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Contribution of astrocyte glycogen stores to progression of spreading depression and related events in hippocampal slices

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Abstract

Spreading depression (SD) is a wave of coordinated cellular depolarization that propagates slowly throughout brain tissue. SD has been associated with migraine aura, and related events have been implicated in the enlargement of some brain injuries. Selective disruption of astrocyte oxidative metabolism has previously been shown to increase the propagation rate of SD *in vivo*, but it is currently unknown whether astrocyte glycogen stores make significant contributions to the onset or propagation of SD. We examined SD in acutely-prepared murine hippocampal slices, using either localized microinjections of KCl or oxygen and glucose deprivation (OGD) as stimuli. A combination of glycogenolysis inhibitors 1,4-dideoxy-1,4-imino-D-arabinitol (DAB) and 1-deoxynojirimycin (DNJ) increased the propagation rates of both high K⁺-SD and OGD-SD. Consistent with these observations, exposure to L-methionine-DL-sulfoximine (MSO) increased slice glycogen levels and decreased OGD-SD propagation rates. Effects of glycogen depletion were matched by selective inhibition of astrocyte TCA cycle activity by fluoroacetate (FA). Prolonged exposure to reduced extracellular glucose (2 mM) has been suggested to deplete slice glycogen stores, but significant modification SD of propagation rate was not observed with this treatment. Furthermore, decreases in OGD-SD latency with this pre-exposure paradigm appeared to be due to depletion of glucose, rather than glycogen availability. These results suggest that astrocyte glycogen stores contribute to delaying the advancing wavefront of SD, including during the severe metabolic challenge of OGD. Approaches to enhance astrocyte glycogen reserves could be beneficial for delaying or preventing SD in some pathologic conditions.

Keywords

spreading depression; glycogen; astrocyte; hippocampal slice; ischemia

1. INTRODUCTION

Spreading depression (SD) is characterized by a wave of severe depolarization that can propagate throughout CNS tissue and result in suppression of electrical activity and disruption of ion homeostasis. These events can provide repetitive large metabolic burdens to both neurons and astrocytes (Hansen, 1985; Somjen, 2001). If SD is generated in otherwise healthy tissue, neuronal ionic gradients and brain function can be fully restored

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after SD (Nedergaard and Hansen, 1988), and there is evidence that such recoverable events are related to migraine aura (Okada et al., 1988; Hadjikhani et al., 2001). However, SD triggered in the context of ischemia can result in irrecoverable injury if metabolic substrates are not resupplied immediately following SD onset (Rader and Lanthorn, 1989; Tanaka et al., 1997), in both animal models (Nedergaard and Astrup, 1986; Hossmann, 1996; Hartings et al., 2003) and in human trauma and stroke patients (Fabricius et al., 2006; Dohmen et al., 2008). Therefore, inhibition of SD-like events following ischemia is being considered a potential therapeutic approach to limit injury progression (Lauritzen et al., 2011). Much work to date has concentrated on targeting neurons to limit these spreading depolarizations, but astrocytes may be a useful additional target.

Although astrocytes may not be the primary direct contributors to generation of SD events (Zhou et al., 2010), pharmacological evidence supports a significant role in regulating SD progression. Selective disruption of astrocyte oxidative metabolism with fluorocitrate (FC) has been shown to increase the rate of SD propagation in rat cortex *in vivo* (Largo et al., 1997). In rat brain slices, the related gliotoxin fluoroacetate (FA) accelerated onset of SD generated by hypoxia (Muller and Somjen, 1999), disrupted prodromal oscillations prior to SD (Larrosa et al., 2006), and with extended exposures, was sufficient to initiate SD under some experimental conditions (Canals et al., 2008).

The aim of the present study was to determine the role of astrocytic glycogen stores in the regulation of SD-like events studied in brain slices. Astrocytes are the primary source of glycogen in the brain (Cataldo and Broadwell, 1986; Wender et al., 2000; Brown, 2004). Glycogen stores are known to be depleted *following* SD events (Selman et al., 2004) and inhibition of glycolysis or glycogenolysis results in decreased glutamate uptake by cultured astrocytes (Swanson, 1992; Sickmann et al., 2009), but the possible contributions of astrocyte glycogen stores in the initiation and/or propagation of SD are currently unclear. A previous study suggested that the latency to single cell anoxic depolarizations in rat hippocampal slices was determined by depletion of astrocytic glycogen (Allen et al., 2005). However, it is not yet known 1) whether the glucose depletion approach used previously did in fact deplete glycogen stores, 2) whether the delay in the onset of a single neuron anoxic depolarization also applies to SD initiation, and 3) whether initiation and/or propagation of coordinated waves of SD are significantly influenced by astrocyte glycogen stores.

In the present study, we have examined these questions by studying SD-like events in murine hippocampal slices. SD-like events were generated either by oxygen glucose deprivation (OGD) or by localized high K^+ stimuli, and astrocyte metabolism was disrupted by using FA, putative inhibitors of glycogen metabolism, or the glucose depletion approach previously suggested to deplete glucose/glycogen stores prior to SD onset. We conclude that availability of astrocyte glycogen stores can modify the latency to SD onset generated in ischemia-like conditions, but that lack of availability of glucose (rather than glycogen) likely explains the effects of low glucose pre-exposure strategies in our preparations. SD propagation rates appear to be significantly regulated by glycogen availability, likely by reducing the rate of extracellular K^+ and/or glutamate accumulation within astrocytes at the advancing wave front of SD generated in both normoxic and ischemic-like conditions.

2. EXPERIMENTAL PROCEDURES

2.1 Slice preparation

Male mice (FVB\N) were obtained from Harlan Laboratories (Indianapolis, IN) at 4-6 weeks of age and were housed in standard conditions (12 hr light/dark cycle) for up to 2 weeks prior to euthanasia. Mice were deeply anesthetized with a mixture of ketamine and xylazine (85 and 15 mg/ml, respectively, s.c.) and decapitated. Brains were rapidly removed and

placed in ice-cold cutting solution (see below for composition). Coronal sections (250 μm) were cut on a Vibratome (Technical Products International, St. Louis, MO) and slices were subsequently transferred to oxygenated room temperature ACSF (see below). Cutting and recording solutions were both 300-305 mOsm/l. After warming to 34°C for one hour, the ACSF was exchanged again and slices were then held at room-temperature. Individual slices were then transferred to a recording chamber and superfused with oxygenated ACSF at 2 ml/min at 35°C.

2.2 Electrical Recording

Extracellular measurements of slow DC shifts characteristic of SD were made using borosilicate glass microelectrodes, filled with ACSF (~5 M Ω) and placed in stratum radiatum ~45 μm below the surface of the slice and approximately 150 μm from the pyramidal cell body layer. In some experiments, Schaffer collateral inputs to the CA1 region were stimulated using a bipolar electrode (25 μm tip) placed on the surface of stratum radiatum. Single shocks (80 μs , 0.1-1.5 mA) were applied using a constant-current stimulus isolation unit (Isoflex, AMPI, Israel). Stimulus intensity was chosen based on an input/output curve generated in each slice, to produce responses ~60% of maximal amplitude (0.4-0.55 mA). Signals were amplified (Neurodata IR-283), digitized (Digidata 1322A, Axon Instruments, Union City, CA) and then acquired using Axoscope software (v 8.1, Axon Instruments).

2.3 Autofluorescence measurements

NAD(P)H autofluorescence was used to assess the inhibition of slice mitochondrial function during OGD exposures, and also to track the progression of high K^+ -SD and OGD-SD. This was performed as previously described (Shuttleworth et al., 2003) with minor modifications. In most experiments, 360 nm excitation was delivered via a fiber optic/monochromator system (Polychrome IV; Till Photonics, Grafelfing, Germany) and reflected onto the slice surface using a dichroic mirror (DMLP 400 nm, Chroma Technology, Brattleboro, VT). Fluorescence emission (>410 nm) was collected with a cooled interline transfer CCD camera (IMAGO, Till Photonics). Image data was background-subtracted to account for camera noise, and presented as the changes in fluorescence intensity/prestimulus fluorescence intensity ($\Delta F/F_0$) from stratum radiatum. All imaging was performed after focusing onto the surface of the slice with a 10X water immersion objective (NA 0.3, Olympus) and fluorescence collected after 2×2 binning of the 640×480 line image. Single images or image pairs were acquired every 2.5 s.

2.4 Tissue glycogen levels

Glycogen measurements were performed following previously-described methods (Cruz and Dienel, 2002). Isolated hippocampal slices were allowed to recover in normal artificial cerebral spinal fluid (nACSF) for at least 4 hours following slicing, to allow glycogen levels recover (Lipton, 1989) prior to different exposure paradigms. Tissues were flash-frozen and added to ice-cold 0.3 ml 65% ethanol/phosphate-buffered saline solution and then homogenized and centrifuged at 9000g for 10 minutes. The supernatant was discarded, and the extraction procedure repeated 4-5 more times, before pellets were freeze dried. Samples were acidified (0.4 ml 0.03N HCl), boiled (45 min), and stored at 4°C. Glycogen levels were determined indirectly, after hydrolysis to glucose. Samples were acidified (final concentration of 0.1 M acetate), divided and incubated (2 hours, 37°C) in either the presence or absence of amylo- α -1,4- α -1,6-glucosidase (30 $\mu\text{g}/\text{ml}$). Samples were centrifuged to remove particulate matter and glucose levels were analyzed by fluorometry (hexokinase/glucose-6-phosphate dehydrogenase procedure, and responses compared with a glucose standard curve. Tissue levels were within the linear detection range of the assay and an approach expected to deplete tissue glycogen (20min OGD, see (Lipton, 1989)) abolished

glycogen signals in slices (-1.18 ± 0.13 nmol/mg protein, $n=2$, $p<.0001$) when compared with controls (11.59 ± 0.53 nmol/mg protein, $n=7$). We used the rough estimate of 150 mg protein per 1 g wet tissue wt to compare our measurement to published work, and found that while our values were lower (~ 2 μ mol/g wet wt vs. 12.5-5.4 μ mol/g wet wt (Cruz and Dienel, 2002)), they were within an order of magnitude of previous work utilizing larger volumes of tissue.

2.5 Reagents and Solutions

Except where noted, all drugs and salts were obtained from Sigma Chemical Co (St Louis, MO). ACSF contained, in mM: 126 NaCl, 2 KCl, 1.25 NaH_2PO_4 , 1 MgSO_4 , 26 NaHCO_3 , 2 CaCl_2 , and 10 glucose, equilibrated with 95% O_2 / 5% CO_2 . Cutting solution contained, in mM: 2 KCl, 1.25 NaH_2PO_4 , 6 MgSO_4 , 26 NaHCO_3 , 0.2 CaCl_2 , 10 glucose, 220 sucrose and 0.43 ketamine. For OGD studies, ACSF was modified by replacement of glucose with equimolar sucrose, and equilibration with 95% N_2 / 5% CO_2 , rather than 95% O_2 / 5% CO_2 . Studies of hypoxia alone, or hypoglycemia alone were performed using ACSF equilibrated with 95% N_2 / 5% CO_2 or replacement of 10 mM glucose by 10 mM sucrose, respectively.

2.6 Statistical analysis

Significant differences between group data were evaluated using unpaired Student's *t* tests or one-way ANOVA. Bonferroni's multiple-comparison test or contrast comparisons were used for *post hoc* analysis in which the effects of multiple treatments were compared against each other. A value of $p<.05$ was considered significant in all cases. Numbers in the study refer to the number of slices, with a maximum of three slices from an individual animal used for each experimental protocol.

3. RESULTS

3.1. Decreasing glycogen availability increased propagation rate

Normoxic SD was generated by localized microinjections of 1 M KCl (high K^+ -SD) and similar events were generated by oxygen-glucose deprivation (OGD-SD) as previously described (Nedergaard and Hansen, 1988; Dietz et al., 2008). Both stimuli produced slow negative DC potential shifts characteristic of SD (Figures 1A&B) and the propagation of events across the tissue were monitored using autofluorescence imaging. High K^+ -SD generated an initial autofluorescence decrease that was tracked at 2.4 ± 0.1 mm/min across area CA1 (Figure 1C). A sustained autofluorescence increase following the transient decrease corresponds to increased oxidative metabolism following the event, as described previously following SD *in vivo* and *in vitro* (Hashimoto et al., 2000; Galeffi et al., 2010). During OGD challenges, oxygen deprivation generated a high initial autofluorescence level prior to onset of the SD-like event. OGD-SD propagation was then tracked by prominent autofluorescence decreases (3.25 ± 0.18 mm/min) that appeared more prolonged than in high K^+ preparations, due to the absence of the substrate-dependent overshoot phase.

To determine whether astrocytic glycogen stores contributed to SD propagation rate, we examined first the effects of pharmacological inhibition of glycogen breakdown using 1,4-dideoxy-1,4-imino-D-arabinitol (DAB, 10 μ M) and 1-deoxynojirimycin hydrochloride (DNJ, 10 μ M) which inhibit glycogen phosphorylase (GP) and glucosidase respectively (Kuriyama et al., 2008; Sadgrove et al., 2008; Walls et al., 2008). As shown in Figure 1E-F, pre-exposure to a combination of these agents (15 min) led to a significant increase in propagation rate of both high K^+ -SD and OGD-SD. Inhibitor concentrations utilized for these studies were based on a previous preliminary report in slice preparations (Sadgrove et al., 2008). To test whether these exposures were maximally effective, an additional set of studies were conducted with significantly higher exposures to the same inhibitors (100 μ M

each inhibitor, 75 min exposure) with OGD-SD. Prolonged exposures did not produce any additional increase in OGD-SD propagation rate, (4.1 ± 0.3 vs. 4.2 ± 0.3 mm/min, 100 μ M, 75 min vs 10 μ M, 15 min, respectively, $n=6$, $p=.83$), suggesting adequate penetration and effectiveness of the inhibitors. The effectiveness of this could be due in part to cooperative effects of the inhibitors, as previously suggested (Kuriyama et al., 2008).

3.2. Increasing glycogen content slows OGD-SD propagation

To assess the impact of increasing the content of glycogen stores on SD propagation, brain slices were exposed to L-methionine-DL-sulfoximine (MSO, 2 mM; 90-180 minutes), an approach which has previously been established to increase astrocyte glycogen content in cell cultures (Swanson et al., 1989). In initial studies, the effectiveness of this approach was evaluated on glycogen content in isolated hippocampal slices. Prolonged MSO exposures produced a small but significant increase in glycogen content to 13.23 ± 0.44 nmol/mg protein ($n=7$) when compared with control concentrations 11.59 ± 0.53 nmol/mg protein ($n=8$, $p=.034$).

For high K^+ -SD, MSO pre-exposures (90-180 min) did not significantly influence SD propagation rate, when compared with interleaved controls (Figure 1E $n=6$, $p=.46$). In contrast, OGD-SD was significantly slowed by MSO (Figure 1F). As discussed below, MSO exposures prevented the onset of OGD-SD in some preparations (3/6), but in those where OGD-SD was generated, the effects on propagation rate appeared dependent on MSO exposure time. Propagation rates approximately halved with 180 minute pre-incubations ($n=3$), compared with interleaved controls ($n=6$, $p=.02$). Shorter (90 min) MSO incubations appeared to reduce SD propagation rate, but these effects were not significant (MSO $n=4$ and control $n=6$, $p=.16$).

In addition to increasing astrocyte glycogen, MSO is a known convulsant as a consequence of decreasing GABA availability (Bentley et al., 1950; Liang et al., 2006). However, unlike the GABA_A antagonist bicuculline (25 μ M, $n=6$), 90 min MSO exposures did not lead to a significant increase in fEPSP duration ($n=7$), and furthermore bicuculline increased, rather than decreased, OGD-SD propagation rates (2.82 ± 0.44 and 5.60 ± 0.91 mm/min, control and bicuculline respectively, $n=3$, $p=.03$). When taken together with effects of DAB/DNJ, these data suggest 1) that basal levels of astrocyte glycogen serve to limit the rate of SD and SD-like propagation, and 2) that supplementation of these stores above basal levels selectively decreases the propagation of SD-like events generated by substrate depletion.

3.3. Selective inhibition of astrocyte oxidative metabolism

Previous work has established that selective inhibition of astrocyte TCA cycle activity accelerated SD propagation (Largo et al., 1997). Since glycogen stores are largely (if not entirely) restricted to astrocytes (Cataldo and Broadwell, 1986; Wender et al., 2000), we next examined whether disruption of astrocyte mitochondrial metabolism matched the effects of glycogenolysis inhibitors, under the same experimental conditions. Selective inhibition of astrocyte oxidative metabolism was achieved using fluoroacetate (FA) (Clarke et al., 1970), and compared with interleaved control slices, as well as slices pre-exposed to sodium acetate (1 mM) which provided an additional metabolic substrate that could feed directly into the TCA cycle (Waniewski and Martin, 1998). For high K^+ and OGD-SD, FA pre-exposure (FA, 1 mM, 25 min) resulted in a significant acceleration of SD compared to both acetate pre-exposures and control conditions (Figure 2A-B, $n=6$, $p<.01$).

3.4. Glycogen availability and OGD-SD onset

Based on the significant effects on SD propagation, we next asked whether glycogen stores affect the initiation of SD-like events. OGD-SD occurs after a substantial (11.3 ± 0.5 min)

delay following the onset of OGD exposure, and this delay presumably reflects the time taken for depletion of metabolic substrates and cellular ATP, sufficient to result in loss of Na^+/K^+ -ATPase activity leading to the induction of a feed-forward SD-like event (Jarvis et al., 2001). High K^+ -SD is initiated virtually instantaneously, as the elevation of extracellular K^+ concentration is sufficient to generate SD, without localized metabolic depletion. Therefore the subsequent studies concentrated on the latency to initiation of SD-like events generated by OGD alone.

Figure 3 shows the time course of development and consequences of OGD-SD in a population of slices. Simultaneous autofluorescence measurements and recordings of fEPSPs were used to assess both mitochondrial redox potential and neuronal viability. Initial NAD(P)H fluorescence increases (contributed to by NADH increases as slice oxidative metabolism is impaired) reached a plateau at $78.1 \pm 2.1\%$ above baseline, (Figure 3A). The passage of OGD-SD across the field of view was visualized as a transient dark band propagating across CA1 at a rate of 2.4 ± 0.4 mm/min (see Fig 1B). In individual slices, this observed propagating wave of NAD(P)H autofluorescence coincided with abolition of postsynaptic fEPSP potentials. Prior to the initiation of SD, there was a brief period of electrical hyperexcitability within the slice that was promptly lost at SD onset (Figure 3Ac), similar to the burst in spontaneous spike firing reported just prior to SD in previous studies (Herreras and Somjen, 1993). This loss of fEPSPs persisted, even after reintroduction of ACSF (Figure 3Ad); suggesting SD triggered by prolonged metabolic challenge in brain slice results in neuronal damage as previously described (Obeidat et al., 2000).

Consistent with previous reports of substrate dependence for single cell anoxic depolarizations (AD), the onset of SD was dependent on the availability of glucose, rather than oxygen. Slices were pre-exposed to either hypoxia or hypoglycemia for 10 minutes prior to 20 min OGD exposure (Figure 3B). While pre-exposure to hypoxia ($n=5$) did not significantly reduce the latency to SD when compared with interleaved controls ($n=6$, $p=.77$), hypoglycemia significantly reduced the time to SD onset ($n=6$, $p=.002$).

Interestingly, pre-exposure to glycogen breakdown inhibitors DAB and DNJ ($10 \mu\text{M}$, 15 min), did not result in any decrease in the time to SD when compared to interleaved controls (Figure 4, $n=6$, $p=.13$). Even prolonged exposures to DAB/DNJ ($100 \mu\text{M}$, 75 min) did not significantly decrease SD latency to SD when compared with interleaved controls (11.7 ± 0.5 min and 11.5 ± 1.0 min respectively, $n=6$, $p=.87$), suggesting depletion of basal glycogen stores within astrocytes does not contribute to OGD-SD onset. However, significantly increasing glycogen content with prolonged MSO exposures had a time-dependent effect on OGD-SD onset (Figure 4). Similar to the time-dependent effects of MSO on propagation rate, preparations subjected to shorter (90 min) 2 mM MSO pre-exposures ($n=4$) did not show a significant difference in the time of SD onset when compared with interleaved controls ($n=6$, $p=.095$). Prolonged MSO pre-exposures (180 min) completely prevented SD in 3 of 6 preparations, and resulted in a significant increase in the latency to SD onset in the remaining tissues when compared with interleaved controls (MSO $n=3$ and control $n=6$, $p=.008$).

3.5. 2mM glucose pre-exposures

It has previously been suggested that extended (>60 min) exposures to reduced extracellular glucose concentrations (2 mM, rather than 10 mM) may effectively deplete astrocytic glycogen stores in hippocampal slices (Allen et al., 2005). We evaluated this possibility with glycogen measurements and effects on SD initiation and propagation. There was a trend towards a decrease in total glycogen between samples exposed to 2 mM glucose versus normal ACSF (9.9 ± 0.64 nmol/mg protein and 11.59 ± 0.53 nmol/mg protein respectively, $n=7$, $p=.065$) and 2 mM glucose pre-exposures induced SD significantly earlier than the

time to SD in interleaved control preparations (Figure 5A, $n=6$, $p=.045$). However, 2 mM glucose pre-exposed preparations showed no significant increase on the rate of SD across the CA1 when compared with interleaved controls during OGD-SD (Figure 5B, $n=6$, $p=.88$) or high- K^+ SD (Figure 5C, $n=4$, $p=.79$). Experiments where slices were pre-exposed to both 2 mM glucose and 10 μ M DAB/DNJ (60 min) showed a decrease in SD latency and an increase in SD propagation rate that was not significantly different from pre-exposure to 2 mM glucose or DAB/DNJ alone suggesting there are no additive effects when inhibiting the utilization of glycogen stores prior to OGD (latency: 9.58 ± 0.48 min and 9.68 ± 0.84 min respectively, $n=6$, $p=.92$ and propagation: 4.6 ± 0.5 mm/min and 4.7 ± 0.4 mm/min respectively, $n=6$, $p=.42$). The contrasting effects of 2 mM glucose pre-exposures with the glycogenolysis inhibitors (see above) suggests that although this procedure may somewhat deplete glycogen stores; the effects on reducing glucose availability may be much more important for regulating the initiation of SD.

4. DISCUSSION

4.1. General

This study addressed the involvement of glycogen stores and astrocytic metabolic function on the initiation and propagation of SD and related events in brain slices. Depletion of glucose availability (rather than glycogen stores) appeared more important for the initial metabolic failure and SD onset in OGD studies. In contrast, maintenance of astrocyte metabolism by glycogen stores appeared significant for limiting the rate of SD propagation through tissues during normoxic and ischemia-like conditions. These findings support the possibility that targeting astrocyte glycogen stores could be of benefit in limiting the spread of SD and related events through peri-infarct tissues in *in vivo* stroke models.

4.2. SD propagation rate

Extracellular accumulation of both glutamate and K^+ likely contribute to the progression of SD (Somjen, 2001), and astrocytic uptake of these mediators is well established (Anderson and Swanson, 2000; Kofuji and Newman, 2004). As mentioned above, previous work has demonstrated the importance of astrocyte oxidative metabolism in regulating SD propagation rate in rat cortex (Largo et al., 1997), raising the possibility that ATP produced by mitochondrial respiration is utilized to maintain electrochemical gradients at the advancing SD wavefront. Our results with FA in hippocampal slice preparations were similar, as a significant acceleration in propagation rate was observed with both high K^+ -SD and OGD-SD. Under ischemia-like conditions, loss of oxygen availability is expected to inhibit utilization of TCA-derived substrates, and pre-exposures to FA likely accelerate depletion of other metabolic reserves (including astrocyte glycogen) prior to the onset of OGD.

The results with pharmacological approaches to limit glycogenolysis with DAB/DNJ suggest that ATP derived from astrocyte glycogen can also significantly contribute to limiting the propagation rate of high K^+ -SD and OGD-SD. In normoxic conditions (high K^+), two observations suggest that adequate metabolic substrates are available to limit SD propagation. Neither supplementation of glycogen stores with MSO, nor provision of an additional oxidative substrate (acetate) significantly slowed SD propagation. In contrast, OGD-SD was significantly slowed by MSO exposures. Glycogen is recognized to be a useful substrate in times of energy depletion, since it does not require ATP for initiation of glycolysis (Brown, 2004), and it is possible that astrocytes may provide glycogen-derived glucose to neurons (Rossi et al., 2007). Glycogen stores are known to be reduced in ischemic tissues prior to SD onset and completely depleted by SD-like events in *in vivo* rat brain (Selman et al., 2004). Therefore, these stores could be a particularly useful target to

limit the progression and/or metabolic consequences of repetitive depolarizing events, which are known to occur in human ischemic brain (Dohmen et al., 2008; Lauritzen et al., 2011).

4.3. SD initiation

OGD-SD occurred in all preparations with a delay of ~11 min. Both complete (0 mM) and partial (2 mM) removal of glucose prior to OGD decreased the latency to SD onset, while removal of O₂ alone had no effect. This is consistent with a dependence on extracellular glucose for the initiation of anoxic depolarization, reported previously in single cells during similar OGD challenges (Allen et al., 2005). The delay to SD onset could be a result of the time required to deplete astrocytic glycogen stores (Allen et al., 2005). However, the combination of pharmacological and substrate depletion studies suggest that this may not be the case. It appears that depletion of tissue glucose availability (rather than glycogen stores) is responsible for setting the time to OGD-SD onset. It remains to be determined whether it is depletion of glucose in all cell types or solely in neurons which is most relevant. It is acknowledged that measured glycogen levels were relatively low in our preparations, and a role of glycogen in SD initiation may be greater with higher store content *in vivo*. Interestingly, increasing glycogen stores resulted in either a significant delay to OGD-SD onset, or complete inhibition of propagating depolarization in half the slice preparations tested. Despite the lack of SD in these preparations, there were still clear signs of neuronal damage. It seems probable that under these conditions, single cell neuronal depolarizations occurred stochastically as substrate availability was exhausted, but astrocytes retained sufficient energy substrates to prevent the accumulation of glutamate and/or K⁺ to threshold levels for SD.

The relevance of these results to *in vivo* studies is not yet determined. It is possible that the regulation of SD onset by astrocytes may be even more apparent *in vivo*, where glycogen concentrations will likely be greater and astrocyte-mediated buffering of K⁺ to blood vessels may be an additional contributor to reducing SD threshold (Newman et al., 1984; Amedee et al., 1997).

4.4. Implications for SD in normoxic and/or ischemic conditions

It is not yet known how changes in SD propagation rates may affect expansion of brain injury. DC shift duration and SD frequency are well established to correlate with injury progression (Dijkhuizen et al., 1999; Lauritzen et al., 2011), but could not be assessed in the current study. Only single SDs could be induced in conditions leading to damage (OGD) and continuous recordings of evoked field potentials limited the ability to reliably measure DC shifts. These parameters would be useful to assess in future studies. In regions where glycogen levels are depleted, SD events may propagate more readily into previously healthy tissue. Astrocytes in penumbral tissues, which will have a range of substrate depletion (Lipton, 1999), may have less glycogen available to provide additional substrate required for sufficient glutamate and/or K⁺ clearance from the extracellular space. It is yet to be determined how maintenance of glycogen levels within astrocytes may influence the propagation of SD through moderately compromised tissue and the stepwise expansion of the ischemic core following injury. It is noted that even in otherwise healthy tissue, normoxic SD leads to a prolonged decrease in extracellular glucose concentrations, and repetitive depolarizations lead to a stepwise depletion of this substrate *in vivo* (Hashemi et al., 2008). It is not yet known whether this significantly contributes to or may be part of a consequence of glycogen depletion.

As noted above, recent clinical recordings have strongly implicated SD-like events in the progression of stroke injury. The current studies emphasize the importance of astrocyte metabolism in preventing the spread of SD-like events both under normoxic and ischemic

conditions, suggesting that these cells may provide a novel therapeutic target for the prevention of SD-like events in clinical settings.

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Abbreviations

ACSF	Artificial cerebrospinal fluid
DAB	1,4-deoxy-1,4-imino-D-arabinitol
DNJ	1-deoxynojirimycin
FA	Fluoroacetate
FC	Fluorocitrate
MSO	L-methionine-DL-sulfoximine
NADH	Nicotinamide adenine dinucleotide (reduced)
OGD	Oxygen and glucose deprivation
SD	Spreading depression
TCA	tricarboxylic acid cycle

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Highlights

We studied contributions of glycogen stores to spreading depression in brain slices
Extended extracellular glucose reductions did not abolish glycogen reserves
Pharmacological reductions of glycogen availability increased propagation rates SD
onset and propagation were delayed or prevented by glycogen increases

Figure 1. Effects of modifying astrocytic glycogen on high K^+ -SD and OGD-SD

(A) Characteristic negative voltage shift produced by the initiation of SD (arrow) by 1M KCl microinjection (B) Similar DC shift potential by OGD exposures. (C-D) Visualization of high K^+ -SD and OGD-SD propagation using NAD(P)H autofluorescence imaging. Advancing wavefront indicated by arrows (see text for details). Inset at top left panel is a bright field image of the preparation, followed by 11 consecutive panels showed at 2.5s intervals (numbers indicate the time from SD onset). R: recording electrode, S: KCl delivery electrode. Scale bar: 500 μ m. (E) Rates of high K^+ -SD after pre-exposure to either DAB/DNJ combinations (15 min) or MSO (90-180 min). Rates in 10 μ M DAB/DNJ were significantly faster than interleaved controls, but no difference was observed between 2mM MSO and interleaved controls. (F) Rates of OGD-SD were significantly increased by DAB/DNJ, and greatly reduced by prolonged (180min) MSO pre-exposures. (n=6, **p<.01 and *p<.05)

Figure 2. Effects of fluoroacetate (FA) or acetate on high K⁺-SD and OGD-SD
(A) Pre-exposures with 1mM FA significantly increased the rate of high K⁺-SD when compared with both control and 1mM acetate pre-exposures. (B) FA also significantly increased OGD-SD propagation rates in preparations pre-exposed to 1mM FA when compared with control and 1mM acetate preparations (n=6, *p<.05).

Figure 3. Effects of substrate removal on OGD-SD initiation

(A) Mean effects of OGD on NAD(P)H autofluorescence signals and evoked fEPSPs recorded simultaneously in the same preparations. OGD-SD occurred on average at 11.3 ± 0.5 min and propagated at an average rate of 2.5 ± 0.4 mm/min. Prior to onset of OGD-SD, NAD(P)H initially increased due to inhibition of mitochondrial respiration, stepped down during the progression of OGD-SD, and then continued to decline to levels below the initial baseline, even after restoration of oxygen and glucose. Representative traces show the effects of OGD on fEPSPs at different time points, pre- and post-SD (a-d). (B) Effects of pre-exposures on OGD-SD latency. Slices were pre-exposed to either 10 min hypoxia alone or 10 min zero glucose alone prior to subsequent OGD exposures. OGD-SD latency was significantly reduced by prior glucose reduction, but not pre-exposure to hypoxia ($n=6$, $**p < .01$).

Figure 4. Effects of modifying astrocyte glycogen on latency to OGD-SD onset

Pre-exposure with glycogenolysis inhibitors, DAB/DNJ (10 μ M, black bars) did not significantly affect the time to OGD-SD compared with interleaved controls. Pre-exposure with 2mM MSO (striped bars) did not significantly effect the time to SD onset with 90 min pre-exposures, but did result in significant delays to OGD-SD latency following 180 min pre-exposures (n=6, *p<.05).

Figure 5. Effects of extended exposures to reduced glucose on OGD-SD and high K^+ -SD
Slices were pre-exposed to modified ACSF with 2mM glucose rather than 10mM for ≥ 60 min prior to subsequent OGD exposures. Reduced glucose pre-exposure resulted in significant reduction in OGD-SD latency (A), but had no effect on rates of propagation for either OGD-SD (B) or high K^+ -SD (C) ($n=6$, $*p<.05$).