to terminal depolarization of 5.5 ± 0.21 min., 4.6 ± 0.3 , and 3.1 ± 0.3 respectively, each significantly different from the others (Fig. 3F). These results suggest that the protection afforded by lower brain temperature is mediated in part by delaying the timing but not magnitude of excitotoxic glutamate accumulation.

Smaller ID current in newborns is due to fewer receptors, not less glutamate

As shown in figure 2, in addition to being slower to develop, the glutamate receptor-mediated ID current in newborn PCs is significantly smaller than the ID current in mature PCs. The smaller amplitude must be due either to less glutamate accumulating around PCs, or to a smaller number of receptors or lower conductance channels responding to the same amount of glutamate. To differentiate between these two possibilities, we compared the amplitude of responses to exogenous glutamate receptor agonists, which are not affected by glutamate uptake, to the amplitude of the ID current in newborn and mature PCs (Fig. 4). To prevent transmitter release evoked by activation of glutamate receptors on cells other than the PC being recorded from, agonist applications were done in the presence of tetrodotoxin (TTX, 0.5 μ M). Responses of newborn PCs to the non-NMDA receptor agonist, AMPA (2 μ M), were about 1/4th the amplitude of responses of mature PCs (amplitude = 271 ± 36 pA and 1029 ± 150 pA respectively, $n=10$ and 6 , $p<0.01$, Fig. 4A&B). The difference in response amplitude to AMPA is very similar to the difference in amplitude of the ID current (Fig. 4C). Although adult PCs do not express significant numbers of functional NMDA receptors, functional NMDA receptors are transiently expressed by newborn PCs with peak expression levels occurring at about 3–4 days postnatal (Rosenmund et al., 1992). Since NMDA receptors have a higher affinity for glutamate than non-NMDA receptors, and they do not desensitize as rapidly, nor to the same degree, it is possible that the ID current we record in newborn PCs is generated by NMDA receptors responding to a lower concentration of glutamate. However, bath application of NMDA (100 μ M) did not generate significant current in either 6–7 day old or 18–21 day old PCs (Fig. 4A&B). Thus, the smaller amplitude ID current in newborn PCs is due to newborn PCs having a smaller number of, or smaller conductance non-NMDA glutamate receptors, responding to a similar amount of glutamate accumulating during ischemia. Indeed, scaling the amplitude of the newborn ID current by the amplitude ratio for AMPA-induced currents in mature and newborn PCs eliminated the difference in ID current amplitudes (Fig. 4C). As will be seen in later figures (Fig. 5&8), the surface area of newborn PCs is likely less than 1/4th of mature PCs, suggesting that despite the smaller number of glutamate receptors, once the ID current develops, the current density, and hence damage should be similar or greater.

Glutamate transporter density is similar in newborn and mature Purkinje cells

Previous immunological studies indicate that glutamate transporter (EAAT) expression increases during development (Ullensvang et al., 1997; Furuta et al., 1997). A lower density of EAAT expression in newborn PCs could explain the delay in ischemia induced glutamate accumulation, if, as pharmacological studies indicate, EAATs are the main source for glutamate release during ischemia. However, immunostaining for the dominant glial and neuronal glutamate transporters in the cerebellum (EAAT1 and EAAT4 respectively) in our

two age groups did not show any obvious differences in expression pattern or density (Fig. 5A, data typical of at least 3 lobes in each of 3 newborn slices and 5 adult slices). To more rigorously quantify glutamate transporter density around PCs we developed two electrophysiological assays. First, as we have done previously (Hamann et al., 2005; Rossi et al., 2000), we compared PC responses to glutamate and AMPA, a glutamate receptor agonist that is not transported by glutamate transporters. The ratio of the response to glutamate and AMPA should be constant from cell to cell unless there are differences in glutamate transporter density, which affect the concentration of glutamate but not AMPA that penetrates into the slice. In particular, the ratio of the magnitude of response to glutamate and AMPA will get smaller with increasing densities of glutamate transporters. In agreement with our immunocytochemical studies we found no significant differences for the response ratio in newborn and mature PCs (ratio = 1.01 ± 0.14 and 1.29 ± 0.24 respectively, $n=14$ and 9 , $p>0.05$, Fig. 5B). As a second quantitative assay we examined PC responses to exogenous glutamate with and without glutamate uptake blocked. The degree to which blocking glutamate uptake enhances the response to exogenous glutamate should be proportional to the density of glutamate transporters. Blocking glutamate uptake with the non-substrate broad-spectrum glutamate transporter antagonist, TBOA ($300\mu\text{M}$) enhanced the PC response to glutamate by a similar degree in newborn and mature cerebellum (TBOA current as percent of total current = 0.50 ± 0.07 and 0.47 ± 0.1 , $n=9$ and 12 respectively, $p>0.05$, Fig. 5C). Taken together these results suggest that there are no significant differences in the density or distribution pattern of glutamate transporters around newborn and mature PCs.

Delay in glutamate release reflects slower loss of ATP

Glutamate release by glutamate transporter reversal in ischemia is thought to be driven by the rundown in ionic gradients that occurs upon loss of ATP, and consequent inhibition of the $\text{Na}^+\text{-K}^+\text{-ATPase}$ (Rossi et al., 2000). Newborn brain has a lower metabolic rate than mature brain (Takahashi et al., 1999; Kinnala et al., 1996), and low temperature slows the metabolic rate (Zhao et al., 2007). Thus, it is possible that the delay in glutamate release in newborn brain and during lowered temperature reflects a slower rate of consumption of ATP. To test this possibility we used a Luciferin-Luciferase based ATP assay to quantify the concentration of ATP in cerebellar slices after different durations of simulated ischemia (Fig. 6). In mature cerebellar slices, ischemia induced a rapid loss of ATP, with concentrations falling below 10% of control values by 5 minutes ($n=6$, Fig. 6A). The rate of decline of ATP in newborn cerebellum was significantly slower, taking more than 1 hour to drop below 10% of control values ($n=11$, Fig. 6A).

Lowering the bath temperature to $\sim 22^\circ\text{C}$ also significantly slowed the rate of decline of ATP for both newborn and mature cerebellum ($n=7$ and 5 respectively, Fig. 6B). There were no significant differences in the rate of ATP loss between males and females at either age (Not shown). With the plots shown in Figure 6, we were able to estimate how far ATP levels had declined by the time that glutamate was released under the various conditions (Fig. 6 gray arrows and dashed lines). The degree to which ATP had declined at the time of glutamate release was between 10 and 20% of control values for both newborn and mature tissue at near body temperature, and for mature tissue at room temperature (Fig. 6A&B). We were unable to accurately estimate the level of ATP at the time of glutamate release for newborn tissue at room temperature because in many experiments glutamate was not released by two hours of ischemia, beyond which time diminished recording quality terminated the experiment. These results are consistent with the hypothesis that the delay in glutamate release in newborns and at lower temperatures is due to slower consumption of ATP, and hence a longer time to reach a critical ATP threshold (10–20%) for glutamate release.

Blocking the Na⁺-K⁺-ATPase reduces the difference in timing of glutamate release

If glutamate release during ischemia is due to glutamate transporter reversal consequent to inhibition of the Na⁺-K⁺-ATPase, and the delay in glutamate release in newborn cerebellum is due to the slower loss of ATP, then bypassing ATP loss, and directly inhibiting the Na⁺-K⁺-ATPase with ouabain should produce similar levels of glutamate release, but the difference in timing should be reduced. Bath application of ouabain (600μM) induced a glutamate receptor-mediated current in both newborn and mature PCs (Fig. 7A&B). For both age groups the magnitude of the ouabain-induced glutamate current was similar to the magnitude of the ischemia-induced glutamate current, but in both cases glutamate release occurred more rapidly with ouabain treatment than with ischemia (Fig. 7C). However, ouabain accelerated glutamate release (compared to ischemia) to a greater degree in newborns, resulting in the difference in timing of glutamate release for ouabain being 57% less than the difference in timing of glutamate release for ischemia (respectively 16 and 38 minutes slower for newborns). This result suggests that about half of the delay in glutamate release in newborn cerebellum is due to the slower rate of consumption of ATP.

Ischemic damage correlates with ATP loss and glutamate release, but blocking glutamate receptors does not protect PCs at either age

While it is well documented that newborn brain tissue is resistant to ischemic brain damage compared to mature tissue, the mechanisms underlying this resistance are not known. To determine if delayed loss of ATP and glutamate release affords protection to newborn PCs we developed an immunocytochemical and propidium iodide based cell death assay (see methods) to examine ischemic damage in newborn and mature cerebellum (Fig. 8&9). Exposing mature cerebellar slices to simulated ischemia for 10 minutes followed by 2 hours recovery essentially obliterated the PCs, as evidenced by complete loss of calbindin staining, and yet no visible propidium iodide staining (Fig. 8A&9). In contrast, 10 minutes of ischemia did not cause any detectable necrosis in newborn PCs (Fig. 8C&9). Exposing newborn cerebellar slices to simulated ischemia for 1 hour, a duration long enough to evoke glutamate release (Fig. 2), caused widespread PC necrosis (Fig. 8D&9). Similar to the protection provided by young age, lowering the temperature significantly reduced PC damage induced by 10 minutes of simulated ischemia in mature slices (Fig. 8B&9). Gender did not affect PC damage, with 10 minutes exposure to simulated ischemia causing severe PC damage in both male and female slices (data not shown, Mean Calbindin staining intensity after 10 minutes ischemia simulation = 20.45 ±15.2 and 9.53±1.3 for n= 7 male and 3 female slices respectively, P>0.05).

Surprisingly, applying glutamate receptor antagonists (NBQX 25μM + AP5 50μM) did not prevent either newborn or mature PC death (Fig. 8A,D&9), although it did appear to slow the damage process in mature PCs as evidenced by a significant increase in Calbindin staining (Fig. 9). These results suggest that the protection afforded to PCs by young age and lowered temperature is mediated by the delayed loss of ATP, but for both newborn and mature PCs, once ATP levels decline to below 20% of control levels, in addition to massive glutamate release, an unidentified non-glutamatergic mechanism is also triggered that is sufficient to kill PCs even when glutamate receptors are antagonized.

Discussion

The principle findings of this study are that young age and lowered temperature, but not female gender dramatically slow the loss of cellular ATP, and delay the time to release of excitotoxic glutamate during cerebellar ischemia. The age- and temperature-related delay in ischemic glutamate release is likely due in part to the slower loss of ATP, because for both ages, glutamate release occurred at a time point when ATP had fallen to a similar degree, ~15% of pre-ischemic levels (Fig. 6). Furthermore, when glutamate release was induced by directly

inhibiting the Na⁺-K⁺-ATPase, thereby bypassing loss of ATP, the difference in timing of glutamate release was reduced to less than half of the difference in timing that occurs during ischemia (Fig. 7).

We found that newborn and mature Purkinje cells have a similar density of glutamate receptors, and are surrounded by a similar density of glutamate transporters (Fig. 4&5). Accordingly, during ischemia, once the apparent threshold of ATP loss occurs, a similar level of glutamate is released, and a similar density of glutamate-gated current is generated in PCs at both ages (Fig. 4C), and at different temperatures (Fig. 3D). Finally, we found that 10 minutes of ischemia, a duration that causes glutamate release in mature but not newborn cerebellum (Fig. 2), caused massive necrotic damage of PCs in mature cerebellum, but no discernible damage in slices from newborns (Fig. 8&9). Prolonging the duration of ischemia in newborn cerebellum to 1 hour, a period of time sufficient to evoke glutamate release (Fig. 2), did cause massive PC necrosis (Fig. 8C&9). Lowering the temperature to 22°C also delayed ATP loss and glutamate release, and significantly reduced PC damage induced by simulated ischemia (Fig. 8&9). Despite the correlation between the timing of glutamate release and induction of PC damage, blocking glutamate receptors failed to prevent PC necrosis at either age (Fig. 8&9), indicating the existence of a non-glutamatergic damage pathway.

Mechanisms of Purkinje cell ischemic damage

Cerebellar Purkinje cells (PCs) are one of the most ischemia-sensitive cells in the brain, being amongst the few cell types to die in response to short episodes of global ischemia, such as occurs during cardiac arrest (Pulsinelli, 1985). The cerebellum is also a frequent site of stroke, and cerebellar stroke is particularly lethal due to consequent swelling-induced compression of the underlying brain stem (Kelly et al., 2001). Despite the extreme sensitivity, frequent involvement and potentially lethal response of PCs to brain ischemia, relatively little is known about how PCs are damaged by ischemia. PCs are unusual in that they do not express significant levels of functional NMDA receptors (Hausser and Roth, 1997; Llano et al., 1991; Rosenmund et al., 1992), which, because of their relatively high permeability to Ca²⁺, are typically a primary source of damaging Ca²⁺ entry during ischemia. Our previous *in vitro* study of young cerebellar slices indicated that activation of desensitization-resistant AMPA receptors drives the terminal ischemic depolarization (Hamann et al., 2005) that in other brain regions is associated with pan-necrosis (Higuchi et al., 2002), and another *in vitro* study examining young cerebellar slices showed that blocking non-NMDA receptors protected PCs from damage induced by 30 minutes of hypoxia (Barenberg et al., 2001). In contrast, several *in vivo* studies of global ischemia in adult rats found that blocking non-NMDA ionotropic glutamate receptors provided only limited protection of mature PCs, ~20% (Balchen and Diemer, 1992; Brasko et al., 1995). This disparity could reflect developmental changes (the slice studies used slices from animals not as fully matured as the *in vivo* studies), but could be due to inadequate block of glutamate receptors with *in vivo* models (it is difficult to ensure adequate antagonism *in vivo*), or may reflect the post-ischemic application protocol used in those *in vivo* studies, which may not be as effective as blocking glutamate receptors during the ischemic episode. Alternatively, the differing outcome may relate to differences in the severity of the insult used in the two studies (hypoxia versus ischemia), with glutamate receptor antagonists providing better protection against hypoxia than ischemia.

Here, by directly comparing newborn and mature tissue (albeit not fully adult) under the same experimental conditions, with well controlled pharmacology, we demonstrate that blocking ionotropic glutamate receptors does not protect either young or old PCs from simulated ischemia (Fig. 8&9). While it is likely that the ischemia-induced massive glutamate-gated current would damage both newborn and adult PCs, our data indicate that an as yet unidentified non-glutamatergic damage mechanism develops in parallel with glutamate release, and that it

alone is capable of causing wide spread PC necrosis (Fig. 8&9). The existence of such a parallel damage pathway may explain why PCs are so sensitive to ischemia despite lacking NMDA receptors, and identifying the underlying mechanism(s) should provide novel therapeutic targets.

In mature PCs, the non-ionotropic glutamatergic damage pathway may be related to the ischemia-induced membrane current that persists in adult but not newborn PCs when glutamate receptors are blocked (Fig. 2A&B). We have tested and ruled out (data not shown) several of the more obvious possible candidate mechanisms including metabotropic glutamate receptor-gated channels, P2X receptor-gated channels, gap junction hemichannels, TRP channels and ASIC channels, all of which can generate substantial membrane currents, are permeable to Ca^{2+} , and have been implicated in ischemic damage in other brain regions (Thompson et al., 2006; Xiong et al., 2004; Lammer et al., 2006; Rossi et al., 2007). Since none of these obvious candidate mechanisms generates the residual non-ionotropic glutamate receptor current, we are currently characterizing its biophysical properties to guide future attempts at identifying its molecular underpinnings.

In contrast to mature PCs, since ionotropic glutamate receptor antagonist block all ischemia-induced currents in newborn PCs (Fig. 2B), the non-ionotropic glutamatergic damage pathway in newborn PCs must not be electrogenic. Consequently, future studies employing Ca^{2+} imaging combined with electrophysiology will be required to identify how newborn PCs are damaged by ischemia.

The role of glutamate transporters in ischemic glutamate release

In our previous *in vitro* studies we determined that ischemic glutamate release onto PCs is reduced by 90% by inhibiting glutamate transporter reversal (Hamann et al., 2005), but *in vivo* studies suggest that both glial and neuronal glutamate transporters protect PCs from ischemic damage (Welsh et al., 2002; Yamashita et al., 2006). These observations present a dilemma: if ischemic glutamate release contributes to PC damage, and glutamate is released primarily by glutamate transporter reversal, how do glutamate transporters protect PCs from ischemic damage? A possible reconciliation comes from our observation that applying glutamate receptor antagonists during the ischemic episode does not prevent ischemic damage (Fig. 8&9), presumably because the potential damage induced by glutamate release during ischemia is redundant with additional parallel damage mechanisms. Thus, preventing glutamate release by transporter reversal would also not afford protection during ischemia. Why then do glutamate transporters protect PCs from ischemic damage *in vivo*? A possible explanation proposed by Welsh et al. (2002) is that the excitotoxic damage that glutamate transporters defend against occurs in the post-ischemic period due to excessive glutamatergic synaptic activity. Indeed, both *in vivo* ischemia studies that demonstrated PC protection with a non-NMDA glutamate receptor antagonist, applied the antagonist in the post-ischemic period (Balchen and Diemer, 1992; Brasko et al., 1995).

Another complication concerning glutamate transporters in ischemic glutamate release stems from our previous finding that for the first 10 minutes of ischemia, removal of extracellular Ca^{2+} reduces ischemic glutamate release onto PCs by about 70% (Hamann et al., 2005). The superadditive block of glutamate release suggests some interaction between removing external Ca^{2+} and blocking glutamate transporter reversal, but no such mechanism is known. Our finding that reducing energy consumption delays the ischemia-induced release of glutamate, suggests a possible explanation for the interaction between removal of external Ca^{2+} and glutamate transporter reversal (Hamann et al., 2005). Specifically, since 1/3rd of the energy consumed by neural processing is thought to be due to vesicular release of glutamate (Attwell and Laughlin, 2001), it is expected that the massive increase in sEPSCs that occurs early during ischemia (Fig 1A) should accelerate consumption of ATP. Thus, removal of external Ca^{2+}

could, by reducing early vesicle release, conserve energy, thereby delaying or reducing the subsequent release of glutamate by transporter reversal.

Mechanisms of neuroprotection

Animal studies indicate that females are more resistant to ischemic damage than males, although the underlying mechanisms are not fully understood (Murphy et al., 2004; Alkayed et al., 1998; Payan and Conrad, 1977). Here we show that gender does not affect ischemia-induced loss of ATP, glutamate release, or PC glutamate current (Fig. 1). Thus, protection afforded by female gender either requires acute presence of female hormones, is mediated by vascular effects, or is downstream of glutamate receptor activation.

Although brain ischemia is a major cause of brain damage in newborns, compared to adult brain, the newborn brain is remarkably resistant to ischemic damage (Towfighi et al., 1997; Vannucci and Hagberg, 2004). Our data shows that a main mechanism of this resistance is energy conservation, resulting in delayed loss of ATP, with a corresponding delay in glutamate release and non-glutamatergic damage pathways (Fig. 2,6&8). Similar to young age, lowered temperature delayed the loss of ATP and release of glutamate (Fig. 3&6). Importantly, with lowered temperature, no other PC currents developed in the absence of glutamate release, suggesting that energy conservation prevents other non-glutamatergic processes from developing as well. Accordingly, as for young age, lowered temperature protected Purkinje cells from damage induced by simulated ischemia (Fig. 8&9). The obvious benefit of preventing multiple parallel damage pathways may be why other naturally evolved ischemia resistance strategies involve energy conservation, including ischemic preconditioning, hibernation and anoxia resistance (Stenzel-Poore et al., 2007; Stenzel-Poore et al., 2003; Storey and Storey, 2007).

Conclusion

The primary mechanism by which young age and lowered temperature protects PCs from ischemic damage is by delaying the loss of ATP, which delays the onset of multiple parallel damage mechanisms, including release of glutamate. Therapeutic approaches aimed at reducing energy consumption during brain ischemia should therefore provide protection against cerebellar ischemic damage in both newborns and adults, and may do so with fewer side effects than blocking glutamate receptors. However, despite their hypometabolic state, even newborn PCs are killed by one hour of ischemia. It would therefore also be useful to identify the apparent non-glutamatergic damage mechanisms, which could be targeted in combination with glutamate receptors for more efficacious protection during cerebellar ischemia, or might be targeted alone with fewer side effects than glutamate receptor antagonists.

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Reference List

1. Alkayed NJ, Harukuni I, Kimes AS, London ED, Traystman RJ, Hurn PD. Gender-linked brain injury in experimental stroke. *Stroke* 1998;29:159–165. [PubMed: 9445346]
2. Amarenco P. The spectrum of cerebellar infarctions. *Neurology* 1991;41:973–979. [PubMed: 2067660]
3. Attwell D, Laughlin SB. An energy budget for signaling in the grey matter of the brain. *J Cereb Blood Flow Metab* 2001;21:1133–1145. [PubMed: 11598490]

4. Balchen T, Diemer NH. The AMPA antagonist, NBQX, protects against ischemia-induced loss of cerebellar Purkinje cells. *Neuroreport* 1992;3:785–788. [PubMed: 1384770]
5. Barenberg P, Strahlendorf H, Strahlendorf J. Hypoxia induces an excitotoxic-type of dark cell degeneration in cerebellar Purkinje neurons. *Neurosci Res* 2001;40:245–254. [PubMed: 11448516]
6. Brasko J, Rai P, Sabol MK, Patrikios P, Ross DT. The AMPA antagonist NBQX provides partial protection of rat cerebellar Purkinje cells after cardiac arrest and resuscitation. *Brain Res* 1995;699:133–138. [PubMed: 8616603]
7. Choi DW, Maulucci-Gedde M, Kriegstein AR. Glutamate neurotoxicity in cortical cell culture. *J Neurosci* 1987;7:357–368. [PubMed: 2880937]
8. Choi DW, Rothman SM. The role of glutamate neurotoxicity in hypoxic-ischemic neuronal death. *Annu Rev Neurosci* 1990;13:171–182. [PubMed: 1970230]
9. Danbolt NC. Glutamate uptake. *Prog Neurobiol* 2001;65:1–105. [PubMed: 11369436]
10. Furuta A, Rothstein JD, Martin LJ. Glutamate transporter protein subtypes are expressed differentially during rat CNS development. *J Neurosci* 1997;17:8363–8375. [PubMed: 9334410]
11. 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]
12. 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]
13. 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]
14. Higuchi T, Takeda Y, Hashimoto M, Nagano O, Hirakawa M. Dynamic changes in cortical NADH fluorescence and direct current potential in rat focal ischemia: relationship between propagation of recurrent depolarization and growth of the ischemic core. *J Cereb Blood Flow Metab* 2002;22:71–79. [PubMed: 11807396]
15. 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]
16. Kinnala A, Suhonen-Polvi H, Aarimaa T, Kero P, Korvenranta H, Ruotsalainen U, Bergman J, Haaparanta M, Solin O, Nuutila P, Wegelius U. Cerebral metabolic rate for glucose during the first six months of life: an FDG positron emission tomography study. *Arch Dis Child Fetal Neonatal Ed* 1996;74:F153–F157. [PubMed: 8777676]
17. Lammer A, Gunther A, Beck A, Krugel U, Kittner H, Schneider D, Illes P, Franke H. Neuroprotective effects of the P2 receptor antagonist PPADS on focal cerebral ischaemia-induced injury in rats. *Eur J Neurosci* 2006;23:2824–2828. [PubMed: 16817887]
18. Lipton P. Ischemic cell death in brain neurons. *Physiol Rev* 1999;79:1431–1568. [PubMed: 10508238]
19. 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]
20. Mercuri E, He J, Curati WL, Dubowitz LM, Cowan FM, Bydder GM. Cerebellar infarction and atrophy in infants and children with a history of premature birth. *Pediatr Radiol* 1997;27:139–143. [PubMed: 9028846]
21. Murphy SJ, McCullough LD, Smith JM. Stroke in the female: role of biological sex and estrogen. *ILAR J* 2004;45:147–159. [PubMed: 15111734]
22. Northington FJ, Ferriero DM, Graham EM, Traystman RJ, Martin LJ. Early Neurodegeneration after Hypoxia-Ischemia in Neonatal Rat Is Necrosis while Delayed Neuronal Death Is Apoptosis. *Neurobiol Dis* 2001;8:207–219. [PubMed: 11300718]
23. Payan HM, Conrad JR. Carotid ligation in gerbils. Influence of age, sex, and gonads. *Stroke* 1977;8:194–196. [PubMed: 847784]
24. Perez Velazquez JL, Frantseva MV, Carlen PL. In vitro ischemia promotes glutamate-mediated free radical generation and intracellular calcium accumulation in hippocampal pyramidal neurons. *J Neurosci* 1997;17:9085–9094. [PubMed: 9364055]
25. Phillis JW, Ren J, O'Regan MH. Transporter reversal as a mechanism of glutamate release from the ischemic rat cerebral cortex: studies with DL-threo-beta- benzoyloxyaspartate. *Brain Res* 2000;868:105–112. [PubMed: 10841893]

26. Pulsinelli WA. Selective neuronal vulnerability: morphological and molecular characteristics. *Prog Brain Res* 1985;63:29–37. [PubMed: 2872695]
27. Rosenmund C, Legendre P, Westbrook GL. Expression of NMDA channels on cerebellar Purkinje cells acutely dissociated from newborn rats. *J Neurophysiol* 1992;68:1901–1905. [PubMed: 1282541]
28. Rossi DJ, Brady JD, Mohr C. Astrocyte metabolism and signaling during brain ischemia. *Nat Neurosci* 2007;10:1377–1386. [PubMed: 17965658]
29. Rossi DJ, Hamann M. Spillover-mediated transmission at inhibitory synapses promoted by high affinity $\alpha 6$ subunit GABA(A) receptors and glomerular geometry. *Neuron* 1998;20:783–795. [PubMed: 9581769]
30. Rossi DJ, Oshima T, Attwell D. Glutamate release in severe brain ischaemia is mainly by reversed uptake. *Nature* 2000;403:316–321. [PubMed: 10659851]
31. Rossi DJ, Slater NT. The developmental onset of NMDA receptor-channel activity during neuronal migration. *Neuropharmacology* 1993;32:1239–1248. [PubMed: 7509049]
32. Stenzel-Poore MP, Stevens SL, King JS, Simon RP. Preconditioning reprograms the response to ischemic injury and primes the emergence of unique endogenous neuroprotective phenotypes: a speculative synthesis. *Stroke* 2007;38:680–685. [PubMed: 17261715]
33. Stenzel-Poore MP, Stevens SL, Xiong Z, Lessov NS, Harrington CA, Mori M, Meller R, Rosenzweig HL, Tobar E, Shaw TE, Chu X, Simon RP. Effect of ischaemic preconditioning on genomic response to cerebral ischaemia: similarity to neuroprotective strategies in hibernation and hypoxia-tolerant states. *Lancet* 2003;362:1028–1037. [PubMed: 14522533]
34. Storey KB, Storey JM. Tribute to P. L. Lutz: putting life on ‘pause’--molecular regulation of hypometabolism. *J Exp Biol* 2007;210:1700–1714. [PubMed: 17488933]
35. Takahashi T, Shirane R, Sato S, Yoshimoto T. Developmental changes of cerebral blood flow and oxygen metabolism in children. *AJNR Am J Neuroradiol* 1999;20:917–922. [PubMed: 10369366]
36. Thompson RJ, Zhou N, MacVicar BA. Ischemia opens neuronal gap junction hemichannels. *Science* 2006;312:924–927. [PubMed: 16690868]
37. Towfighi J, Mauger D, Vannucci RC, Vannucci SJ. Influence of age on the cerebral lesions in an immature rat model of cerebral hypoxia-ischemia: a light microscopic study. *Brain Res Dev Brain Res* 1997;100:149–160.
38. Ullensvang K, Lehre KP, Storm-Mathisen J, Danbolt NC. Differential developmental expression of the two rat brain glutamate transporter proteins GLAST and GLT. *Eur J Neurosci* 1997;9:1646–1655. [PubMed: 9283819]
39. Vannucci RC, Connor JR, Mauger DT, Palmer C, Smith MB, Towfighi J, Vannucci SJ. Rat model of perinatal hypoxic-ischemic brain damage. *J Neurosci Res* 1999;55:158–163. [PubMed: 9972818]
40. Vannucci RC, Vannucci SJ. Perinatal hypoxic-ischemic brain damage: evolution of an animal model. *Dev Neurosci* 2005;27:81–86. [PubMed: 16046840]
41. Vannucci SJ, Hagberg H. Hypoxia-ischemia in the immature brain. *J Exp Biol* 2004;207:3149–3154. [PubMed: 15299036]
42. Welsh JP, Yuen G, Placantonakis DG, Vu TQ, Haiss F, O’Hearn E, Molliver ME, Aicher SA. Why do Purkinje cells die so easily after global brain ischemia? Aldolase C, EAAT4, and the cerebellar contribution to posthypoxic myoclonus. *Adv Neurol* 2002;89:331–359. [PubMed: 11968459]
43. Xiong ZG, Zhu XM, Chu XP, Minami M, Hey J, Wei WL, MacDonald JF, Wemmie JA, Price MP, Welsh MJ, Simon RP. Neuroprotection in ischemia: blocking calcium-permeable acid-sensing ion channels. *Cell* 2004;118:687–698. [PubMed: 15369669]
44. Yamashita A, Makita K, Kuroiwa T, Tanaka K. Glutamate transporters GLAST and EAAT4 regulate postischemic Purkinje cell death: an in vivo study using a cardiac arrest model in mice lacking GLAST or EAAT4. *Neurosci Res* 2006;55:264–270. [PubMed: 16647773]
45. Zhang Y, Lipton P. Cytosolic Ca^{2+} changes during in vitro ischemia in rat hippocampal slices: major roles for glutamate and Na^{+} -dependent Ca^{2+} release from mitochondria. *J Neurosci* 1999;19:3307–3315. [PubMed: 10212290]
46. Zhao H, Steinberg GK, Sapolsky RM. General versus specific actions of mild-moderate hypothermia in attenuating cerebral ischemic damage. *J Cereb Blood Flow Metab* 2007;27:1879–1894. [PubMed: 17684517]

Figure 1.

Gender does not affect ischemia-induced glutamate release. **A**, Representative response of a voltage-clamped ($V_h = -60\text{mV}$) female PC to simulated ischemia. **B**, Mean peak amplitude of ischemia-induced current and mean amplitude of current blocked by glutamate receptor antagonists (NBQX $25\mu\text{M}$ + AP5 $50\mu\text{M}$), as in **A**, in slices of cerebellum from female (black) and male (white) rats. **C**, Mean time to peak of ischemia-induced current.

Figure 2. Ischemia-induced glutamate current is delayed and smaller in newborn PCs. **A**, Representative response of a voltage-clamped ($V_h = -60\text{mV}$) mature (18–21 day old) PC. Note dotted gray line extension of baseline current highlighting residual non-glutamatergic current induced by ischemia. **B**, Representative response of a newborn (6–7 day old) PC ($V_h = -60\text{mV}$). Note dotted gray line extension of baseline current, highlighting complete block of ischemia-induced current. **C**, Mean time to onset and peak of ischemia-induced current in mature (black) and newborn (white) PCs. **D**, Mean peak amplitude and I_{glu} in mature (black) and newborn (white) PCs.

Figure 3.

Low temperature delays the onset of ischemia-induced glutamate release. **A**, Representative response of a mature PC ($V_h = -60\text{mV}$) at near body temperature (32–34°C, top) and at room temperature (22–23°C, bottom). **B**, Representative response of a newborn PC ($V_h = -60\text{mV}$) at near body temperature (top) and at room temperature (bottom). **C**, Mean time to peak amplitude for mature and newborn PCs at near body (black) and room (white) temperature. **D**, Mean peak amplitude and Iglu for mature and newborn PCs at near body (black) and room (white) temperature. **E**, Representative field potential recording in the dendritic field of the Purkinje cells, shows the terminal depolarization that is driven by the large glutamate-mediated current. **F**, Mean time to peak for the terminal depolarization, as in E, for different temperatures.

Figure 4.

Fewer AMPA receptors on newborn PCs account for smaller ischemia-induced current. **A**, representative responses of mature and newborn PCs ($V_h = -60$ mV) to NMDA (100 μ M) and AMPA (2 μ M). **B**, Mean amplitude of response to AMPA and NMDA (as in A) for mature (black) and newborn (white) PCs. **C**, Mean amplitude of ischemia-induced glutamate current (Iglu from Fig. 2D) for mature and newborn PCs. **D**, Mean ischemia-induced Iglu replotted with values for newborn PCs scaled by the ratio of responses to AMPA (determined from data shown in Fig. 4b).

Figure 5.

Newborn and mature PCs are surrounded by similar densities of glutamate transporters. **A**, 3-D projections of confocally acquired images of immunocytochemistry for dominant neuronal glutamate transporter EAAT4 (green) and dominant glial glutamate transporter GLAST/EAAT1 (red). **B**, Mean amplitude of mature and newborn PC response to glutamate (120μM) and AMPA (2μM), and the mean ratio $I(\text{glu})/I(\text{AMPA})$ of those response amplitudes in each cell. **C**, Representative responses of mature and newborn PCs ($V_h = -60\text{mV}$) to glutamate (2μM), followed by the broad-spectrum glutamate transporter antagonist, TBOA (300μM), and then the AMPA receptor antagonist, NBQX (25μM). Mean value of TBOA-induced current as a percent of total current is plotted in the bar chart (right).

Figure 6.

Young age and lowered temperature slow the rate of ischemia-induced loss of ATP. **A**, Plot of [ATP] as a percent of controls versus time under control conditions or ischemia for newborn and mature cerebellar slices at near body temperature (32–34°C). **B**, Same as in A, except at room temperature (22–23°C). In both A&B, the gray arrows point to the average time at which the glutamate-mediated ID current occurs (from Fig. 2C), and the gray dashed line indicates the estimated [ATP] at that time point.

Figure 7.

Difference in timing of glutamate release is smaller for ouabain than ischemia. **A**, Representative response of a mature PC ($V_h = -60\text{mV}$) to ouabain ($600\mu\text{M}$). **B**, Representative response of a newborn PC ($V_h = -60\text{mV}$) to ouabain. **C**, Mean time to peak current induced by ischemia or ouabain for mature (black) and newborn (white) PCs.

Figure 8.

Duration of ischemia required to kill PCs parallels loss of ATP and glutamate release, but damage is not prevented by glutamate receptor antagonists. **A–D**, 3-D projections of confocally acquired images of propidium iodide (red) and immunocytochemistry for calbindin (green), for mature (**A&B**) or newborn (**C&D**) cerebellum under control conditions (**Ai, Bi, Ci & Di**) or after ischemia for 10 min. (**Aii,iii, Bii & Cii**) or 60 min. (**Dii&iii**), either alone (**Aii, Bii, Cii & Dii**) or with (**Aiii & Diii**) glutamate receptor antagonists (NBQX 25μM + AP5 50μM). Scale bar in Aiii is 50μm and applies to all images.

Figure 9.

Young age and low temperature, but not glutamate receptor antagonists protect Purkinje cells against simulated ischemia. Mean intensity of Calbindin signal for PC and molecular layer for the various conditions shown in figure 8. Values are from at least 3 lobules in 3 separate slices from at least 2 different animals. At least 1 slice (typically 2) from each animal was treated to all possible conditions. Note, block = 25 μ M NBQX + 50 μ M AP5.