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PERSISTENT ASTROGLIAL SWELLING ACCOMPANIES RAPID REVERSIBLE DENDRITIC INJURY DURING STROKE-INDUCED SPREADING DEPOLARIZATIONS

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Abstract

Spreading depolarizations are a key event in the pathophysiology of stroke, resulting in rapid dendritic beading which represents acute damage to synaptic circuitry. The impact of spreading depolarizations on the real-time injury of astrocytes during ischemia is less clear. We used simultaneous *in vivo* 2-photon imaging and electrophysiological recordings in adult mouse somatosensory cortex to examine spreading depolarization-induced astroglial structural changes concurrently with signs of neuronal injury in the early periods of focal and global ischemia. Astrocytes in the metabolically compromised ischemic penumbra-like area showed a long lasting swelling response to spontaneous spreading depolarizations despite rapid dendritic recovery in a photothrombotic occlusion model of focal stroke. Astroglial swelling was often facilitated by recurrent depolarizations and the magnitude of swelling strongly correlated with the total duration of depolarization. In contrast, spreading depolarization-induced astroglial swelling was transient in normoxic healthy tissue. In a model of transient global ischemia, the occurrence of a single spreading depolarization elicited by a bilateral common carotid artery occlusion coincided with astroglial swelling alongside dendritic beading. With immediate reperfusion, dendritic beading subsides. Astroglial swelling was either transient during short ischemic periods distinguished by a short-lasting spreading depolarization, or persistent during severe ischemia characterized by a long-lasting depolarization with the ultraslow negative voltage component. We propose that persistent astroglial swelling is initiated and exacerbated during spreading depolarization in brain tissue with moderate to severe energy deficits, disrupting astroglial maintenance of normal homeostatic function thus contributing to the negative outcome of ischemic stroke as astrocytes fail to provide neuronal support.

Keywords

two-photon microscopy; *in vivo* imaging; dendrites; astrocytes; ischemic penumbra

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INTRODUCTION

Spreading depolarizations occurring in patients with malignant hemispheric stroke, subarachnoid hemorrhage, spontaneous intracerebral hemorrhage or traumatic brain injury contribute to lesion progression in metabolically compromised brain tissue (Dreier, 2011; Lauritzen et al., 2011). In focal stroke, spreading depolarization starts spontaneously in the low perfusion area of the ischemic core as a depolarization wave of neurons and glia. Spreading depolarization causes profound disruption of ion gradients associated with neuronal and astroglial swelling, dendritic beading and spine loss (Andrew et al., 2007; Murphy et al., 2008; Obeidat et al., 2000; Risher et al., 2010; 2011). Membrane repolarization and recovery of ion gradients depends on activity of the Na^+/K^+ -ATPase, which is severely impaired in the ischemic core. The resultant inadequate energy supply leads spreading depolarization to become terminal depolarization throughout the core, where neurons do not repolarize (Dreier, 2011). The duration of this spreading depolarization wave becomes shorter as it propagates throughout the penumbra along gradients of perfusion, oxygen and glucose and into naive tissue (Aitken et al., 1998; Somjen, 2004). At this point, it evolves into classic cortical spreading depression (Leão, 1944) and does no harm because energy resources are not severely compromised. Spontaneously recurring spreading depolarizations initiate at the edge of the ischemic core and persist throughout the metabolically compromised ischemic penumbra for days after the initial injury (Dohmen et al., 2008; Hartings et al., 2003). In contrast to the ischemic core, where dendrites remain beaded (Zhang and Murphy, 2007), penumbral dendrites undergo rapid beading coinciding with the occurrence of spontaneous spreading depolarizations, recovering their structure when there is sufficient nearby capillary flow (Risher et al., 2010; 2011). Nevertheless, prolonged depolarization of recurring spreading depolarizations further elevates metabolic stress in penumbra, facilitating acute dendritic injury (Risher et al., 2010).

Recent advances in the study of astrocytes have shown that they are tightly coupled with neurons, combining with axons and dendrites to form the “tripartite synapse” (Araque et al., 1999). Astrocytes also form close associations with the microvasculature of the brain, providing a bridge between neurons and the blood supply as part of the “neuro-vascular unit” (Haydon and Carmignoto, 2006). Therefore, stroke-induced disruption of synaptic circuitry should also be studied with regards to astroglial damage and not just neuronal injury. It has been proposed that the failure of glia to provide support for neurons makes neurons more susceptible to spreading depolarization induced injury (Largo et al., 1996), but the real-time *in vivo* astroglial response to spreading depolarization and whether this response depends on the degree of metabolic compromise in the affected region remain to be elucidated. Here we have used *in vivo* 2-photon laser scanning microscopy (2PLSM) to reveal simultaneous dynamic structural changes in astrocytes and dendrites as they respond to spreading depolarization. A photothrombotic stroke model of focal ischemia (Risher et al., 2010) was used to generate spontaneous spreading depolarizations in a metabolically-compromised penumbra-like area that resulted in a significantly different astroglial response to that seen following KCl-induced spreading depolarization in normal healthy tissue. A transient bilateral common carotid artery occlusion (BCCAO) model of global ischemia (Murphy et al., 2008) was employed to assess acute effects of a single occurrence of spreading depolarization on astrocytes and neurons as well as recovery during reperfusion and repolarization.

MATERIALS AND METHODS

Transgenic mice

All animal procedures follow National Institutes of Health guidelines and underwent yearly review by the Animal Care and Use Committee at Georgia Health Sciences University. In

total 48 male and female mice (average age ~9 months) were used in this study. Founders of the *B6.Cg-Tg(Thy1-EGFP)MJs/J* colony [GFP-M] were kindly provided by Dr. J. Sanes (Harvard University). Mice of GFP-M strain display bright fluorescence in a fraction of pyramidal neurons of the neocortex aiding dendritic imaging (Feng et al., 2000). Founders of the *FVB/N-Tg(GFAP-EGFP)GFEA-FKi* colony [GFAP-EGFP] were kindly provided by Dr. H. Kettenmann (Max Delbrück Center for Molecular Medicine, Berlin, Germany). Mice of this strain display EGFP fluorescence in astrocytes from multiple areas of the CNS (Nolte et al., 2001). Hybrid mice, with a small proportion of both fluorescent neocortical pyramidal neurons and fluorescent astrocytes, were generated by crossing between [GFP-M] and [GFAP-GFP] strains (Risher et al., 2009). In total we have used 19 GFAP-EGFP and 12 hybrid [GFP-M]/[GFAP-EGFP] mice. In 10 [GFP-M] and 2 wild-type mice, the astrocyte-specific stain sulforhodamine 101 (SR101; Invitrogen) was used to achieve robust and specific labeling of astroglia (Nimmerjahn et al., 2004). In an additional 5 [GFAP-EGFP] mice SR101 was used to co-label EGFP-expressing astrocytes for direct comparison of astroglial structural changes revealed by EGFP and SR101 fluorescence. Labeling was accomplished via topical application of 100 μ M SR101 through a craniotomy (as described below) followed by washout after 30 minutes.

Preparation of mice for *in vivo* imaging

Craniotomy for the optical window followed standard protocol (Risher et al., 2009; 2010; 2011). Mice were anesthetized with an intraperitoneal injection of urethane (1.5 mg/g body weight) with heart rate monitored (450–650 beats/min) using MouseOx® pulse oximeter (STARR Life Sciences). Depth of anesthesia was assessed by toe pinch and heart rate monitoring, maintained with 10% of the initial urethane dose if necessary. A short ~1 cm L-shaped glass capillary (1.2 mm diameter) was inserted into the trachea and secured with two sutures to minimize potential breathing problems that may result from prolonged anesthesia. Oxygen saturation level remained >90% for duration of experiments indicating that mice were respiring properly. Hydration was maintained by intraperitoneal injection of 100 μ l 0.9% NaCl with 20 mM glucose at 1 hour intervals. A 0.1 ml bolus of 5% (w/v) Texas Red Dextran (70 kDa) (Invitrogen) in 0.9% NaCl was injected into the tail vein for blood flow visualization. A dental drill (Midwest Stylus Mini 540S, Dentsply International) with ¼ bit was used to thin the circumference of a 2–4 mm diameter circular region of the skull over somatosensory cortex centered at stereotaxic coordinates –1.8 mm from bregma and 2.8 mm lateral, and the thinned bone was lifted up with forceps. An optical chamber was constructed by covering the intact dura with a thin layer of 1.5% agarose prepared in a cortex buffer containing (in mM) 135 NaCl, 5.4 KCl, 1 MgCl₂, 1.8 CaCl₂, and 5 HEPES, pH 7.3. The chamber was left open to facilitate access with microelectrodes. The cortical slow direct current (DC) potential was recorded with glass microelectrode (filled with 0.9% NaCl, resistance 1–2 M Ω) inserted through dura to the site of imaged dendrites and astrocytes within layer I of somatosensory cortex. The Ag/AgCl pellet reference electrode (A–M Systems) was installed under the skin above the nasal bone. In some experiments spreading depolarization was induced by pressure-injecting ~50 nL of 1 M KCl with Picospritzer (Parker Instruments) through a micropipette (resistance 1 M Ω) inserted through the dura to the depth of 200–300 μ m within 2 mm away from the site of imaged astrocytes. Signals were recorded with MultiClamp 200B amplifier, filtered at 1 kHz, digitized at 10 kHz with Digidata 1322A interface board and analyzed with pClamp 10 (Molecular Devices). All chemicals were from Sigma Chemical unless indicated otherwise.

2 photon laser scanning microscopy (2PLSM)

2PLSM images were collected with IR-optimized 40 $\times$ /0.8NA water immersion objective (Carl Zeiss) using LSM 510 NLO META multiphoton system (Zeiss) mounted on the motorized upright Axioscope-2FS microscope (Zeiss). The scan module was directly

coupled with the Spectra-Physics Ti:sapphire broadband mode-locked laser (Mai-Tai) tuned to 910 nm for 2-photon excitation. Three-dimensional (3D) time-lapse images were taken at 1 μm increments (with approximately 20 sections per z-stack) using 3 $\times$ optical zoom, yielding a nominal spatial resolution of 6.86 pixels/ μm (12 bits/pixel, 0.91 μs pixel time) across a 75 $\times$ 75 μm imaging field. Emitted light was detected by internal photomultiplier tubes (PMTs) of the scan module with the pinhole entirely opened.

Image analysis

Image Examiner (Zeiss) was used together with NIH ImageJ for image analysis and processing. The Scientific Volume Imaging (Hilversum, Netherlands) Huygens Professional image deconvolution software was used to process images in figures. Dendritic beading was identified as the appearance of rounded regions extending beyond the diameter of the parent dendrite separated by “interbead” segments. Dendritic recovery was defined as the disappearance of rounded “beaded” regions. Since the axial resolution of 2PLSM is relatively poor ($\sim 2 \mu\text{m}$), small astroglial processes were not readily measurable and therefore, volume changes of fine astroglial processes were not quantified. To simplify the interpretation of morphometric data imposed by the lower axial resolution of 2PLSM as compared to the lateral resolution ($\sim 0.4 \mu\text{m}$), we used two-dimensional Maximum Intensity Projections (MIPs) of image stacks (approximately 20 μm in thickness) to assess relative changes in the areas of individual astroglial somata as was described by us before (Andrew et al., 2007; Risher et al. 2009). This analysis of MIP images assumed that astroglial soma volume is changing uniformly in all directions, as based on viewing astrocytes along the z-axis in control and at each experimental time point. Such morphometric analysis of changes in the lateral dimensions was adequate to determine relative volume changes, which underestimated the actual volume changes assuming they are approximately isotropic. Therefore, as in our previous studies by Andrew et al. (2007) and Risher et al. (2010), three techniques were used for analyses of astrocytes: (1) MIP images were digitally traced by hand to measure the area of astroglial soma profiles in control and for each time point. Alternatively, MIP images were filtered with a median filter (radius=1), background subtracted, and thresholded using ImageJ. The thresholded image with the astrocytic somata was then outlined and automatically measured with the “analyze particles” function of ImageJ. The relative change in soma profiles traced either manually or automatically yielded similar results. (2) Control and experimental MIP images were pseudocolored red and green, aligned and overlaid. Overlapping areas projected as yellow, whereas different areas remained green or red. (3) Control profiles were traced and filled to create a mask, which revealed peripheral areas of swelling when overlaid upon experimental images.

BCCAO stroke model

BCCAO was implemented as described in Murphy et al. (2008). After installation of a cranial window, the mouse was placed on its back, an incision was made above the thyroid gland and the CCAs were separated from the surrounding tissue and fitted with sutures. The sutures were crossed in an X pattern across the trachea and the incision closed with the sutures exposed to allow tensioning during imaging. Ischemia was induced on the microscope stage by pulling carefully on the sutures and securing them with tape to the microscope nosepiece. Once the onset of spreading depolarization was detected, reperfusion was accomplished by immediately removing the tape, thereby relieving the tension of the sutures. 2PLSM imaging was performed for up to 4 h following reperfusion.

Photothrombotic stroke model

Photothrombotic occlusion surrounding an ischemic penumbra-like “area at risk” was implemented as described recently (Risher et al., 2010). A bolus of Rose Bengal was injected through the tail vein (0.03 mg/g, diluted to 10 mg/ml in 0.9% NaCl). With LSM 510

software, the perimeter (100 μm wide) of a square-shaped region of interest (1270 $\times$ 1270 μm) was irradiated with the 514 nm laser line through 10x/0.3NA water immersion objective resulting in Rose Bengal activation. The central square (1070 $\times$ 1070 μm) was not illuminated by the laser. Photoactivation was stopped immediately upon occurrence of initial photothrombosis-induced spreading depolarization recorded in the vicinity of imaged dendrites and astrocytes. 2PLSM imaging was performed for up to 5 h following photothrombosis.

Statistics

SigmaStat (Systat) was used for statistical analyses. A paired t-test and repeated measures (RM) ANOVA followed by Tukey post-hoc test and Wilcoxon signed rank test were used to compare means and the median values over different time points, respectively. The linear regression analysis was applied to quantify the strength of the relationship between two variables. The significance criterion was $p < 0.05$. Data are presented as mean $\pm$ s.e.m.

RESULTS

Using high resolution 2PLSM imaging, we compared the neuronal and astroglial response to spontaneously recurring spreading depolarizations in the metabolically compromised ischemic penumbra-like area at risk by using the photothrombotic occlusion model. As seen previously (Risher et al., 2010; 2011), dendrites in the penumbra-like zone undergo a rapid cycle of beading and recovery that coincides with the passage of each spreading depolarization. Here we performed photothrombotic occlusion on our hybrid [GFP-M]/[GFAP-EGFP] mice ($n=10$ animals) to observe what happens to astrocytes concurrently with dendrites during these depolarizations. There was a significant swelling of astroglial somata during the initial photothrombosis-induced spreading depolarization in the penumbra-like area at risk (Fig. 1A). This swelling far outlasted the expected dendritic response of rapid beading and recovery. In addition, we have also performed photothrombotic occlusion on 7 [GFAP-EGFP] mice and 4 [GFAP-EGFP] mice with SR101 co-stained astrocytes. In all 21 animals astrocytes experienced a significant increase in soma size following the initial photothrombotically induced spreading depolarization along with an apparent swelling of fine astroglial processes (Fig. 1B), but the latter observation was not quantified because small processes were not readily measurable especially along the z-axis. When images were taken almost immediately (2.3 ± 0.2 min) after the onset of the initial spreading depolarization, an increase in astroglial cross-section soma area was detected in 60 out of 64 astrocytes (94%) with an average increase by $15.8 \pm 1.8\%$ above the control value ($p < 0.001$) (Fig. 1C). The size of astroglial somata was linearly correlated with time after the onset of the initial induced spreading depolarization indicating continuous swelling of both EGFP-expressing astrocytes and astrocytes labeled with SR101 (Fig. 1D). These results further confirmed that the observed glial swelling was due to actual structural changes and not due to redistribution of GFP during ischemia. The cross-section soma area increased from $17.5 \pm 2.2\%$ ($p < 0.001$, Fig. 1E) during the first 5 min after the onset of the initial induced spreading depolarization to $31.4 \pm 4.8\%$ during the next 30–60 min after the depolarization onset ($p < 0.001$ relative to control, $^{\dagger}p < 0.05$ relative to 0–5 min bin, Fig. 1E). Furthermore, astrocytes showed little to no structural recovery during these acute experiments (up to 4 h) (Fig. 1E), remaining swollen well after the onset of the initial induced spreading depolarization.

Occlusion of cortical microcirculation in the zone surrounding the penumbra-like area at risk produces recurrent spontaneous spreading depolarizations riding on a shallow negative ultraslow potential (Risher et al., 2010) (Fig. 1A, top right) indicating metabolically compromised tissue at risk for permanent injury (Oliveira-Ferreira et al., 2010; Dreier et al. 2012). Because terminal dendritic injury in this model was significantly correlated with the

total duration of spontaneous spreading depolarizations (Risher et al., 2010), we have investigated whether the degree of astroglial swelling also depended on the total duration of spreading depolarizations. First, the duration of each spreading depolarization was measured as the time interval between the points corresponding to half the DC amplitude and the same potential during recovery, and then the total duration for all depolarizations was calculated. Since the longest total duration of spreading depolarization was 19.8 min (average, 8.9 ± 1.5 min, $n=39$ depolarizations), we have selected images taken beyond this time point at an average of 25.1 ± 0.7 min after initial induced spreading depolarization, with a total of 37 astrocytes from 15 animals available for this analysis. In 11 of 15 mice the initial photothrombosis-induced spreading depolarization was followed by spontaneous recurrent spreading depolarizations ($n = 24$ recurrent spreading depolarization events), with a maximum of 6 recurrent spreading depolarizations recorded during the first 27 min after stroke. The majority of imaged astrocytes were swollen, reaching $27.4 \pm 4.1\%$ above the control cross-section soma area ($p < 0.001$, paired t-test). Additionally, the extent of astroglial soma swelling was strongly correlated with the total duration of spreading depolarizations (Fig. 2A, $r = 0.64$, $p < 0.001$), strengthening the idea that accumulated stress resulting from the prolonged duration of recurrent spreading depolarizations aggravated astroglial swelling. To clearly delineate whether additional astroglial soma swelling can occur during spontaneous recurrent spreading depolarizations we searched the data set for images taken just prior to and immediately after a recorded spontaneous recurrent spreading depolarization (Fig. 2B). Overlaid soma images from before, during (Fig. 2B, middle right panel), and after depolarization (Fig. 2B, bottom right) have revealed further cell body swelling facilitated by spontaneous recurrent spreading depolarization. Importantly, astrocytes swelled by an additional $11.1 \pm 2.4\%$ ($p < 0.005$, Fig. 2C) as measured at 2.0 ± 0.3 min after spontaneous recurrent spreading depolarization onset. Therefore, astroglial soma size in the penumbra like area at risk could increase in a stepwise manner coinciding with the occurrence of recurrent spontaneous spreading depolarizations.

To further support our conclusion that the observed astroglial swelling was in response to depolarization and not just due to ischemic interruptions in blood flow, we then tested the effect of depolarization on astrocytes in normal, healthy neocortex. We elicited a spreading depolarization far away from the site of imaging with KCl microinjection in 4 [GFAP-EGFP] mice. The spreading depolarization then invaded the imaging field, as recorded with a microelectrode placed next to imaged astrocytes, causing a rapid reversible astroglial swelling (Fig. 3A, top). Overlaying MIP images prior to, during and after passage of spreading depolarization facilitated visual comparison between images of astrocytes at different time points (Fig. 3A, middle). These transient responses of EGFP-expressing astrocytes to spreading depolarization in the normoxic neocortex were also replicated in the wild-type mouse with astrocytes labeled with SR101 (Fig. 3B). Furthermore, both EGFP-expressing astrocytes ($n=10$) and SR101 labeled astrocytes ($n=5$) undergo reversible swelling after the passage of a second KCl-evoked spreading depolarization (Fig. 3D). Astroglial cross-section soma area rapidly increased by $15.6 \pm 2.9\%$ ($p < 0.001$) above the control at 1.2 ± 0.2 min after the onset of the first KCl-induced spreading depolarization before returning to the control value at 9.5 ± 1.9 min after repolarization (11.4 ± 2.4 min after the depolarization onset) (Fig. 3D). Similarly, the second KCl-induced spreading depolarization resulted in rapid astroglial swelling by $11.5 \pm 2.7\%$ ($p < 0.02$) above the pre-spreading depolarization value as measured at 1.5 ± 0.4 min after the depolarization onset. Astrocytes quickly recovered to the pre-spreading depolarization value as assessed at 1.3 ± 1.8 min after repolarization (9.7 ± 2.2 min after the depolarization onset) (Fig. 3D). We conclude, that in contrast to the persistent swelling seen in the metabolically compromised ischemic penumbra-like area at risk with spontaneously recurring spreading depolarizations, astroglial swelling was transient following spreading depolarization in normoxic tissue. This finding suggests that though spreading depolarization is involved in initiating the astroglial

swelling, the metabolic state of the tissue determines whether the swelling will be readily reversible or not.

To further test this hypothesis we examined astroglial changes during BCCAO-induced spreading depolarization using 2 hybrid [GFP-M]/[GFAP-EGFP] mice, 9 [GFAP-EGFP] mice, 10 [GFP-M] mice with SR101 labeled astrocytes and one wild-type mouse with SR101 staining. Abrupt reduction in blood flow during global ischemia results in a severe energy deficit with the cortical ATP level rapidly declining to near zero values (Hossmann, 2006). Here the spreading depolarization may become terminal in the absence of reperfusion. In this respect it resembles the ischemic core when compared to focal stroke. DC potential recovers upon reperfusion, but the spreading depolarization wave lasts longer in those experiments where neurons are more severely energetically compromised (Oliveira-Ferreira et al., 2010; Dreier et al. 2012). Murphy et al. (2008) previously used transient BCCAO to show that dendritic injury is precisely coordinated with the occurrence of spreading depolarization, which we confirmed here (Fig. 4A). Immediately upon detection of spreading depolarization, dendrites became beaded as nearby blood flow was either slow or stalled ($n = 12$ mice). With immediate reperfusion, dendrites recovered their form in 11 of 12 animals and remained stable throughout the imaging period (up to 4 h). Astroglial swelling occurred alongside dendritic beading (Fig. 4B). The powerful propagating spreading depolarization front triggered swelling in all 73 imaged astrocytes in 20 animals, with astroglial cross-section area increasing by $20.9 \pm 1.9\%$ ($p < 0.001$, Fig. 4C) as measured at 2.3 ± 0.3 min after depolarization onset. As with a short-lasting normoxic spreading depolarization (Fig. 3C, 1.36 ± 0.44 min average duration, $n=10$ depolarizations), astroglial swelling appeared to be reversible during short-lasting BCCAO-induced spreading depolarization (average duration; 4.1 ± 1.0 min, $n=9$) as shown in the image sequence presented in Fig. 4D. As expected, dendritic beading was also reversible when monitored in 6 mice alongside astroglial swelling. After the initial swelling, somata size of EGFP-expressing astrocytes as well as SR101-labeled astrocytes was negatively correlated with time after the onset of spreading depolarization, indicating transient swelling (Fig. 4F). Indeed, the cross-section soma area increased by $16.3 \pm 1.9\%$ ($p < 0.001$, Fig. 4G) during spreading depolarization as measured at 2.4 ± 0.6 min after the depolarization onset, and the astroglial soma size returned to the control values as measured at 1.7 ± 0.3 min after repolarization.

Our findings reveal that astrocytes in the metabolically challenged penumbra in focal stroke showed a long lasting swelling response. The ultraslow potential component of DC could be used as an indirect spatial and temporal mark for the energy status of neurons (Oliveira-Ferreira et al., 2010; Dreier et al. 2012). Therefore, we searched the data set for experiments with a more robust BCCAO-induced metabolic compromise, as indicated by a large negative ultraslow potential component of DC (Fig. 5A, note that the DC trace duration of this long-lasting depolarization is ~ 30 times longer than the duration of the short-lasting depolarization shown in Fig. 4E). Under these conditions astrocytes swelled during BCCAO-induced spreading depolarization and remained swollen, as shown on the 2PLSM image sequence in Fig. 5A. Dendritic beading was reversible in 5 of 6 mice when both dendrites and astrocytes were simultaneously monitored. There was a weak positive correlation ($r = 0.25$, $p < 0.001$) between the size of astroglial somata and time after the spreading depolarization onset, indicating persistent swelling of both EGFP and SR101-demarcated astrocytes (Fig. 5B). The astroglial swelling response occurred with the onset of spreading depolarization, with astroglial soma area increasing by $24.1 \pm 2.8\%$ above control during the first 5 min after spreading depolarization onset (Fig. 5C). The soma swelling was persistent, remaining significant throughout the imaging time window of our acute experiments (reaching $29.8 \pm 4.3\%$ above control astroglial cross-section soma area by 88.2 ± 5.8 min post-spreading depolarization) (Fig. 5C). These findings suggest that, during

transient global ischemia, spreading depolarization is involved in initiating astroglial swelling that is only reversible in tissue that is not severely metabolically compromised.

DISCUSSION

We used real-time *in vivo* imaging to elucidate simultaneous effects of spreading depolarization on astrocytes and dendrites in metabolically stressed ischemic neocortex. In the penumbra-like zone, each occurrence of spreading depolarization was precisely coordinated with acute dendritic beading, which was reversible as long as there was sufficient local capillary blood flow (Risher et al., 2010; 2011). Astrocytes responded differently from dendrites, swelling in conjunction with spreading depolarization with the increased soma size persisting for the duration of the acute experiment even when nearby dendrites were seen to recover. The present work provides the first direct demonstration of such persistent astroglial swelling in the ischemic penumbra. The degree of astroglial swelling was correlated with the total duration of depolarization. Furthermore, the size of astroglial somata increased in a stepwise manner following spontaneous spreading depolarization. In contrast, in healthy metabolically uncompromised neocortex, spreading depolarization induced reversible astroglial swelling. Consistent with a previous report by Murphy et al. (2008), a single occurrence of spreading depolarization in a transient BCCAO model of global ischemia was sufficient to induce dendritic beading that was reversible with immediate reperfusion. As shown in the current study, spreading depolarization-induced astroglial swelling in this global ischemia model was either transient or persistent, depending on the severity of ischemic periods and, therefore, the metabolic status of the tissue.

Under normoxic conditions astrocytes undergo a passive transient swelling in response to the depolarization-elicited rise of $[K^+]_o$ (Zhou et al., 2010) as we observed here in the case of EGFP-expressing astrocytes and astrocytes labeled with SR101. In contrast with astrocytes in normal healthy neocortex, our experiments with photothrombotic occlusion have shown that metabolically compromised astrocytes in the penumbra-like area do not recover their structure following focal ischemia but instead remain swollen. Astroglial swelling was frequently facilitated by the passage of spontaneous spreading depolarizations, whereas dendrites rapidly beaded and recovered in conjunction with recurring spontaneous depolarizations (Risher et al., 2010; 2011). Astrocytes are relatively resistant to hypoxia but become quickly injured if hypoxia is combined with acidosis and $[K^+]_o$ increase (Chesler, 2005). It is conceivable that spreading depolarization-induced acidosis and increased $[K^+]_o$ combine with hypoxia to compromise astroglial viability, promoting damage to neighboring neurons and thus, contribute to a stepwise expansion of the infarct.

A transient global ischemia-elicited spreading depolarization induced astroglial swelling that was either reversible or persistent for the duration of the imaging (up to 3h). The transient or persistent nature of the astroglial swelling was correlated with occurrence of the ultraslow component of the DC potential. This negative ultraslow potential is thought to be generated by local factors related to increased cell death (Oliveira-Ferreira et al., 2010; Dreier et al. 2012). The generators of the ultraslow negativity are unknown but non-cellular mechanisms are thought to contribute (e.g. passive diffusion of ions that were released in the affected regions during rupture of the neuronal membrane may establish sustained electric potential differences across the extracellular space). Here, we have used the ultraslow potential component of DC as an indirect spatial and temporal mark for the energy status of the neurons and cortical tissue and as an electrophysiological sign of cell death (Oliveira-Ferreira et al., 2010; Dreier et al. 2012). Astrocytes were permanently swollen in animals with the ultraslow component of the DC potential, likely reflecting severe energy deficit. In this respect, such an outcome was similar to previously reported persistent swelling of

EGFP-expressing astrocytes during cardiac arrest and subsequent terminal spreading depolarization (Risher et al., 2009). Furthermore, astrocytes in the metabolically compromised ischemic penumbra-like area showed a long-lasting swelling response to spontaneous spreading depolarizations, with recurrent spreading depolarizations superimposed on a shallow negative ultraslow component of the DC (Oliveira-Ferreira et al., 2010; Risher et al., 2010). Spreading depolarization-induced astroglial swelling was transient during short-lasting depolarizations in some BCCAO experiments and in normoxic healthy tissue. Therefore, the spreading depolarization-induced transient or persistent nature of astroglial swelling was likely dependent on the energy status of surrounding tissue as judged from the ultraslow potential component of the DC.

Multiple mechanisms may participate in astroglial swelling in ischemic tissue with compromised sodium pump activity and spreading depolarization-induced ion gradient breakdown. Spreading depolarization-elicited astroglial swelling during ischemia likely reflects the clearing of $[K^+]_o$ (Zhou et al., 2010) with associated water influx through aquaporins (Amiry-Moghaddam and Ottersen, 2003; Kimelberg, 2005; Leis et al., 2005). However, such water influx through aquaporins triggers Ca^{2+} signaling in astrocytes and results in activation of signaling cascades that could compromise astrocyte viability and worsen the pathological outcome (Thrane et al., 2011). Thus, astroglial swelling can be harmful and decrease the ability of astrocytes to maintain normal homeostatic function (Kimelberg, 2005; Rossi et al., 2007). Astroglial swelling may be a precursor to several detrimental consequences of ischemia, since swelling activates one or more membrane permeability pathways and leads to the release of neuroactive and neurotoxic molecules, including the excitatory amino acids glutamate and aspartate (Abdullaev et al., 2006; Kimelberg et al., 1990), ATP (Mongin and Kimelberg, 2002; Liu et al., 2008; Orellana et al., 2011) and D-serine (Schell et al., 1995; Katsuki et al., 2004). Such release of ATP and glutamate through astroglial connexin 43 hemichannels could mediate neuronal death through activation of neuronal pannexin 1 channels (Orellana et al., 2011). Furthermore, spreading depolarization-induced astroglial swelling shown here at such early time points after stroke may also be a prelude to devastating events occurring later on, as swelling eventually results in rupture of the plasmalemma and organelles during delayed astroglial necrosis (Gurer et al., 2009). Loss of astrocytes would then be a crippling blow to the continued survival of neurons, since it has been well-established that neurons are dependent on the support provided by their glial neighbors both during development and following brain injury (Barreto et al., 2011).

Alternatively, astroglial swelling may be reflective of the protective work of these cells, including the removal of extracellular K^+ and glutamate (Mongin and Kimelberg, 2005), increased glycogen metabolism (Hertz et al., 2007; Seidel and Shuttleworth, 2011) and the release of glutathione to defend neurons from oxidative injury (Dienel and Hertz, 2005). Equally, the release of ATP (Kimelberg et al., 2006) and subsequent extracellular accumulation of adenosine (Pascual et al., 2005) could provide neuroprotection by delaying the onset of spreading depolarization (Canals et al., 2008). This dual role of astroglial swelling may underlie the transition between injury and repair in tissue at risk and the ability of astrocytes to protect surrounding neurons may likely depend on the level of metabolic compromise.

Here we focused on the initial changes in astroglial morphology in the early minutes to hours following ischemic injury and future longitudinal imaging studies will be necessary to fully elucidate whether these swollen astrocytes will recover or die at later time points and also how these early astroglial changes are related to the long-term survival of neurons. Nevertheless, the work presented here strongly indicates that spreading depolarization-

elicited disturbance of ion gradients greatly contributes to significant morphological changes in astrocytes that may affect the pathogenesis of stroke.

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Figure 1.

Long lasting spreading depolarization-induced astroglial swelling in the ischemic penumbra-like area at risk following focal photothrombotic stroke. **A**, MIP images of an astrocyte (asterisk) along with nearby dendrites (arrows) and blood vessels (red; labeled with Texas Red Dextran) in layer I of mouse somatosensory cortex. Each numbered image corresponds with a time point indicated on the respective DC potential recording at the imaging site (top right). Negative deflections in the DC potential represent spreading depolarizations. Note that spontaneous SD is superimposed on a shallow ultraslow DC negativity (asterisk) assumed to be an electrophysiological index of cell death. The dendrites bead during initial photothrombotically-induced spreading depolarization and rapidly recover (<2 min). By

contrast, the astrocyte swells with no recovery seen. A spontaneous spreading depolarization occurring 4 min later is accompanied by another round of dendritic beading and recovery (shown at 9 min), while the astrocyte continues to swell. **B**, Paired single section 2PLSM images showing an astrocyte in the penumbra-like area at risk before (control, red) and after initial photothrombotically-induced spreading depolarization (green). Astroglial process swelling is especially evident. Control and after induced spreading depolarization (Post-SD) images are overlaid (bottom) with arrows pointing to green areas illustrating swelling beyond control morphology. **C**, Summary from 64 astrocytes in 20 animals showing the increase in astroglial soma size immediately after initial photothrombotically-induced spreading depolarization (post spreading depolarization images were taken 2.3 ± 0.2 min after initial depolarization onset). $*p < 0.001$, paired t-test. **D**, Somata of EGFP-expressing astrocytes (green dots) and astrocytes labeled with SR101 (red dots) continued to increase in size after initial spreading depolarization onset. Values are shown as percent of control cross-section soma area for each astrocyte. Regression line is shown (black) ($r = 0.43$, $p < 0.001$; 66 astrocytes from 21 animals). **E**, Same data as in (**D**) presented as a bar graph showing a long lasting increase in astroglial soma size following the initial induced spreading depolarization. Values are shown as percent of control for each astrocyte. $*p < 0.001$ relative to control, $^{\dagger}p < 0.05$ relative to 0–5, one-way RM ANOVA.

Figure 2.

Astroglial swelling in the ischemic penumbra-like area at risk is exacerbated by spontaneous recurrent spreading depolarizations. **A**, Data from 39 spreading depolarizations in 15 mice showing a positive strong correlation between astroglial soma size and the total duration of spreading depolarization. Values are shown as percent of control, representing the cross-section soma area recorded at 25.1 ± 0.7 min after the onset of the initial spreading depolarization. Regression line is shown ($r = 0.64$, $p < 0.001$, 37 astrocytes). **B**, Astroglial soma size increases with occurrence of spontaneous recurrent spreading depolarization. Numbered MIP image sequence of swollen astrocyte (asterisk), dendrites (arrow) and blood vessels (red) taken shortly before (Pre-SD, 1), during (2) and after (Post-SD, 3) spontaneous recurrent spreading depolarization at time points indicated by a corresponding number on the DC potential recording of spontaneous recurrent spreading depolarization shown to the right. Image 2 demonstrates dendritic beading and further astroglial swelling during passage of spontaneous spreading depolarization. Dendrites rapidly recover after spreading depolarization, but astrocyte remains swollen (image 3). Overlays showing the merged Pre-SD/During SD and Pre-SD/Post-SD images reveal green areas (arrows) of additional recurrent spreading depolarization-induced astroglial swelling. **C**, Summary from 15 astrocytes in 6 animals showing step-wise increase in astroglial soma size during the occurrence of a recurrent spontaneous spreading depolarization. Data points were only used when images were taken immediately prior to and after a recorded recurrent spontaneous

spreading depolarization (Pre-SD images were taken 5.3 ± 1.3 min and Post-SD images were taken 2.0 ± 0.3 min after recurrent spreading depolarization onset). $*p < 0.005$, paired t-test.

Figure 3.

Astrocytes reversibly swell in response to spreading depolarization in normoxic healthy neocortex. **A**, Top: MIP images of an astrocyte before, during, and after KCl-induced spreading depolarization (shown in **C**) in normoxic somatosensory cortex. The astroglial soma and processes swell transiently during spreading depolarization before returning to control morphology. Bottom: *Left*, overlay showing the merged pre-SD/during SD images for the same astrocyte as above. *Center and Right*, overlay images of another astrocyte for merged Pre-SD/During SD and for Pre-SD/Post-SD images. Green areas (arrows) represent swelling beyond the pre-SD morphology. Chevron (left image) points to a swollen astroglial

endfoot during SD. **B**, An astrocyte labeled with SR101. Yellow mask showing the Pre-SD cross-section soma area is placed over the same cell During and Post-SD. SD evokes transient swelling (arrows). **C**, Example spreading depolarization recording from microelectrode placed next to an imaged astrocyte. **D**, Summary from 15 astrocytes from 5 animals showing reversible astroglial soma swelling in response to two subsequent KCl-induced spreading depolarizations in normoxic somatosensory cortex. Average duration of the first depolarizations was 0.99 ± 0.38 min and the second was 1.73 ± 0.82 min, as measured between the points of half the DC amplitude during depolarization onset and recovery. Astrocytes transiently swell in response to both spreading depolarizations. ** $p < 0.001$ and * $p < 0.02$ relative to Pre-SD and Post-SD for each spreading depolarization, one-way RM ANOVA.

Figure 4.

Astrocytes swell in response to BCCAO-induced spreading depolarization. **A**, *In vivo* 2PLSM high-magnification MIP images of dendrites (green) in layer I of mouse somatosensory cortex along with nearby blood vessel (red). The vessel constricts and dendrites become beaded during BCCAO, coinciding with the occurrence of spreading depolarization. When reperfusion is initiated immediately after the detection of spreading depolarization, dendritic beading is reversed as blood flow returns. **B**, MIP images of an astrocyte (asterisk) along with nearby dendrites (arrows) and blood vessel (red) in layer I of mouse somatosensory cortex. As in **A**, the dendrites bead and recover in concert with

BCCAO-induced spreading depolarization. The astroglial soma profile increases following BCCAO as its process tree becomes more diffuse. **C**, Summary from 73 astrocytes in 20 animals showing the increase in astroglial soma size evoked by BCCAO-induced spreading depolarization (during spreading depolarization images were taken 2.3 ± 0.3 min after depolarization onset). $*p < 0.001$, Wilcoxon Signed Rank Test. **D**, Images of two astrocytes corresponding to the various time points shown in **E** during short-lasting spreading depolarization recorded from microelectrode placed next to imaged astrocytes. The astroglial soma and processes swell during short-lasting spreading depolarization and then return to control morphology during post-spreading depolarization reperfusion and DC recovery. **F**, Data from 9 BCCAO-induced short-lasting spreading depolarizations (4.1 ± 1.0 min average duration) in 9 animals showing that astroglial soma size decreases after an initial swelling. Regression line is shown (black) ($r = -0.35$, $p < 0.001$; 30 astrocytes). **G**, Same data as in (**F**) presented as a bar graph reveal transient astroglial swelling during short-lasting BCCAO-induced spreading depolarization. Images were acquired immediately after onset of spreading depolarization at 2.4 ± 0.6 min (During SD) and after repolarization at 1.7 ± 0.3 min (Post-SD). Data points acquired after repolarization were at 8.8 ± 1.0 min after spreading depolarization onset. Values are shown as percent of control for each astrocyte. $*p < 0.001$, relative to Control and Post-SD, one-way RM ANOVA.

Figure 5.

A, Single section image sequence of astrocyte corresponding to the various time points shown on the electrophysiological recording of long-lasting spreading depolarization (left). The astroglial soma and processes swell during spreading depolarization and remain swollen during the late ultraslow potential (asterisk). Continued swelling is also seen at 3 h post-spreading depolarization. **B**, Data from 13 BCCAO-induced long-lasting spreading depolarizations in 13 animals show that astroglial somata remain swollen after spreading depolarization onset. Values are shown as percent of control, representing the cross-section soma area of each astrocyte. Regression line is shown (black) ($r = 0.25$, $p < 0.001$, 49 astrocytes). **C**, Same data as in **(B)** plotted as a bar graph showing a persistent increase in astroglial soma size following the long-lasting BCCAO-induced spreading depolarization. Values are shown as percent of control cross-section soma area of each astrocyte. $*p < 0.001$ relative to control, one-way RM ANOVA.