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Insulin-Like Growth Factor-1 Abrogates Microglial Oxidative Stress and TNF- α Responses to Spreading Depression

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Abstract

Spreading depression (SD), the most likely cause of migraine aura and perhaps migraine, occurs with increased oxidative stress (OS). SD increases reactive oxygen species (ROS), and ROS, in turn, can signal to increase neuronal excitability, which includes increased SD susceptibility (SDS). SD also elevates tumor necrosis factor- α (TNF- α), which increases neuronal excitability. Accordingly, we probed for the cellular origin of OS from SD and its relationship to TNF- α , which might promote SD, using rat hippocampal slice cultures. We observed significantly increased OS from SD in astrocytes and microglia but not in neurons or oligodendrocytes. Since insulin-like growth factor-1 (IGF-1) mitigates OS from SD, we determined the cell types responsible for this effect. We found that IGF-1 significantly decreased microglial but not astrocytic OS from SD. We also show that IGF-1 abrogated the SD-induced TNF- α increase. Furthermore, TNF- α application increased microglial but not astrocytic OS, an effect abrogated by IGF-1. Next, we showed that SD increased SDS, and does so via TNF- α . This work suggests that microglia promote SD via increased and interrelated ROS and TNF- α signaling. Thus, IGF-1 mitigation of microglial ROS and TNF- α responses may be targets for novel therapeutics development to prevent SD, and perhaps migraine.

INTRODUCTION

Migraine headache is a disabling neurological disorder affecting 11% of adults worldwide, with 3% experiencing chronic daily headache (Rasmussen *et al.*, 1991; Stovner *et al.*, 2007). However, existing therapies for high frequency and chronic migraine offer only a modest benefit (Mack, 2011).

Spreading depression (SD), the most likely cause of migraine aura and perhaps migraine pain (Moskowitz *et al.*, 1993; Lauritzen and Kraig, 2005), is a large negative DC shift in the interstitial space that slowly propagates and is associated with transient neuronal silence (Leão, 1944). SD is initiated by excessively increased neuronal excitability (i.e., increased excitation, reduced inhibition, or both) synchronously involving a sufficient volume of gray matter (Bureš *et al.*, 1974; Somjen, 2001). The increase in neuronal excitability necessary to initiate SD can be elicited by reactive oxygen species (ROS; Grinberg *et al.*, 2012a), and by the proinflammatory cytokine tumor necrosis factor- α (TNF- α ; Cipolla *et al.*, 2012).

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ROS are modulators of neuronal excitability (Kishida and Klann, 2007) and appear to be a critical signaling component of SD. Not only do ROS increase SD susceptibility, SD itself also increases ROS and oxidative stress (OS; Viggiano *et al.*, 2011; Grinberg *et al.*, 2012a). OS occurs as a result of an imbalance between pro- and antioxidants. Recent findings suggest that ROS (Grinberg *et al.*, 2012a), like TNF- α (Cipolla *et al.*, 2012), may be the consequence of SD that results in post-SD aberrantly increased neuronal excitability (Beattie *et al.*, 2002) and decreased inhibition (Stellwagen *et al.*, 2005). Determining the cellular sources of ROS generated by SD is thus an important next step in determining the mechanisms underlying this phenomenon.

Here we induced SD in rat hippocampal slice cultures, then exposed them to a fluorogenic probe for ROS, combined with immunolabeling for specific brain cell types. We found OS generated from SD significantly increased in astrocytes and microglia but not neurons or oligodendrocytes. Since we previously showed that insulin-like growth factor-1 (IGF-1, which functions as a neuroprotective environmental enrichment mimetic in brain; Liu *et al.*, 2001), reduces excitability from ROS and the OS of SD (Grinberg *et al.*, 2012a), we investigated which cell types were involved in this effect. We found that IGF-1 reduced the OS from SD in microglia but not astrocytes. Next, we showed that TNF- α increased OS in microglia (but not astrocytes), and that this effect was abrogated by IGF-1. Since IGF-1 appeared to decrease microglial activation by SD and TNF- α , we next probed for TNF- α involvement in SD susceptibility. We found TNF- α decreased threshold to SD, and that SD itself decreased threshold to subsequent SD, an effect dependent on TNF- α signaling. These findings suggest that controlling microglial activation is an important target for development of novel migraine therapeutics. This work has appeared in preliminary forms (Dibbern, 2012; Mitchell *et al.*, 2010b; Grinberg *et al.*, 2012b).

MATERIALS AND METHODS

Hippocampal slice culture preparation

All procedures involving animals were approved by the Institutional Animal Care and Use Committee at the University of Chicago and conducted in accordance with ARRIVE guidelines, and the guidelines of the National Institutes of Health.

Rat hippocampal slice cultures are well-suited for the study of cell type-specific OS from SD. In this preparation, electrophysiological function, and the reactive states of astrocytes and microglia parallel *in vivo* conditions (Kunkler and Kraig, 1997; Kunkler *et al.*, 2004; Hulse *et al.*, 2008), suggesting a similar baseline level of ROS. Additionally, this preparation allows for the precise control of environmental conditions of an intact brain tissue (Pusic *et al.*, 2011).

Untimed pregnant Wistar female rats (12 total pregnant female rats, ten pups per litter; Charles River, Wilmington, MA, USA) were single-housed with Enviro-dri[®] paper bedding (Shepherd, Watertown, TN, USA) and Nestlets (#NES3600, Ancare, Bellmore, NY, USA) added for nesting materials. Litters were culled to ten pups at birth. Nine to ten day old male and female rat pups were used to make 350 μ m thick hippocampal slice cultures, as previously described (Kunkler and Kraig 1997; Mitchell *et al.*, 2010a). Slice cultures were maintained in serum-based media until 18 days *in vitro*, then changed to serum-free media. Serum-free media composition, per 100 mL: Neurobasal medium (97 mL; #21103, Invitrogen, Carlsbad, CA, USA); Gem-21, (2.0 mL; #400-160-010; Gemini Bioproducts, Sacramento, CA, USA); Glutamax (1 mM; #35050, Invitrogen); Gentamicin (1 μ g/mL; #15710-064, Invitrogen); D-glucose (45%; 680 μ L; #G8769, Sigma, St. Louis, MO, USA); ascorbic acid (0.5 mM; #A4544, Sigma); Fungizone, (1 mg/mL; #15295, Invitrogen); NaCl (41 mM; #S6546, Sigma); Mg₂Cl₂ (0.8 mM; #M1028, Sigma); CaCl₂ (1.6 – 2.4 mM;

#21115, Sigma). Importantly, this serum-free media contained reduced glutathione as well as cysteine, glycine, and glutamate which are essential to maintain cellular glutathione levels. Also, the media contained ascorbate and tocopherols and the antioxidant enzymes catalase and superoxide dismutase (Gemini Bio-Products Material Data Safety Sheet; Brewer *et al.*, 1993).

At 21 days *in vitro*, cultures were screened for vitality using Sytox Green (#S7020; Invitrogen, Carlsbad, CA; Hulse *et al.*, 2008). All electrophysiological and pharmacological manipulations were performed between 21 and 35 days *in vitro* in serum-free media (Pusic *et al.*, 2011).

Electrophysiology and OS detection

Slice culture electrophysiological recordings were performed in a PDMI recording chamber in static serum-free media, as previously described (Pusic *et al.*, 2011). Briefly, slice culture vitality was evaluated by assessing the maximal field potential response to bipolar electrode stimulation at the dentate gyrus (100 μ s pulses at 0.2 Hz) and recording at the CA3 pyramidal neuron layer. Slice cultures were used if field potentials were ≥ 3 mV with stimuli of 10 – 20 μ A.

SDs were induced with stimuli of ten 100 μ s pulses at 10 Hz, with stimulus intensity of $\sim 1,000$ μ A. SDs were triggered every seven to nine minutes, for a total of six SDs over an hour. Sham controls consisted of half-maximal field potentials evoked with the same periodicity. We chose six SDs so as to model the neuronal hyperexcitability seen in migraine (Pietrobon and Moskowitz, 2012) that we have previously recapitulated in this preparation (Grinberg *et al.*, 2012).

SD induction, as described here, is a non-injurious perturbation that does not result in pyramidal neuron death, as previously shown (Kunkler *et al.*, 2004; Grinberg *et al.*, 2011). For OS experiments, immediately following either SD or sham paradigm, slice cultures were placed in CellROX™ Deep Red Reagent (Invitrogen, #C10422), a cell-permeant, fixable fluorogenic probe of ROS, for 24 hours. Because we previously found neuronal inhibitory drive to be decreased many hours after SD induction (Grinberg *et al.*, 2011) and microglial movement to be increased 24 hrs but not three days after SD (unpublished observations) we chose to examine cumulative OS over 24 hours instead of looking at the pro-oxidants produced simply during the repolarization of SD. In this way, the OS associated with the post-SD progressive inflammatory reaction and increased excitability would be registered.

SD threshold was determined with stimulation similar to SD induction above (ten 100 μ s pulses, 10 Hz), but beginning with current intensity needed for a half-maximal field potential, then doubling stimulus intensity until SD was elicited (Pusic *et al.*, 2011). The current that evoked an SD was recorded as the threshold. For experiments in Figure 4, following threshold determination, an additional five SDs were induced (for a total of six) and the slice cultures placed back into normal incubation conditions, with or without addition of pharmacological agents. Three days later, SD threshold was determined once again.

Pharmacological manipulations

For OS experiments, some slices were placed in media supplemented with IGF-1 (100 ng/mL; #4326-RG; R&D Systems, Minneapolis, MN, USA) three days prior to manipulations, as we have previously found this time/dose elicits a robust response (Grinberg *et al.*, 2012a). For OS experiments, 100 ng/mL TNF- α (#510RT, R&D Systems; Mitchell *et al.*, 2011) was applied along with CellROX™ reagent for 24hrs.

For SD threshold experiments, slices were exposed to TNF- α for three days prior to threshold determination. For a different set of experiments, following SD induction, slices were placed in media supplemented with 200 ng/mL sTNFR1 (#425R1, R&D Systems; Hulse *et al.*, 2008) for three days before re-determining SD threshold.

Fixative determination

To determine the optimal fixative for OS detection coupled to cell-specific labeling, hippocampal slices were exposed to CellROX™ with or without pro-oxidant-inducing lipopolysaccharide (LPS; 1 μ g/mL; #L6761, Sigma) overnight. LPS induces pro-oxidant production via activation of NADPH oxidase and inducible nitric oxide synthase (Li *et al.*, 2005). Slices were then collected into one of three fixatives: paraformaldehyde-lysine-periodate [PLP; 10 mL 16% paraformaldehyde (#30525-894, Alfa Aesar, Ward Hill, MA, USA), 1.096 g lysine, 0.420 g Na₂HPO₄, 0.17 g sodium periodate (#L6001, #59638, #311448, Sigma), pH 6.2]; 4% paraformaldehyde [10 mL 16% paraformaldehyde, 20 mL phosphate-buffered saline (PBS), 10 mL distilled water]; or 10% buffered formalin phosphate [4% formaldehyde w/w; sodium phosphate, monobasic, monohydrate 0.4% w/v; sodium phosphate, dibasic, anhydrous 0.65% w/v; stabilizer methanol 1.5% w/v (#SF100-4; Fisher Chemicals; Fair Lawn, NJ)] for overnight fixation at 4°C.

CellROX™ fluorescence intensity of control vs. LPS – exposed slices was determined as previously described (Grinberg *et al.*, 2012a). Briefly, the average fluorescence intensities of a uniform CA3 area of interest were recorded using MetaMorph software (v. 7.0.4; Molecular Devices, Sunnyvale, CA), captured using a Cool Snap *fx* CCD camera (Photometrics, Tucson, AZ) on an inverted Leica DM-IRBE microscope (Leica Mikroskopie und Systeme, Wetzlar, Germany) at 10x magnification (1.70 mm²) after system calibration (Hulse *et al.*, 2008; Pusic *et al.*, 2011).

Oxidized protein quantification

Immunohistochemical detection of protein oxidation (Frank *et al.*, 2002) was used to confirm CellROX™ as a marker of OS from SD. Briefly, protein carbonyls were derivatized by 2,4-DNPH to produce 2,4-dinitrophenyl hydrazones. These dinitrophenyl (DNP) groups that now label oxidized proteins were then immunohistochemically detected using an anti-DNP antibody tagged with AlexaFluor488 (#A11097; Invitrogen). Hippocampal slices were collected for detection of protein oxidation 24 hrs following SD induction so as to confirm that SD has a long-lasting effect on tissue redox status. Fluorescence intensity in hippocampal slices was assessed as previously described (Grinberg *et al.*, 2012a).

Cell-specific labeling

Immunohistochemistry was used to label neurons, astrocytes, and oligodendrocytes. Following 24 hour CellROX™ incubation, hippocampal slice cultures were fixed in 10% buffered formalin phosphate overnight. Slices were first transferred to blocking solution (90 mL PBS, 0.75 mL Triton X-100, 10 mL goat serum), followed by primary antibody (1:1000 mouse-anti-NeuN; 1:1000 mouse-anti-GFAP; or 1:160 mouse-anti-RIP; Millipore, #MAB377, #MAB3402, and #MAB1580, respectively). Secondary antibody for all cell types was 1:100 AlexaFluor® 488 goat-anti-mouse IgG (#A11029, Invitrogen). Following immunostaining, slices were mounted onto gelatin-coated slides and coverslipped using ProLong® Gold Antifade (#P36930; Invitrogen).

Isolectin GS-IB₄ was used to label microglia (AlexaFluor®488; from *Griffonia simplicifolia*, #I21411, Invitrogen) at 50 μ g/mL in PBS supplemented with 0.1 mM each CaCl₂, MgCl₂, and MnCl₂ (Sigma, #21115, #M1028, and #M1787, respectively). Slices were mounted and coverslipped as above.

All cell labeling included negative controls, where primary antibodies or isolectin were excluded to assure that there is no non-specific labeling.

Confocal microscopy

Confocal images were acquired using a Leica TCS SP5 II AOBS laser scanning confocal microscope (University of Chicago Integrated Light Microscopy Core Facility). Confocal imaging section thickness was 772 nm to minimize the possibility of cell body overlap in the z-axis. CellROX™ fluorescence was acquired with excitation wavelength 633 nm and detecting at 648 – 712 nm. AlexaFluor488 was detected with excitation wavelength 488 and detecting at 501 – 542 nm.

Image processing and quantification

All image pairs (i.e. sham vs. experimental) were adjusted equally, converted to RGB using green for cell-specific labeling and red for CellROX™, and overlapped using Image J (J 1.43i; National Institutes of Health public access). Figures were produced using CorelDraw (v.X3; Corel, Ottawa, ON, Canada) and Photoshop (v.CS2; Adobe, San Jose, CA) software.

Cell-specific OS red-green overlap images of CellROX™ and cell type labeling were color-inverted for better detection of cell-specific ROS signal –i.e. where cell type labeling is pink and OS labeling is blue. A blinded investigator selected OS-labeled cell bodies in these images using MetaMorph software. Then, matching OS-only images were thresholded, and integrated optical density of the selected cell type-specific OS quantified. Thresholding between sham and experimental group images was always equivalent. Three to five confocal images were taken per slice and cell type-specific OS quantified and averaged for each hippocampal slice culture.

This methodology allows for quantification of the cumulative OS contributed by a particular cell type per area of interest. Since microglial proliferation has been described following SD (Tamura *et al.*, 2004), we also quantified the average OS per microglial cell and the total number of microglia per area of interest/group (see Supporting Information for details).

TNF- α quantification

TNF- α protein in hippocampal slices was determined as previously described (Hulse *et al.*, 2008). Briefly, hippocampal slices were collected six hours after induction of SD, and total protein determined in homogenized slices using the MicroBCA assay (#23235; Pierce, Rockford, IL, USA). TNF- α levels were determined using the Bio-Plex Pro™ Reagent Kit (#171-304070, #171L1025M; Bio-Rad, Hercules, CA, USA).

Statistical analysis

Data were analyzed using SigmaStat software (v.3.5; Systat Software, Chicago, IL). All data were subject to normality testing (p -value to reject: 0.05) and equal variance testing (p -value to reject: 0.05). Controls in each experiment were set to 1.0 with experimental data scaled proportionally. Experimental groups consisted of biological replicates of $n = 3$. Statistical tests are noted in the figure legends.

RESULTS

CellROX™ fixative selection

We first optimized tissue fixation for CellROX™ detection of OS from LPS exposure. The rationale for this experiment was based on the plan to couple OS measurements to specific cell types within brain. PLP fixative is ideal for immunostaining against microglial CD11b

(Caggiano *et al.*, 1996), whereas other fixatives (e.g., 4% paraformaldehyde) reduce detection of this antigen. Importantly, appropriate fixative choice was also essential for detection of OS using CellROX™ (Fig. 1c; $n = 9$ slices/group). Only use of 10% buffered formalin phosphate provided fixation that allowed detection of a significant ($p < 0.001$) change in OS (control: 1.00 ± 0.08 , LPS: 1.67 ± 0.08). Other fixatives either resulted in high control levels, such as with PLP (control: 1.78 ± 0.06 , LPS: 1.81 ± 0.09) or muted fluorescence in LPS-exposed slices, such as with 4% paraformaldehyde (control: 1.21 ± 0.06 , LPS: 1.20 ± 0.07). Accordingly, we used 10% buffered formalin phosphate for all experiments and as a result opted to label microglia with isolectin GS-IB₄.

SD increased OS

We recently showed that OS increases following SD using CellROX™ (Grinberg *et al.*, 2012a). Here we confirm this finding via an alternate label of OS: protein carbonyl levels (Fig. 1d). 24 hours following SD, oxidized protein levels were significantly ($p = 0.039$) increased when compared to sham controls (1.00 ± 0.08 and 1.38 ± 0.17 for sham and SD slices, respectively; $n = 8$ and 3 slices, respectively.) Unfortunately, while tissue slice detection of protein carbonyl is possible (Frank *et al.*, 2002), this technique does not allow for fixation and subsequent determination of cell type-specific OS levels.

SD did not affect OS in neurons or oligodendrocytes

We next looked at cell type-specific oxidative stress in hippocampal slices. Surprisingly, neuron-specific OS did not change following SD (Fig. 2a). There was a non-significant ($p = 0.63$; $n = 4$ slices/group) OS increase in SD-exposed neurons (1.38 ± 0.65) when compared to sham controls (1.00 ± 0.34). Furthermore, both the SD-exposed and the sham control neurons rarely showed strong CellROX™ labeling.

There was also no significant ($p = 0.96$; $n = 3$ slices/group) difference in oligodendrocyte-specific OS between sham control (1.00 ± 0.87) and SD-exposed (0.95 ± 0.48) slices (Fig. 2b). As in neurons, oligodendrocytes did not show substantial CellROX™ labeling. In fact, many of the oligodendrocytes imaged registered no staining (i.e., values of zero), therefore requiring transformation of experimental values via the use of $(n + 1)$ for statistical comparison of low experimental values (Snedecor and Cochran, 1989).

SD increased OS in astrocytes and microglia

Following double-label CellROX™ and immunohistochemistry for glial fibrillary acidic protein (GFAP), a marker of most astrocytes (Walz and Lang, 1998), SD significantly ($p = 0.019$) increased OS in astrocytes (1.00 ± 0.28 and 7.55 ± 1.70 in sham and SD-exposed astrocytes, respectively; $n = 3$ slices/group; Fig. 2c). The CellROX™ fluorescence was diffusely distributed around the astrocyte somata. GFAP labeling also appeared to be increased in SD-exposed astrocytes when compared to sham controls, consistent with astrogliosis from SD (Kraig *et al.*, 1991).

SD also significantly ($p = 0.018$) increased microglial OS (1.00 ± 0.22 and 16.27 ± 1.34 in sham and SD-exposed microglia; $n = 3$ slices/group; Fig. 2d). Consistent with previous findings, SD-exposed microglia appeared to be activated to a primed state, characterized by larger cell bodies and shorter and thicker branches, when compared to sham (Gehrman *et al.*, 1993; Caggiano and Kraig, 1996). Microglial OS appeared consistently perinuclear, brighter and more compartmentalized than in astrocytes.

IGF-1 mitigated microglial but not astrocytic OS from SD

When slices were pretreated with IGF-1 for three days prior to SD induction, no change in astrocyte-specific OS from SD was observed ($p = 0.65$; 1.00 ± 0.11 and 0.85 ± 0.28 for SD

and SD + IGF-1 astrocytes, respectively; $n = 3$ slices/group; Fig. 3a). However, microglial-specific OS in slices pretreated with IGF-1 was significantly ($p = 0.018$) decreased when compared to slices exposed to SD alone (0.08 ± 0.02 and 1.00 ± 0.24 , respectively; $n = 3$ slices/group; Fig. 3b). The morphology of microglia from slices treated with IGF-1 also appeared to be less activated than microglia exposed to SD alone (Gehrman *et al.*, 1993; Caggiano and Kraig, 1996).

OS per microglial cell increased with SD, an effect abrogated by IGF-1

We found that the microglial contribution to the total levels of tissue OS was significantly increased by SD (Fig. 2d). This consisted of a significant increase in OS per microglial cell that could be abrogated by IGF-1 (Fig. S1a), and a trend toward an increase in the number of microglia per area of interest (Fig. S1b). This trend is consistent with previous findings of microglial proliferation following SD (Tamura *et al.*, 2004).

Increased TNF- α protein from SD was abrogated by IGF-1

Here we confirm previous findings that TNF- α protein increases with SD (Kunkler *et al.*, 2004) and extend these results to show that IGF-1 abrogates this effect of SD. TNF- α protein significantly ($p = 0.01$) increased with SD and was abrogated by IGF-1 preincubation (1.00 ± 0.04 , 1.62 ± 0.20 , 0.71 ± 0.13 for sham, SD, and SD + IGF-1 slices, respectively; $n = 3$ –4 slices/group; Fig. 4a).

TNF- α increased microglial OS, an effect abrogated by IGF-1

SD occurs with a microglia-specific increase in TNF- α (Hulse *et al.*, 2008), a proinflammatory cytokine that can increase ROS production (Han *et al.*, 2009; Shoji *et al.*, 1995). Here we confirmed TNF- α – mediated increase in microglial OS and examine whether IGF-1 could mitigate this cytokine response (Fig. 4b). Twenty-four hour TNF- α application significantly increased ($p = 0.005$) microglial OS and this increase in microglial OS was abrogated by preincubation in IGF-1 (1.00 ± 0.19 , 18.25 ± 6.22 , 1.92 ± 0.49 in control, TNF- α , and TNF- α + IGF-1 – treated hippocampal slice cultures, respectively; $n = 6$ –7 slices/group). However, TNF- α application did not induce astrocytic OS (Fig. S2).

TNF- α increased SD susceptibility

Since we previously showed that ROS application increased susceptibility to SD (Grinberg *et al.*, 2012a), we examined whether application of TNF- α and its subsequent increase in microglial OS (see above) similarly affected SD threshold. We found that TNF- α application significantly decreased SD threshold (Fig. 4c). Slices exposed to TNF- α for three days had significantly ($p = 0.002$) decreased threshold to SD (0.52 ± 0.03 , $n = 10$) when compared to controls (1.00 ± 0.10 , $n = 37$).

SD increased susceptibility to subsequent SD, in a TNF- α – dependent manner

SD increases SD-promoting cytokine and ROS production, and here we extended these findings to determine whether SD itself will promote subsequent SD. We found SD led to a significantly increased susceptibility to subsequent SD when threshold was reevaluated three days later in the same slice (Fig. 4d). SD threshold was significantly ($p < 0.001$) reduced three days after (0.15 ± 0.03 , $n = 13$) initial induction of SD (1.00 ± 0.10 , $n = 17$). This SD-induced increase in susceptibility to subsequent SD could be abrogated by sTNFR1 application after initial SD induction (Fig. 4e). SD threshold to subsequent SD (1.00 ± 0.10 , $n = 17$) was significantly ($p < 0.001$) increased via application of 200 ng/mL sTNFR1 (345 ± 29 ; $n = 15$) for three days.

Microglial OS from SD is blocked by sTNFR1

Since TNF- α increased microglial OS (Fig. 4b), we examined whether TNF- α was necessary for the increase in microglial OS from SD. We induced SD and incubated in CellROX™ as above, with some incubations also supplemented with 200 ng/mL sTNFR1 to absorb TNF- α . TNF- α was necessary for the increased microglial OS from SD: sTNFR1 application significantly decreased ($p = 0.036$) microglial OS from SD (1.00 ± 0.27 and 0.13 ± 0.03 for SD and SD + sTNFR1, respectively; $n = 3$ slices/group; Fig. 4f). That is, inclusion of sTNFR1 with SD returned microglial OS to a level comparable to that seen in sham controls (Fig. 2). Thus, increased TNF- α is a predominant signal responsible for increased microglial OS from SD.

DISCUSSION

Here we show for the first time that increased OS from SD is specific to astrocytes and microglia in rat hippocampal slice cultures. We also show that IGF-1 mitigation of OS from SD is specific to microglia, and that increased microglial OS from SD is mediated by TNF- α signaling. Lastly, we demonstrate that SD promotes subsequent SD, and does so via TNF- α .

CellROX™ is unique in that it is a fixable and highly photostable ROS marker. Since CellROX™ reacts collisionally with pro-oxidants, thus competing with endogenous antioxidants, it effectively functions as a marker of excessive ROS, and thus OS. We confirmed CellROX™ labeling of OS by also assessing oxidized protein carbonyl content (Fig. 1d), which showed a similar increase in OS from SD using carbonyl levels as we previously observed using CellROX™ (Grinberg *et al.*, 2012a). Additionally, while GFAP is a marker of most astrocytes (Walz and Lang, 1998), we have no evidence of how OS might change in GFAP⁺ astrocytes. However, both GFAP⁺ and GFAP⁺ astrocytes have been recently shown to have very similar metabolic gene expression (Lovatt *et al.*, 2007). Therefore, our results are likely to reflect the OS response to SD of astrocytes in general. Finally, it is important to note that we measured cumulative CellROX™ at 24 hrs following SD, and not simply during the SD repolarization.

OS is the result of an imbalance between pro-oxidants and antioxidant systems, and can result in oxidative damage and increased neuronal excitability (Pellmar, 1987; Kishida and Klann, 2007). Cells generate ROS as byproducts of oxidative metabolism as well as during normal physiological signaling processes (Thannickal and Fanburg, 2000; Murphy, 2009; Sorce and Krause, 2009). In order to protect against the potentially damaging effects of pro-oxidants, cells produce antioxidant enzymes and utilize small molecule antioxidants such as glutathione and ascorbate (Thannickal and Fanburg, 2000; Dringen, 2005).

SD results in a rise in brain tissue OS (Grinberg *et al.*, 2012a) and a rise in hydrogen peroxide (Viggiano *et al.*, 2011), which unlike most other ROS can cross cell membranes, travel long distances, and can dismutate into the highly disruptive hydroxyl radical (Cardoso *et al.*, 2012). Since SD results in a 10-fold increase in local brain metabolic rate (Bureš *et al.*, 1974; Dienel and Hertz, 2001), and a small fraction of all oxygen utilized by mitochondria become ROS (Murphy, 2009), we suggest that in part, the increased OS from SD arises from oxidative metabolism. SD also results in increased production of inflammatory mediators and activation of genes involved in inflammatory pathways such as TNF- α , matrix metalloproteinase-9, interleukin-1 β , interleukin-6, cyclooxygenase-2, and nitric oxide synthase (Jander *et al.*, 2001; Kunkler *et al.*, 2004; Thompson and Hakim, 2005; Hulse *et al.*, 2008). Along with ROS directly (Zhou *et al.*, 2009), these signaling molecules can activate inflammatory pro-oxidant production pathways (Han *et al.*, 2009).

Surprisingly, SD increased astrocytic but not neuronal OS. Due to the high metabolic load of SD (Bureš *et al.*, 1974; Dienel and Hertz, 2001), some OS was expected in neurons, the principal cells traditionally believed to bear the metabolic burden of activity (for review see: Wong-Riley, 1989; 2012) and until recently were believed to be the principal cells involved in brain glucose oxidation (Lovatt *et al.*, 2007). Furthermore, astrocytes are widely believed to have a higher antioxidative potential than neurons, and neurons are associated with high vulnerability to OS-induced injury (Wilson, 1997; Dringen, 2000; Bélanger *et al.*, 2011). However, since we saw increased astrocytic and not neuronal OS, our findings may provide *functional* evidence to confirm and extend the recent anatomical evidence for the high metabolic capacity and abundant mitochondria of astrocytes (Lovatt *et al.*, 2007; Bélanger *et al.*, 2011).

Additionally, astrocytes express NADPH oxidase isoforms, and ROS produced by NADPH oxidase play important roles in astrocytic survival, signaling, and production of inflammatory mediators (Sorce and Krause, 2009). Caggiano and Kraig (1998) also observed that astrocytic nitric oxide synthase levels increase following SD in rat brain. CellROX™ is reported to robustly detect a range of pro-oxidants, including superoxide anion, hydroxyl radical, peroxynitrite, and to a lesser extent, nitric oxide (Invitrogen communication). Thus, the above sources of pro-oxidants may all be contributing to the astrocytic CellROX™ signal.

While the literature shows oligodendroglia to be very sensitive to OS (Thorburne and Juurlink, 1996; Dewar *et al.*, 2003), their capacity to generate ROS is unclear. Indeed, our results show that these cells do not contain significant levels of OS after SD. However, there is evidence to indicate that oligodendrocytes are affected *by* SD and its incurred OS (Tamura *et al.*, 2004; Pusic and Kraig, 2011). Thus, the lack of CellROX™ labeling of oligodendrocytes suggests that SD-induced demyelination (Pusic and Kraig, 2011) may be mediated by other cell types, perhaps microglia (Pusic and Kraig, unpublished observations).

Some microglial ROS production was expected following SD, since SD induces both morphological and functional changes consistent with microglial activation. SD induces microgliosis (Gehrman *et al.*, 1993; Caggiano and Kraig, 1996) and is associated with upregulation of proinflammatory factors (Caggiano and Kraig, 1996; Kunkler *et al.*, 2004). Following SD, microglia increase production of TNF- α and other proinflammatory cytokines (Jander *et al.*, 2001; Kunkler *et al.*, 2004; Hulse *et al.*, 2008), which can signal to induce ROS production by mitochondria and NADPH oxidase (Shoji *et al.*, 1995; Han *et al.*, 2009; Zhou *et al.*, 2009). We have described microglia to be the predominant cell type producing TNF- α after SD, as well as cold-preconditioning (Hulse *et al.*, 2008; Mitchell *et al.*, 2011) in hippocampal slice cultures. Microglia have also recently been described to move long distances following SD (Grinberg *et al.*, 2011), an effect that likely also includes NADPH oxidase activity at the leading edge of lamellipodia (Ushio-Fukai, 2009), which could increase dispersion of ROS and TNF- α to distant regions of brain. Future studies will identify the specific pro-oxidant species involved in SD-induced OS and which species are altered by IGF-1 treatment.

SD-induced microgliosis and astrogliosis have previously been observed to be separable phenomena. Pharmaceutical treatments such as dexamethasone exposure, inactivation of lipoxygenase, or increased nitric oxide signaling inhibits microgliosis but not astrogliosis (Caggiano and Kraig, 1996). Here we extend these observations by showing IGF-1 too can influence OS from SD in microglia but not astrocytes (Fig. 3). Furthermore, we show that TNF- α increased microglial but not astrocytic OS (Fig. 4, S2). While astrocytes possess TNF receptors and have well-described responses to TNF- α (Buffo *et al.*, 2010) their

production of pro-oxidants in response to TNF- α , outside of primary culture conditions, has been limited.

We show that IGF-1 decreases microglial OS by altering TNF- α signaling, which is necessary and sufficient for increased microglial but not astrocytic OS (Fig. 4, S2). IGF-1 receptor activation increases nuclear factor kappa B, promoting the TNF pro-survival complex I formation over that of the TNF pro-apoptotic complex II (Wang *et al.*, 2003; Han *et al.*, 2009). While astrocytes have been well-characterized as capable of producing TNF- α (Buffo *et al.*, 2010), our previous findings show that microglia are the predominant cell type responsible for TNF- α expression after SD (Hulse *et al.*, 2008) and cold-preconditioning (Michell *et al.*, 2011), which are non-injurious but proinflammatory events in hippocampal slice cultures. The IGF-1 receptor is found on neurons, oligodendroglia, astrocytes and endothelial cells (Mendez *et al.*, 2006). However, while microglia produce IGF-1 during development and in response to injury (Scheepens *et al.*, 2000), to our knowledge there is no direct evidence that microglia possess the receptor for IGF-1. It is thus possible that IGF-1 alteration of microglial responses to SD involves other brain cell types or is mediated by microglial IGF-II receptor or perhaps insulin receptor cross-reactivity (Sugimoto *et al.*, 2002; Suh *et al.*, 2010).

We have previously shown that two sequelae of SD, TNF- α and OS, can excessively increase neuronal excitability (Cipolla *et al.*, 2012; Grinberg *et al.*, 2012a). SD has previously been shown to decrease neuronal inhibition (Kruger *et al.*, 1996; Grinberg *et al.*, 2012a), and migraine is a disorder characterized by deficient regulation of cortical excitability (Pietrobon and Moskowitz, 2012). We thus hypothesized that SD itself may increase susceptibility to subsequent SD. Indeed, here we show that SD decreases electrical threshold to subsequent SD (Fig. 4d). Furthermore, we show that this effect is dependent upon TNF- α signaling (Fig. 4c-f).

Though the pathogenesis of migraine remains controversial and incompletely defined, there is substantial evidence for SD involvement in both migraine with and without aura (Moskowitz *et al.*, 1993; Lauritzen and Kraig, 2005). Furthermore, SD increases neuronal excitability, consistent with the cortical excitability phenotype seen in migraineurs (for review see Pietrobon and Moskowitz, 2012). Our findings support the hypothesis that recurrent SD activates microglia to increase neuronal excitability and prime brain for subsequent SD (Kraig *et al.*, 2010). We have previously shown IGF-1 significantly decreases SD susceptibility (Grinberg *et al.*, 2012a). Here, we show IGF-1 abrogated the *microglial* responses to SD that would otherwise excessively increase neuronal excitability. This work further supports IGF-1 as a novel therapeutic for mitigation of SD, and thus perhaps migraine. Since IGF-1 is an endogenously-produced compound that can easily enter brain (Liu *et al.*, 2001) and is likely to have a high benefit/risk ratio, our findings support further investigation into its efficacy as a potential migraine therapeutic.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. YYG, MED, VAL, and RPK were involved in experimental design, acquisition and interpretation of data. YYG and RPK wrote the manuscript. All authors reviewed the final version of the manuscript and approved its submission.

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YYG and RPK, along with Aya D. Pusic, Heidi M. Mitchell, and Marcia P. Kraig have filed an international patent application (PCT/US2012/047683) entitled "Treatments for Migraine and Related Disorders" on July 20, 2012 which partially involves development of IGF-1 as a therapeutic for migraine.

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Abbreviations

GFAP	Glial fibrillary acidic protein
IGF-1	insulin-like growth factor-1
OS	oxidative stress
ROS	reactive oxygen species
SD	spreading depression
TNF-α	tumor necrosis factor- α

Figure 1. Experimental paradigm used to assess cell type-specific oxidative stress from spreading depression

(a) Six spreading depressions (SD) were evoked over the course of an hour by stimulating at the dentate gyrus and recording at CA3. (b) Sham stimuli consisted of six half-maximal field potentials induced using the same periodicity as SD. (c) For optimal fixative determination, hippocampal slice cultures were exposed to CellROX™ ± lipopolysaccharide (LPS) overnight before fixation-harvest. Only 10% buffered formalin phosphate (10% BFP) resulted in a significant ($*p < 0.001$) increase in CellROX™ fluorescence intensity. Neither paraformaldehyde-lysine-periodate (PLP) nor 4% paraformaldehyde (4% PF) fixation resulted in a significant increase in oxidative stress from LPS as judged by relative CellROX™ fluorescence above control (C). (d) Oxidized protein carbonyl levels, a well-established marker of OS, were significantly ($*p = 0.039$) increased in SD-exposed slices, when compared to controls, thus confirming CellROX™ results. Images show CA3 area (dotted line) fluorescence from exemplary control (C) and SD slices. Cal bar, 100 μ m. All values are mean $\pm$ S.E.M., with groups compared via (c) ANOVA plus Holm-Sidak *post-hoc* testing and (d) Student's *t*-test.

Figure 2. Oxidative stress from spreading depression preferentially increased in astrocytes and microglia

Hippocampal slice cultures were incubated in oxidative stress marker CellROX™ for 24 hours following sham stimulation (Sham) or spreading depression (SD). (a) Neurons were then labeled using anti-NeuN antibody (pink). Neuron-specific OS (blue) intensity in SD-exposed slices was not different from sham controls ($p = 0.63$). (b) Oligodendrocytes were labeled using anti-RIP antibody (pink). Oligodendrocyte-specific OS (blue) intensity was not different in SD-exposed slices, when compared to sham controls ($p = 0.96$). (c) Astrocytes were labeled using glial fibrillary acidic protein (pink). Astrocyte-specific OS (blue) in SD-exposed slices was significantly increased when compared to sham controls (* p

= 0.019). (d) Microglia were labeled using isolectin GS-IB4 (pink), and microglia-specific OS (blue) was significantly ($*p = 0.018$) higher in SD-exposed slices, when compared to sham controls. Arrowheads point to exemplary cells. All values are mean $\pm$ S.E.M., with groups compared via Student's *t*-test. Cal bar, 30 μ m.

Figure 3. IGF-1 abrogated microglial but not astrocytic oxidative stress from spreading depression

Hippocampal slice cultures were exposed to 10 nM IGF-1 for three days prior to induction of spreading depression (SD). Immediately following SD, slices were incubated for 24 hours in CellROX™ (blue), then labeled for astrocytic marker GFAP (a; pink) or microglial marker isolectin IB4 (b; pink) and quantified (arrowheads point to exemplary cells). Astrocyte-specific oxidative stress following SD was not different in IGF-1 – exposed slices, when compared to sham controls ($p = 0.65$). In contrast, microglia-specific oxidative stress following SD was significantly ($*p = 0.018$) decreased in IGF-1 – exposed slices. All values are mean $\pm$ S.E.M., with groups compared via Student's t -test. Cal bar, 30 μ m.

Figure 4. Spreading depression (SD) promoted subsequent SD via TNF- α signaling, which can be abrogated by IGF-1

(a) TNF- α protein was significantly ($*p = 0.01$) increased in spreading depression (SD)-exposed hippocampal slices cultures, an effect that was abrogated by preincubation in 10 nM IGF-1. (b) Microglia-specific oxidative stress was significantly ($*p = 0.006$) increased by 24 hr application of 100 ng/mL TNF- α , an effect that was abrogated by a 3-day preincubation in 10 nM IGF-1. Exemplary images of microglia (pink) from TNF- α – exposed slices and TNF- α + IGF-1 – exposed slices are shown, with CellROX™ fluorescence (blue). Cal bar, 15 μ m. (c) SD threshold was significantly ($*p = 0.002$) decreased in slices exposed to 100 ng/mL TNF- α for three days. (d) SD threshold was significantly ($*p < 0.001$) decreased in slices after induction of six SDs three days prior. (e) This increased SD susceptibility shown in (d) was abrogated by sTNFR1 exposure. The decreased SD threshold for subsequent SDs seen in (d) was significantly increased ($*p < 0.001$) when slices were exposed to 200 ng/mL sTNFR1 immediately following initial SD induction. (f) sTNFR1 also significantly ($*p = 0.036$) decreased microglial oxidative stress from SD. All values are mean $\pm$ S.E.M., with groups compared via (a) ANOVA plus Holm-Sidak *post-hoc* testing and (b-f) Student's *t*-test.