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ORIGINAL ARTICLE

Insulin-like growth factor-1 lowers spreading depression susceptibility and reduces oxidative stress

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Abstract

Spreading depression (SD), the likely cause of migraine aura and perhaps migraine, is triggered by widespread and unfettered neuronal hyperexcitability. Migraine and the initiating hyperexcitability of seizure, which involve oxidative stress (OS), are likely interrelated. Environmental enrichment (EE) decreases seizure and can reduce migraine. EE's well-characterized neuroprotective effect involves insulin-like growth factor-1 (IGF-1). Accordingly, we asked if IGF-1 could mitigate the hyperexcitability that initiates SD using rat hippocampal slice cultures. We demonstrate that IGF-1 significantly decreased SD susceptibility and related OS. We mimicked OS of SD and observed that IGF-1 abolished hyperexcitability from OS. Application of an antioxidant significantly decreased SD susceptibility and co-administration of an antioxidant with IGF-1 produced no additive effect, whereas an oxidizer significantly increased SD, and this effect was abrogated by IGF-1. Moreover, IGF-1 significantly decreased baseline OS, despite seemingly paradoxically increasing CA3 bursting. These results suggest that IGF-1 increased endogenous antioxidants to levels sufficient to buffer against the OS of SD. Insulin similarly mitigated SD susceptibility, but required a far greater dose. Since brain IGF-1 increases with EE, and, like insulin, independently functions as an EE mimetic, we suggest that EE mimetics are a novel source of therapeutics for SD, and by extension, migraine.

Keywords: antioxidants, hippocampus, IGF-1, migraine, reactive oxygen species, slice culture.

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Migraine and its maladaptive transformation to high frequency and chronic migraine (HFCM) are immense healthcare burdens affecting 11% of the adult population worldwide, with 3% experiencing chronic daily headache (Rasmussen *et al.* 1991; Stovner *et al.* 2007). In the United States, these maladies result in annual medical and lost worktime costs of $30 billion (Hu *et al.* 1999; Goldberg 2005), yet existing therapies, largely centering on the use of anticonvulsants, are only modestly effective for treatment of HFCM (Mack 2011).

Spreading depression (SD) is a slowly propagating loss of neuronal activity that is the most likely cause of migraine aura and perhaps migraine pain (Moskowitz *et al.* 1993; Lauritzen and Kraig 2005). SD is associated with increased brain hydrogen peroxide concentration, likely as the result of the increased metabolic demands associated with this phenomenon (Viggiano *et al.* 2011). SD is preceded (Bureš *et al.* 1974; Somjen 2001), and importantly, followed by increased synaptic activity, as recently shown (Grinberg *et al.* 2011). Synchronous and excessively increased brain excitability in a sufficient brain volume is necessary to trigger SD (Bureš *et al.* 1974; Somjen 2001; Kunkler *et al.* 2005). Furthermore, without sufficient time for compensatory adaptation, recurrent epochs of excessively increased synaptic activity from repeated SDs may lower SD initiation threshold, and therefore be a determinant of HFCM (Kraig

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Abbreviations used: EE, environmental enrichment; HFCM, high frequency and chronic migraine; IGF-1, insulin-like growth factor-1; OS, oxidative stress; SD, spreading depression.

et al. 2010). Experiments performed using hippocampal slice cultures support this suggestion (Mitchell *et al.* 2010a).

In contrast, physiologically increased neuronal activity from environmental enrichment [(EE); i.e., increased physical, intellectual, and social volitional opportunities], which necessarily occurs with sufficient time for adaptive change, has the opposite effect. EE is a well-recognized preconditioning stimulus that induces neuroprotection (Will *et al.* 2004). Notably, EE occurs with physiologically increased neuronal activity and increased hippocampus-based learning and memory (van Praag *et al.* 2000; Kraig *et al.* 2010) and EE reduces excessive hyperexcitability from seizures (Young *et al.* 1999; Kraig *et al.* 2010). Furthermore, there is evidence that EE reduces SD (Guedes *et al.* 1996) and improves migraine (Darabaneanu *et al.* 2011).

Since insulin-like growth factor-1 (IGF-1) is a primary determinant of neuroprotection from EE (Carro *et al.* 2001), we hypothesized that IGF-1 might act as an EE-mimetic that would reduce SD susceptibility. Our results here show that exposure to IGF-1 triggered a significant reduction in SD susceptibility, a protective effect that involves reduced oxidative stress (OS) and reduced hyperexcitability. Furthermore, insulin, which enhances hippocampus-based memory when delivered to the brain via nasal administration (Stockhorst *et al.* 2004; Craft *et al.* 2011) had a similar protective effect against SD, but at a dose orders of magnitude greater than required for IGF-1. Moreover, while IGF-1 decreased the hyperexcitability for SD, it also increased spontaneous CA3 area bursting activity, consistent with the electrophysiological changes in hippocampus-based learning (Yanovsky *et al.* 1995). Our results provide the first evidence that EE-based signaling (i.e., involving IGF-1) can lead to the development of novel therapeutics to prevent SD, and by extension, perhaps recurrent and HFCM. This work has appeared in preliminary form (Grinberg and Kraig 2011).

Materials and Methods

Culture preparation, maintenance, and electrophysiology

Experimental procedures were approved by the Institutional Animal Care and Use Committee at The University of Chicago Medical Center and followed Animal Research: Reporting of *In Vivo* Experiments guidelines.

Slice cultures were prepared (Mitchell *et al.* 2010b) from 9-day-old male or female pups of Wistar rats (Charles River, Wilmington, MA, USA) and SD induced as previously described (Grinberg, *et al.*, 2011; Pusic *et al.* 2011). All electrophysiological procedures were performed in serum-free media, containing (per 100 mL): Neurobosal 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 μg/mL; #15710-064; Invitrogen); D-glucose [(45%), 680 μL; #G8769; Sigma, St. Louis, MO, USA]; ascorbic acid (0.5 mM; #A4544; Sigma); Fungizone, (1 mg/mL; #15295; Invitrogen); NaCl (41 mM; #S6546; Sigma); Mg_2Cl_2 (0.8 mM; #M1028; Sigma); $CaCl_2$ (1.6 – 2.4 mM; #21115; Sigma). The normalcy of slice culture electrophysiological function was verified by recording CA3 area field potentials evoked from bipolar electrical stimuli applied to the dentate gyrus (100 μs pulses, ≤ 0.2 Hz). The recording microelectrode was moved along the long axis of pyramidal neurons at the genu of CA3 until field potential excitatory post-synaptic responses were maximal (Fig. 1). Slices with CA3 field post-synaptic potential responses ≥ 3 mV (with applied currents of 10–20 μA) were used for experiments. The Matlab commands 'filter' and 'butter' (Matlab 7.1; Mathworks, Natick, MA, USA) were used to filter the digitized data with a second-order high-pass Butterworth filter with a 1 Hz cutoff frequency for the signals shown in Fig. 5.

The SD threshold was determined by progressively increasing the amount of current applied [10 pulses, 10 Hz (100 μs/pulse)], starting with the current needed to produce a half-maximal field potential from a single 100 μs pulse (Fig. 1). If a given current intensity did not trigger SD, the amount of current applied was doubled and re-applied 1–2 min later until SD occurred. Applied currents ranged from 10 to 10 000 nC. Unless otherwise stated, experimental agents were applied 'acutely' (i.e., 15–30 min before electrophysiological procedures). For OS measurements, SD was triggered six times over an hour (i.e., ∼ every 9 min).

While the number of SDs that can occur with migraine and HFCM has not been established, others suggest that chronic migraine may occur with only one SD per day. However, this experimental paradigm results in decreased brain excitability in anesthetized animals (Sukhotinsky *et al.* 2011), suggesting that the number of SDs and recovery period are critical to establishing a migraine model (Kraig *et al.* 2010). We chose six SDs so as to replicate the hyperexcitable phenotype seen in the brain of migraneurs (Palmer *et al.* 2000; Mulleners *et al.* 2001; Welch 2005; Brennan 2011) and after recurrent SD in hippocampal slice cultures (Mitchell *et al.* 2010a). Following SD, slice cultures were returned to normal incubation conditions with fresh media until fixation-harvest 24 h later.

Slice culture excitability change from hydrogen peroxide exposure was assessed by noting the CA3 area field potential response to a single dentate gyrus 100 μs half-maximal current pulse applied 30 min after exposure.

Experimental manipulations

IGF-1 (40 or 100 ng/mL; #4326-RG; R&D Systems, Minneapolis, MN, USA) or insulin (400 μg/mL; #0355; Invitrogen) was added to media either acutely or 3 days before (and during) electrophysiological studies of SD threshold responses. For experiments involving SD and OS, slice culture IGF-1 was refreshed after SD. Hydrogen peroxide (50 or 200 μM; #H1009; Sigma) or ascorbate (2 mM) were added to serum-free media acutely before (and during) electrophysiological recordings. This hydrogen peroxide concentration was chosen to mimic concentrations produced by SD *in vivo* (Viggiano *et al.* 2011) and the ascorbate concentration is fourfold that of the serum-free media, consistent with physiological levels (Rice 1999). Sham control cultures only experienced normal media.

Measurement of oxidative stress

Oxidative stress of the CA3 area was determined using CellROX™ Deep Red Reagent (#C10422; Invitrogen), a fixable fluorogenic probe that fluoresces (near-infrared) when oxidized. After SD, slice

cultures were incubated for 24 h in normal media supplemented with CellROX™ (5 μM) followed by fixation using 10% phosphate-buffered formalin (#SF100-4; Fisher Chemicals, Fair Lawn, NJ, USA) for 24 h. Then, slice cultures were mounted on gelatin-coated glass slides and coverslipped using Prolong® Gold antifade reagent (#P36930; Invitrogen).

To indirectly assess antioxidant content, slice cultures were exposed to a standard load of OS, namely 50 μM/L menadione (#47775; Sigma-Aldrich) for 1 h. Menadione participates in redox cycling reactions at the mitochondrial electron transport chain, leading to production of superoxide anions (Thor *et al.* 1982). Slices were then incubated in 5 μM CellROX™ for 30 min, followed by fixation in 10% phosphate-buffered formalin for 24 h. Hydrogen peroxide could not be used as a mimetic of SD-induced OS here, since we found that it interferes with the CellROX™ reporter molecule (data not shown). Cell death, as measured by Sytox Green (Hulse *et al.* 2008; Mitchell *et al.* 2010b), was not observed following hydrogen peroxide or menadione treatment at harvest times described above. There was also no pyramidal neuron death following SD induction, IGF-1 exposure, or any combination thereof (data not shown).

Fluorescence intensity of CellROX™ was measured using a Cool Snap *fx* CCD camera (Photometrics, Tucson, AZ, USA) on an inverted Leica DM-IRBE microscope (Leica Mikroskopie und Systeme, Wetzlar, Germany) and MetaMorph (v. 7.0.4) software (Molecular Devices, Sunnyvale, CA, USA). Fluorescence intensity (i.e., average fluorescence intensity/area) was registered for a uniform CA3 area of interest (used throughout experiments) at 10× magnification (i.e., 1.70 mm^2). Before acquisitions, the imaging system was calibrated to register uniform full image intensity (1500/4096) to a standard (480 nm excitation; 527 nm emission) 125 μM/L acridine orange (100 mg/L; #A6014; Sigma) solution imaged through a hemacytometer.

Statistical analysis

Data were analyzed using SigmaStat (V. 3.5) software (Systat Software, Chicago, IL, USA). Control data for each experiment were set to 1.0 with related group data scaled proportionally to allow for inter-experimental comparisons. All experimental group measurements were compared to same-day sham and/or control cultures and reported as mean ± standard error of mean. Specific statistical tests used are indicated in the figure legends. CorelDraw (v. X3; Corel, Ottawa, ON, Canada) and Photoshop (v. CS2; Adobe, San Jose, CA, USA) were used to produce figures.

Results

IGF-1 (and insulin) significantly increased SD threshold

Hippocampal slices were exposed to IGF-1 either acutely (i.e., 15–30 min), for 3 days, or for 7 days prior to assessing the SD threshold. The 7-day IGF-1 exposure was performed phasically to better mimic anticipated effects of EE [i.e., exercise–rest intervals (Will *et al.* 2004; Kraig *et al.* 2010)], where slices were exposed to IGF-1-supplemented media in the day and returned to regular media at night. Acute, 3-day,

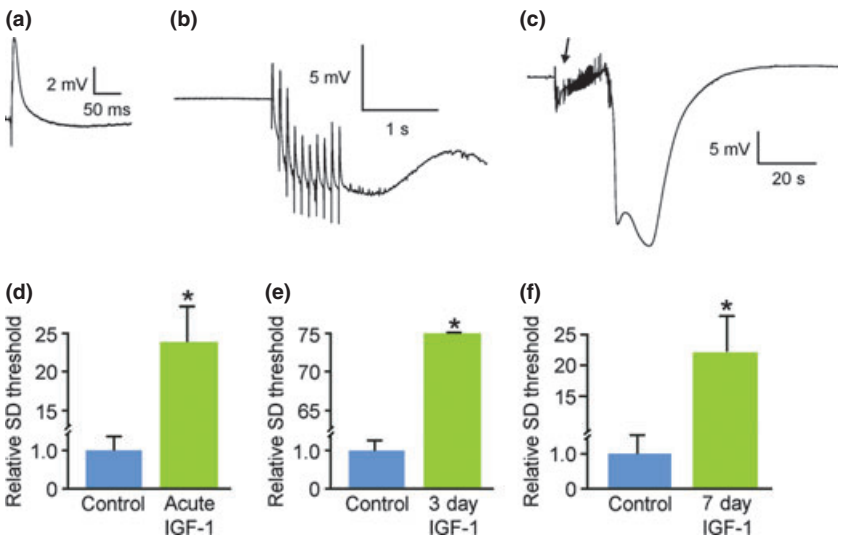

Fig. 1 IGF-1 reduced spreading depression (SD) susceptibility in hippocampal slice cultures. (a) Exemplary CA3 area evoked field potential. Experiments begin with establishment of the current intensity needed to evoke maximal field potential responses; stimuli of half-maximal intensity were then used to elicit subsequent field potentials. Only those cultures with CA3 pyramidal neuron post-synaptic responses of at least 3 mV were used for experiments. (b) CA3 response to dentate gyrus bipolar stimulation (10 pulses at 10 Hz, 500 μA) was used to elicit a SD, as shown in (c). (c) The spreading depression shown here was induced by the stimulation/response (arrow) shown in (b). (d) Average current necessary to induce SD (SD threshold) was significantly (*$p = 0.001$) higher when slice cultures were exposed to 40 ng/mL IGF-1 acutely ($n = 6$ and 7 for control and experimental slices, respectively). (e) Similarly, average SD threshold was also significantly (*$p < 0.001$) increased when slice cultures were exposed to IGF-1 for 3 days prior to SD ($n = 8$ and 7 for control and experimental slices, respectively). (f) Finally, average SD threshold was significantly (*$p < 0.001$) increased when slice cultures were exposed to IGF-1 for 7 days prior to SD ($n = 11$ and 6 for control and experimental slices, respectively.) Comparisons between groups made via Student's *t*-test.

and 7-day exposure to IGF-1 all significantly increased SD threshold compared to control by 24, 75, and 22-fold (Fig. 1). Furthermore, 3-day exposure to insulin [(400 μg/mL); but not lower insulin doses, i.e., 6, 12, and 100 μg/mL (n = 3–9/group)] resulted in a significantly (p = 0.03) higher SD threshold versus control [i.e., 22.60 ± 9.60 (n = 8) and 1.00 ± 0.20 (n = 9), respectively]. However, the insulin dose needed for this protective effect was 15 500-fold higher than IGF-1 (i.e., 70 μM versus 4.5 or 10 nM), suggesting that IGF-1 has greater therapeutic utility against SD. Accordingly, we focused our subsequent work on IGF-1.

IGF-1 significantly reduced OS from SD

Since SD may increase OS (Viggiano *et al.* 2011), OS can enhance brain excitability (Muller *et al.* 1993; Gulati *et al.* 2005; Waldbaum and Patel 2010), and IGF-1 is involved in antioxidant signaling (see Discussion), we next tested whether IGF-1 treatment altered SD-induced OS. Results show that acute, 3-day, and 7-day treatment with IGF-1 significantly reduced OS from SD (Fig. 2). Seven-day exposure was again phasic, as described for SD threshold studies above. While acute treatment with IGF-1 led to a 20% decrease in OS from SD, 3-day exposure to IGF-1 afforded an even greater level of protection, with a 30% decrease in OS from SD, and 7 days offered a 73% decrease in OS from SD.

SD susceptibility is modulated by OS

Slices were exposed to either ascorbic acid or hydrogen peroxide and SD threshold was assessed. Ascorbate (2 mM) significantly increased the SD threshold, while hydrogen peroxide (50 μM) significantly decreased the SD threshold (Fig. 3). Co-exposure to IGF-1 and a higher dose of hydrogen peroxide (200 μM) led to a significant decrease in the SD threshold when compared with IGF-1 alone. However, 50 μM hydrogen peroxide co-exposed with IGF-1 was an insufficient oxidant stress to overwhelm the protective effect of IGF-1 on SD susceptibility (Fig. 3). Finally, co-incubation of slice cultures with ascorbate and IGF-1 (n = 8) did not significantly raise the threshold for SD versus IGF-1 alone (n = 7; p = 0.28 with relative SD threshold levels of 7.39 ± 6.16 and 1.00 ± 0.31, respectively).

IGF-1 eliminated effects of SD-mimetics on excitability and OS

We further assessed the ability of IGF-1 to reduce slice culture excitability by decreasing OS. First, we mimicked OS from SD by application of hydrogen peroxide. This exog-

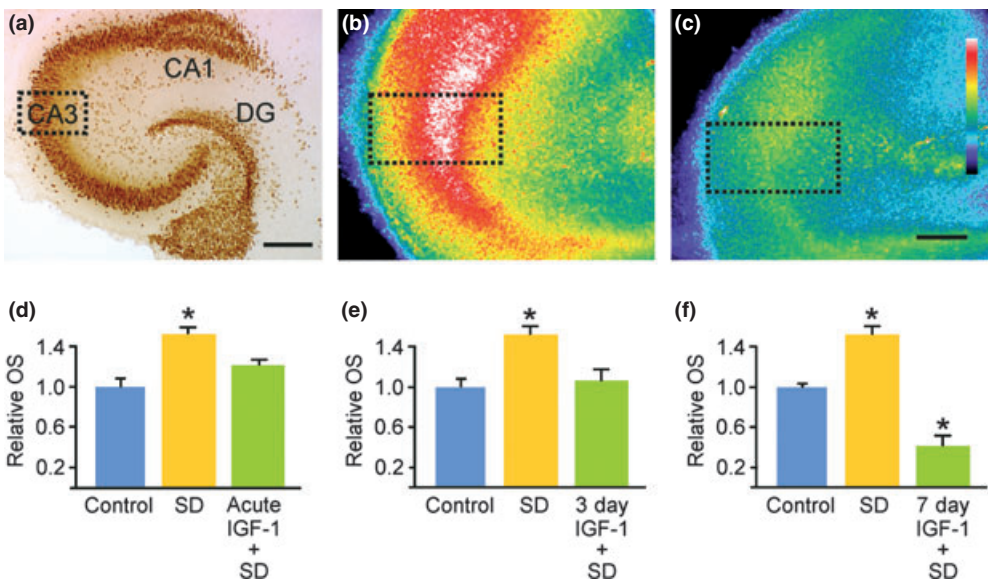

Fig. 2 IGF-1 decreased oxidative stress from spreading depression. (a) NeuN immunohistochemical labeling of a hippocampal slice culture, for cytoarchitectural reference and to show the CA3 area of interest (dotted line box) used for quantification of oxidative stress (OS) via CellROX™ fluorescence intensity. (b, c) Representative CellROX™-labeled hippocampal slices exposed to SD (b) and to 3-day IGF-1 incubation followed by spreading depression (SD) (c). Dotted line boxes illustrate CA3 areas of interest used for relative OS quantifications. (d) OS was significantly (*p = 0.008) increased from controls after hippocampal slice cultures were exposed to SD, and this effect was abrogated when exposed to IGF-1 acutely (n = 21, 12, and 9 for control, 'SD', and 'SD + IGF-1' slices, respectively). (e) Similarly, the significantly (*p = 0.007) increased OS induced by SD was abrogated when slices were exposed to IGF-1 for 3 days prior to SD (n = 21, 8, and 6 for 'Control', 'SD', and 'SD + IGF-1' slices, respectively). (f) The significant increase in OS from SD (*p < 0.001), when compared with controls, was significantly reduced (*p < 0.001) in slices exposed to IGF-1 for 7 days prior to SD induction (n = 21, 12 and 3 for 'Control', 'SD' and 'SD + IGF-1' slices, respectively). Note: IGF-1 exposure was continued for the additional 24 h CellROX™ incubation. Scale bars = 400 μm (a) and 200 μm (b and c). Comparisons between groups were made via ANOVA plus Holm–Sidak *post hoc* testing.

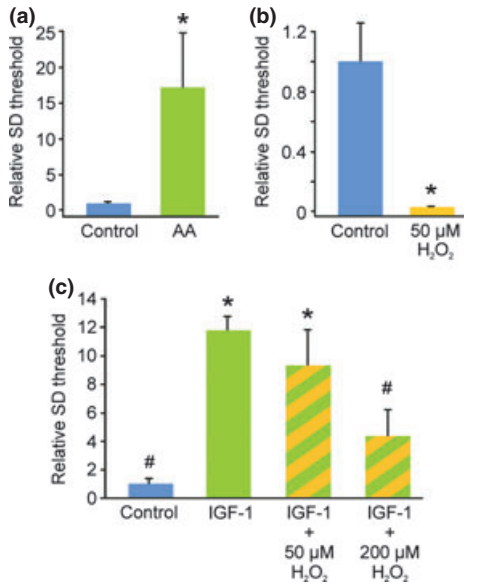

Fig. 3 The exemplary antioxidant ascorbate reduced, whereas the oxidizer hydrogen peroxide increased, spreading depression susceptibility, with the latter effect abrogated by IGF-1. (a) Average current necessary to induce spreading depression (SD; i.e., SD threshold) was significantly (*$p = 0.018$) higher when slice cultures were acutely exposed to ascorbate (AA; $n = 8$) when compared with controls ($n = 12$). (b) In contrast, average current necessary to induce SD was significantly lower (*$p < 0.001$) when slice cultures were exposed to 50 μM hydrogen peroxide (H_2O_2; $n = 8$ and 11 for control and experimental slices, respectively). (c) While IGF-1 triggered a significant protection from SD susceptibility (*$p < 0.0001$) and this effect continued when co-administered with 50 μM H_2O_2, the higher dose of 200 μM H_2O_2 abrogated this effect to a non-significant difference from control ($n = 14$, 16, 5, and 9 for control, IGF-1, IGF-1 + 50 μM H_2O_2, and IGF-1 + 200 μM H_2O_2, respectively). When compared with IGF-1, SD thresholds of controls and IGF-1 + 200 μM H_2O_2 were significantly decreased (#$p \leq 0.00001$). Comparisons between groups were made via Student's *t*-test (a, b) or ANOVA plus Holm–Sidak *post hoc* testing (c).

enously induced OS significantly increased evoked slice hyperexcitability (Fig. 4), like that seen from SD (Mitchell *et al.* 2010a). Both 3-day and acute exposure to IGF-1 abrogated this hydrogen peroxide-induced hyperexcitability. Second, we additionally mimicked OS from SD by slice exposure to menadione (Fig. 4). As expected, this treatment triggered a significant increase in slice OS, an effect that was abrogated by acute and 3-day exposure to IGF-1. In fact, 3-day exposure to IGF-1 alone could significantly reduce baseline OS from control levels. Furthermore, 7-day exposure to IGF-1 also significantly reduced baseline OS levels by 26% when compared with controls ($p = 0.001$; $n = 11$ and 9 for controls and 7-day IGF-1, respectively). The latter is important because exposure to IGF-1 alone, which led to the significant reductions in baseline OS (Fig. 4), triggered a significant increase in spontaneous CA3 bursting (Fig. 5).

Discussion

Here we show that IGF-1 mitigated SD susceptibility and decreased its associated OS. Furthermore, we show that OS induced hyperexcitability and increased SD susceptibility, and that IGF-1 mitigated these effects. Finally, our results reveal that IGF-1 treatment lowered baseline levels of OS and simultaneously increased spontaneous activity of CA3 pyramidal neurons. These are the first results to indicate that a neuroprotective EE mimetic, IGF-1, prevents SD.

Evidence suggests that EE leads to a physiological increase in neuronal excitability that prevents SD. While SD increases aberrant hyperexcitability (Kruger *et al.* 1996) that makes brain tissue more susceptible to future SD (Mitchell *et al.* 2010a; Grinberg *et al.* 2011), EE increases physiological neuronal excitability associated with improved learning and memory (Eckert and Abraham 2010; Kumar *et al.* 2011). EE protects from the aberrant hyperexcitability of seizure (Young *et al.* 1999; Kraig *et al.* 2010), and has been shown to reduce SD (Guedes *et al.* 1996). Furthermore, the neuroprotective effects of EE have also recently been shown to include migraine (Darabaneanu *et al.* 2011). While various molecular mechanisms of how EE produces these neuroprotective effects have been characterized (Gagné *et al.* 1998; van Praag *et al.* 2000; Ekstrand *et al.* 2008; Herring *et al.* 2010; Kempermann *et al.* 2010), the role of IGF-1 is particularly noteworthy.

IGF-1 mediates the neuroprotective effects of EE (Carro *et al.* 2001), and improves learning and memory (Sonntag *et al.* 2000). With EE, IGF-1 production is increased and its active uptake by the brain increases in an activity-dependent manner (Nishijima *et al.* 2010). Once in the brain, IGF-1 increases spontaneous hippocampal neuronal activity and improves hippocampus-dependent learning and memory test performance (Lupien *et al.* 2003; Xing *et al.* 2007; Miltiadous *et al.* 2011). Mechanisms by which IGF-1 affects neuronal activity may include increasing ionic conductances (Blair and Marshall 1997; Kanzaki *et al.* 1999), modulating neurotransmitter receptor activity (Gonzalez de la Vega *et al.* 2001; Ramsey *et al.* 2005), and decreasing generation of reactive oxygen species (Csiszar *et al.* 2008; Pérez *et al.* 2008). Important to our work here, increased neuronal activity enhances neural antioxidant production (Papadia *et al.* 2008). Furthermore, IGF-1 has been shown to similarly increase antioxidant production in multiple peripheral tissues (Jallali *et al.* 2007; Csiszar *et al.* 2008). Here we show that IGF-1 reduced OS in control hippocampal slices, a preparation which shows spontaneous, physiological neuronal activity. In fact, this spontaneous activity increased after IGF-1 exposure, a phenomenon that should elevate metabolic activity and therefore the generation of reactive oxygen species. Despite this, we found that net OS significantly declined. We speculate that this results from neural activity-dependent signaling involving increased antioxidant production, as first shown by Papadia *et al.* (2008). While beyond the scope of

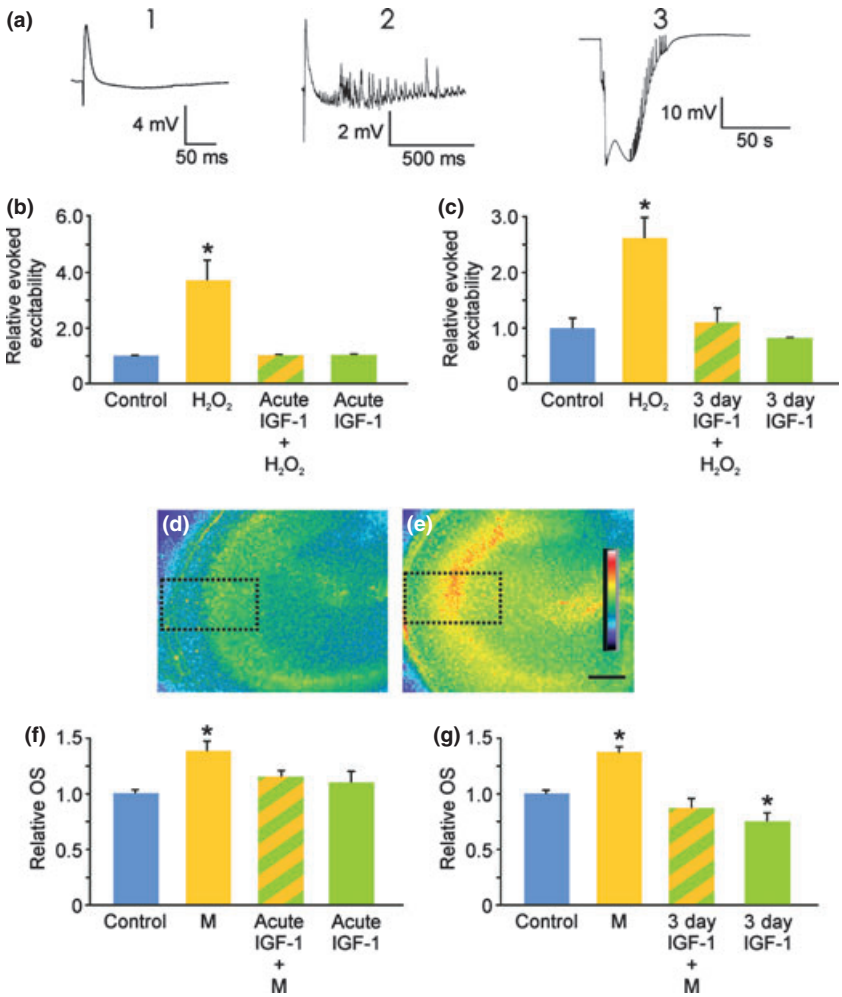

Fig. 4 IGF-1 decreased CA3 oxidative stress and its related hyperexcitability. (a) Slice culture excitability in response to oxidative stress (OS) was further characterized by classifying evoked potential changes to a single current pulse that normally triggered a half-maximal field potential response where a normal field potential (FP; left) was rated '1'; a FP that included stimulus-related bursting activity (center) was rated '2'; and a stimulus that resulted in spreading depression (right) was rated is a '3'. Relative evoked excitability was determined as a sum of responses seen (e.g., responses of '2' and '3' yielded an overall excitability score of 5). Responses were measured 30 min after exposure to hydrogen peroxide (H_2O_2). (b) Exposure to H_2O_2 ($n = 7$) triggered a significant (*$p < 0.001$) increase in evoked excitability compared with control ($n = 8$) and this increase was abrogated by acute application of IGF-1 ($n = 4$) to a non-significant difference from control. IGF-1 exposure alone ($n = 4$) had no significant impact on slice CA3 area-evoked excitability (i.e., showed a response of '1'). (c) Three-day exposure to IGF-1 had a similar impact on slice culture OS-increased excitability mimicked by application of H_2O_2. H_2O_2 significantly (*$p < 0.001$) increased slice excitability ($n = 15$) compared with control ($n = 18$). Pretreatment with IGF-1 for 3 days ($n = 9$) reduced the H_2O_2-induced increased excitability to a non-significant difference from control. (d, e) Exemplary images of control (d) slice culture OS compared with increased slice OS induced by exposure to menadione (e). Calibration bar, 200 μm. Dotted boxes indicate CA3 areas of interest used for relative OS quantifications (f, g). (f) Exposure to menadione (M; $n = 12$) triggered a significant (*$p < 0.001$) increase in OS compared with control ($n = 18$). Acute treatment with IGF-1 ($n = 15$) reduced OS from menadione to a non-significant ($p = 0.15$) difference from control ($n = 18$). Acute IGF-1 exposure alone ($n = 18$) did not reduced slice culture OS from control. (g) Pretreatment with IGF-1 for 3 days ($n = 15$) also reduced menadione-induced significant increase in OS (*$p < 0.001$; $n = 12$) to a non-significant ($p = 0.148$) difference from control ($n = 15$). In addition, IGF-1 pretreatment alone ($n = 18$) significantly (*$p = 0.008$) reduced slice culture OS compared with control.

our current report, future studies should be designed to directly confirm that IGF-1 can lead to increased antioxidant production in the brain.

We show that SD induces increased tissue OS, as other work has suggested (Viggiano *et al.* 2011). OS increases hyperexcitability (Muller *et al.* 1993; Gulati *et al.* 2005; Waldbaum and Patel 2010). We confirm and extend these findings to show that OS-induced CA3 hyperexcitability can lead to SD. Furthermore, we show that IGF-1 mitigated the amount of OS generated by SD, SD susceptibility, OS-induced SD susceptibility, as well as the hyperexcitability of OS. Finally, the impact of IGF-1 protection from SD-

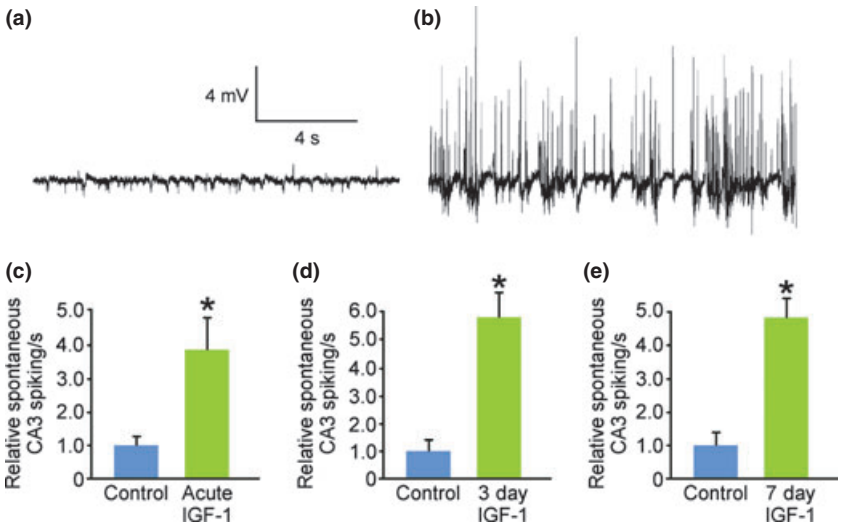

Fig. 5 IGF-1 increased spontaneous neuronal spiking activity. (a) Exemplary recording of unstimulated control CA3 pyramidal layer spontaneous electrophysiological activity. (b) Exemplary recording of 3-day IGF-1-exposed CA3 pyramidal layer spontaneous electrophysiological activity. (c) Acute IGF-1 treatment ($n = 9$) triggered a significant (*$p = 0.03$) increase in spontaneous CA3 pyramidal neuron spiking compared with control ($n = 6$). (d) Three-day IGF-1 treatment also ($n = 7$) triggered a significant (*$p = 0.001$) increase in spontaneous CA3 pyramidal neuron bursting compared with control ($n = 6$). (e) Similarly, 7-day IGF-1 treatment also ($n = 6$) triggered a significant (*$p < 0.001$) increase in spontaneous CA3 pyramidal neuron bursting compared with control ($n = 7$). Comparisons between groups made via ANOVA plus Holm–Sidak *post hoc* testing.

induced OS increased with time. Together, these results suggest that the effects of IGF-1 on OS (and, therefore, SD susceptibility) may involve an adaptive response, consistent with physiological-conditioning hormesis (Radak *et al.* 2008). These effects may help to entrain brain tissue away from the unfettered hyperexcitability needed for SD and toward physiological excitability and decreased OS.

IGF-1 is highly protective in stroke (Liu *et al.* 2004; Rizk *et al.* 2007; Fletcher *et al.* 2009). SDs occur spontaneously in the penumbra of stroke. The number and cumulative duration of the SDs occurring there are proportional to the growth in infarct volume (Mies *et al.* 1993; Dijkhuizen *et al.* 1999; Nakamura *et al.* 2010). Our results may indicate that the mechanism by which IGF-1 decreases stroke size involves decreasing the spontaneous SDs occurring in the penumbra of stroke.

Although we demonstrate the proof of principle that the EE-mimetic IGF-1 decreases OS and SD susceptibility, a complete analysis of the optimal dose, duration, and frequency of treatment requires further study. As a first step, we chose to administer 7-day IGF-1 phasically to better mimic the inherently phasic effects of EE, as well as to avoid potentially harmful effects of prolonged tonic application of agents, such as those seen with corticosterone (De Kloet *et al.* 1999; Zoladz and Diamond 2009).

We suspect that the 24-fold reduction in SD susceptibility seen with acute IGF-1 exposure that further increased to 75-fold at 3 days before settling to 22-fold at 7 days reflects an adaptive, damped oscillatory response, commonly seen in biological systems (Stark *et al.* 2007; Paszek *et al.* 2010; Wang *et al.* 2012). In contrast, the progressive reduction in OS from IGF-1 by 20%, 30%, and 73% at these time points suggests that a maximal steady-state has not yet been reached. Thus, whether OS too would show a damped oscillatory or a more simple sigmoid response pattern remains unclear. However, collectively, these results suggest that with optimal dosing, the ability of IGF-1 to protect against SD via reduced OS can be expected to be at least 20-fold.

While insulin is already recognized as an agent that increases learning and memory (Stockhorst *et al.* 2004; Craft *et al.* 2011), its ability to influence SD has not been previously examined. We show that insulin protects against SD and hypothesize that it does so, like IGF-1, via its actions as an EE-mimetic (i.e., by increasing processes associated with improved learning and memory, such as CA3 bursting). However, while insulin mitigated SD susceptibility, we show that IGF-1 has this effect at a 15 500-fold smaller dose, suggesting that the effects of insulin may occur via cross-reactivity with the IGF-1 receptor. We thus conclude that neuroprotective EE-mimetics are promising targets against SD, and by extension migraine and HFCM, with IGF-1 shown here to be a novel and potentially effective therapeutic.

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