Published in final edited form as:

Epilepsy Res. 2012 May ; 99(3): 293–305. doi:10.1016/j.epilepsyres.2011.12.011.

Hypoxia enhances high-voltage-activated calcium currents in rat primary cortical neurons via calcineurin

Kun Xiang^{1,2}, Brian L. Behrle^{1,2,3}, Elizabeth I. Tietz², and L. John Greenfield Jr^{1,2,3,*}

¹Department of Neurology, University of Toledo College of Medicine, Toledo, OH, USA

²Department of Physiology & Pharmacology, University of Toledo College of Medicine, Toledo, OH, USA

³Department of Neurology, University of Arkansas for Medical Sciences Little Rock, AR, USA

Abstract

Hypoxia regulates neuronal ion channels, sometimes resulting in seizures. We evaluated the effects of brief sustained hypoxia (1% O₂, 4 h) on voltage-gated calcium channels (VGCCs) in cultured rat primary cortical neurons. High-voltage activated (HVA) Ca²⁺ currents were acquired immediately after hypoxic exposure or after 48 h recovery in 95% air/5% CO₂. Maximal Ca²⁺ current density increased 1.5-fold immediately after hypoxia, but reverted to baseline after 48 h normoxia. This enhancement was primarily due to an increase in L-type VGCC activity, since nimodipine-insensitive residual Ca²⁺ currents were unchanged. The half-maximal potentials of activation and steady-state inactivation were unchanged. The calcineurin inhibitors FK-506 (in the recording pipette) or cyclosporine A (during hypoxia) prevented the post-hypoxic increase in HVA Ca²⁺ currents, while rapamycin and okadaic acid did not. L-type VGCCs were the source of Ca²⁺ for calcineurin activation, as nimodipine during hypoxia prevented post-hypoxic enhancement. Hypoxia transiently potentiated L-type VGCC currents via calcineurin, suggesting a positive feedback loop to amplify neuronal calcium signaling that may contribute to seizure generation.

Keywords

Calcium channel; phosphatase; patch-clamp electrophysiology; FK-506; cyclosporine A

1. Introduction

Brain neurons can tolerate moderate to severe hypoxia for several hours (Erecinska and Silver, 2001; Scheufler et al., 2002) as long as glucose is present to meet minimal metabolic demands via glycolysis (Kahlert and Reiser, 2000; Vannucci and Vannucci, 2000; Vannucci and Vannucci, 2001). However, hypoxia can predispose to seizures, which occur in 9% of

© 2011 Elsevier B.V. All rights reserved.

*Contact information: Dept. of Neurology, University of Arkansas for Medical Sciences, 4301 W. Markham St., Slot 500, Little Rock, AR 72205, Phone: (501) 686-7236, Fax: (501) 686-7850.

Current address of first author: Kun Xiang: Dept. of Internal Medicine, University of Toledo College of Medicine, Toledo, OH 43614

Disclosures

None.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

patients after stroke (Bladin et al., 2000) and up to 36% of patients after cardiopulmonary arrest (Krumholz et al., 1988). The risk of epilepsy after perinatal hypoxia is 5-fold greater than in normal neonates (Bergamasco et al., 1984; Jensen, 2002). Thus, understanding the cellular mechanisms mediating hypoxia-induced neuronal dysfunction is clinically relevant and could lead to new treatments for hypoxia-induced seizures and epilepsy.

Transmembrane ion channels play important roles in oxygen (O_2)-regulated cellular processes (Hammarstrom and Gage, 1998; Jiang et al., 1994; Tai and Truong, 2007). We previously reported that transient hypoxia reduces inhibitory GABA_A receptor (GABA_AR) function in NT2-N neurons (Gao et al., 2004) and primary cortical neurons (Wang and Greenfield, Jr., 2009) and that the reduction in cortical neurons was blocked by the L-type voltage-gated calcium channel (L-VGCC) antagonist, nitrendipine (Wang and Greenfield, Jr., 2009). L-VGCCs play a critical role in mediating intracellular Ca^{2+} signaling, activity-dependent synaptic plasticity and neuronal survival (Berridge, 1998; Catterall, 2000), and are likely involved in post-hypoxic signaling mechanisms. However, the sensitivity of neurons to hypoxia varies by neuron type and brain region, and cortical neurons are more resistant to O_2 deprivation than hippocampal or cerebellar neurons (Krnjevic, 2008). For example, Li et al. (Li et al., 2007) reported a delayed reduction in Ca^{2+} currents in hippocampal CA1 but not CA3 neurons, associated with increased cell death in CA1 neurons. Moreover, regulation of L-VGCCs after deprivation of both O_2 and glucose may differ markedly from hypoxia alone. The effects of short term hypoxia on high voltage activated (HVA) Ca^{2+} currents in primary rat cortical neurons have not been thoroughly investigated. Regulation of cortical neurons HVA Ca^{2+} currents by hypoxia may contribute to cortical neuron hyperexcitability and thus predispose to seizures.

At least four types of HVA VGCCs (L-, N-, P/Q- and R-type) have been identified in neurons, with diverse regulatory mechanisms that are specific to VGCC subtypes (Catterall, 2000). One important regulatory factor is the Ca^{2+} /calmodulin-dependent phosphatase, calcineurin (also known as protein phosphatase 2B). Both positive and negative regulation of VGCC activity by calcineurin have been reported, varying across cell types (Groth et al., 2003) and model systems (Zeilhofer *et al.*, 2000; Norris et al., 2002; Oliveria *et al.*, 2007; Norris et al., 2008), suggesting that the physiological regulation of VGCCs by calcineurin is complex and variable depending on the neurons involved. Since seizure generation is a predominantly cortical activity, it is critical to understand how seizure-inducing stimuli like hypoxia affect cortical neuron VGCCs and the possible role of calcineurin in this response.

Here, we evaluated HVA Ca^{2+} currents in rat primary cortical neurons in culture using whole-cell voltage clamp recordings after exposure to 1% O_2 for 4 h. HVA Ca^{2+} currents were increased immediately (0–2 h) after 4 h hypoxia but returned to baseline when recorded after 48 h normoxic recovery. The increase in HVA current was blocked by nimodipine (NIM), and hence L-VGCC-dependent. Inhibition of calcineurin activity with FK-506 or cyclosporine A (CsA) blocked the post-hypoxic increase in L-VGCC current. Our results suggest that O_2 deprivation transiently increases L-VGCC activity in cortical neurons via a calcium dependent process requiring L-VGCC activation and calcineurin, suggesting a positive feedback loop to amplify neuronal calcium signaling after hypoxia. These findings may have clinical significance, since hypoxia-induced increases in intracellular Ca^{2+} after stroke or cardiopulmonary arrest may contribute to post-hypoxic neuronal hyperexcitability, cell death or epileptogenesis.

2. Methods

2.1 Ethical approval

Experimental protocols involving the use of vertebrate animals were approved by the University of Toledo College of Medicine Institutional Animal Care and Use Committee (IACUC) and conformed to United States National Institutes of Health guidelines.

2.2 Tissue preparations

2.2.1 Cell cultures—Primary cultures of cortical neurons were prepared from E18 fetal Sprague-Dawley rats according to a protocol slightly modified from established techniques (Porter et al., 1997). Briefly, E18 rat fetuses were removed under sterile conditions after euthanization of the dam. Fetal cortices were dissected in sterile Hank's Balanced Salt Solution (HBSS) and digested for 5 min at 37°C using 0.25% trypsin-ethylene diaminetetraacetic acid (EDTA) in HBSS, then repeatedly washed in HBSS. Tissues were further triturated in Spinner's modification of Eagle's minimum essential medium (SMEM) containing both horse serum (HS, 5%) and fetal bovine serum (FBS, 5%). The cell suspension was plated onto 35 mm plastic culture dishes (Corning Inc., Corning, NY) pre-coated with poly-D-lysine at $2\text{--}3 \times 10^5$ cells/ml in SMEM plus 5% FBS and 5% HS. 5-Fluoro-2'-deoxyuridine (FUDR) and uridine were added 48 h after plating to prevent non-neuronal cell proliferation. Half of the medium was exchanged for fresh SMEM/HS three times a week. Cells were maintained in humidified atmosphere with 5% CO₂ at 37 °C for 2 weeks and used for experiments between 13–15 days *in vitro* (DIV).

2.2.2 Hypoxia and reoxygenation—Culture medium (SMEM/HS) was deoxygenated by bubbling for 10 min with 95% N₂/5% CO₂ and warmed to 37°C. Regular SMEM/HS in culture dishes were then replaced with deoxygenated SMEM/HS and placed in an O₂- and CO₂-controlled incubator (Innova CO-48, New Brunswick Scientific Co. Inc.) pre-equilibrated to 1% O₂, 5% CO₂ at 37 °C for 4 h. After hypoxic exposure, the medium was replaced with fresh aerated SMEM/HS and neurons were either studied immediately (within 2 h of termination of hypoxia) or returned to the normoxic incubator (95% air/5% CO₂, 37 °C) for 48 h prior to recording (within $\pm$ 2 h after 48 h recovery). Control neurons were similarly handled but maintained in a normoxic environment and solutions.

2.3 Electrophysiology

2.3.1 HVA Ca²⁺ current recording—HVA currents were recorded under whole-cell voltage-clamp conditions at room temperature. The external solution contained (in mM): NaCl 110; HEPES 10; TEA chloride 25; KCl 5.4; CaCl₂ 5; 4-AP 5; MgCl₂ 1; D-glucose 25; TTX 1 μ M pH 7.4. The patch pipettes (4–6 M Ω) contained (in mM): CsF 110; TEA chloride 25; phosphocreatine 20; phosphocreatine kinase 50 units/mL; EGTA 10; HEPES 10; NaCl 5; MgCl₂ 2; CaCl₂ 0.5; BaCl₂ 0.5; MgATP 2; NaATP 0.1; pH 7.3. Currents were recorded with an Axoclamp 200B amplifier (Molecular Devices, Union City, CA) using a Digidata 1440A AD/DA converter and pClamp 10.2 acquisition software (Molecular Devices, Union City, CA). Current signals were low-pass filtered at 1 kHz and digitized at 10 kHz. After establishing the whole-cell configuration, neurons were allowed to stabilize for 5 min before current recording protocols were initiated. Neurons were voltage clamped at -80 mV. Peak HVA calcium currents (pA) were activated with a protocol modified from (Norris et al., 2002) and (Sochivko et al., 2003) using 200 ms voltage steps to voltage levels from -75 to $+40$ mV in 5 mV increments. The steady-state inactivation of Ca²⁺ currents was evaluated with a 200 ms test pulse to $+10$ mV, preceded by a 1500 ms conditioning pre-pulse ranging from -80 mV to $+10$ mV in 5 mV increments. Currents were corrected for linear, non-specific leak currents and capacitive transients. Junction potentials were nulled in the bath using the pipette offset control. Series resistance was typically under 10 M Ω , compensated

by at least 80%, and did not differ between control and post-hypoxic cells. Neurons in which the access resistance changed >20% during the course of an experiment were excluded from statistical analyses. Cell membrane capacitance was measured shortly after obtaining whole-cell configuration using the “Membrane Test” function of Clampex 10.2. Current density was calculated as a function of cell membrane capacitance (pF) as an estimate of cell size.

2.3.2 Ca²⁺ current analysis—Whole-cell calcium currents were analyzed off-line using Clampfit 10.2 software. Current density (pA/pF) was defined as peak current amplitude divided by membrane capacitance and plotted as a function of membrane potential (mV). Activation curves were constructed using individual conductance values for each cell from the current/voltage plot using the equation $g = I/(V_t - V_r)$, where V_t was the test voltage, V_r was the reversal potential extrapolated from individual I/V curves (usually near +60 mV) and I was the measured current (Sochivko et al., 2003). The conductance was then normalized to the maximum conductance ($g_{\max}$) and plotted against activating voltage. Activation and inactivation curves were constructed from peak currents elicited by the current/voltage or steady state inactivation protocols by fitting normalized peak conductance, $g/g_{\max}$, with a Boltzmann equation: $g/g_{\max} = 1/(1 + \exp[(V - V_h)/k])$, where V_h was the potential where g was half of $g_{\max}$ and k was a factor proportional to the slope at V_h . Curve fitting was performed using Prism 4.0 software (Graph Pad Software Inc., San Diego, CA). Data are reported as mean $\pm$ standard error of the mean (SEM). Statistical analyses were performed using Student's *t*-test or ANOVA with *post hoc* Bonferroni correction for multiple comparisons. Asterisks denote significant differences between compared groups at $p < 0.05$.

2.4 Drug solutions and drug application

FK-506 (tacrolimus), cyclosporine A (CsA), rapamycin (RAP), okadaic acid (OKA) and nimodipine (NIM) were dissolved in DMSO and stored in light-tight vials at -20°C . Drugs were diluted 1000-fold in extracellular buffer to the specified concentration, the DMSO concentration in the recording solution not exceeding 0.1%. VGCC antagonists were dissolved in external recording solution and delivered using a pressure-driven, modified U-tube “multipuffer” system (Greenfield, Jr. and Macdonald, 1996) with continuous, rapid perfusion of external solution when drug was not being applied. For incubation studies, drugs were added to the deoxygenated medium and were present during 4 h hypoxia treatment. Culture medium was then exchanged with several washes of drug-free medium and recording solution immediately prior to the experiment. For intracellular application studies, drugs were dissolved in the pipette solution on the day of experiment. All other drugs were dissolved in dH₂O for stock solutions and diluted in external buffer to final concentration. Tetraethylammonium chloride (TEA), 4-aminopyridine (4-AP), NIM, cadmium chloride, CsA, and OKA were all from Sigma-Aldrich Chemical Co. (St Louis, MO). FK-506 and RAP were from Alexis Biochemicals Inc. (Lausen, Switzerland). Tetrodotoxin (TTX) was obtained from Alamone Laboratories (Jerusalem, Israel).

2.5 Abbreviations

AKAP: protein kinase A anchoring protein; CsA: cyclosporine A; DIV: days in vitro; dH₂O: distilled water; FUDR: 5-Fluoro-2'-Deoxyuridine; FK506: tacrolimus; GABA_AR: GABA type A receptor; HIF-1 α : hypoxia-inducible factor 1 alpha; HS: horse serum; HVA: high voltage activated; OKA: okadaic acid; PI3K phosphoinositol 3-kinase; RAP: rapamycin; SMEM: Spinner's modification of Eagle's minimum essential medium; SEM: standard error of the mean; TEA chloride: tetraethylammonium chloride; TRP channel: transient receptor potential channel; VGCC: voltage-gated calcium channel; 4-AP: 4-aminopyridine

3. Results

3.1 Pharmacological characterization of HVA Ca^{2+} currents in rat cortical neurons

Cultured rat primary cortical neurons were inspected by phase-contrast microscopy prior to recordings to confirm normal morphology and viability after 4 h exposure to 1% O_2 . Consistent with our previous reports (Gao et al., 2004; Wang and Greenfield, Jr., 2009), this hypoxic exposure did not result in obvious somatic swelling or dendrite retraction, features commonly associated with neuronal injury, and showed no increase in cell death or injury as measured by trypan blue staining or LDH release (Gao et al., 2004; Wang and Greenfield, Jr., 2009).

VGCC blockers were used for pharmacological identification of the elicited currents. At maximal activation (+10 mV), the selective L-VGCC antagonist nimodipine (NIM, 10 μM) reduced HVA Ca^{2+} currents to $55.9 \pm 5.2\%$ of baseline ($n=5$) indicating that L-type currents represented a major component of HVA Ca^{2+} current in investigated neurons (Fig. 1). Sample traces from a typical experiment are shown in Fig. 1A. The nimodipine-resistant current was likely attributable to other HVA Ca^{2+} currents, including N, P/Q and R-type (Catterall, 2000), as well as TTX-insensitive sodium currents and non-specific cation currents. T-type channel currents were unlikely to contribute significantly as no rapidly desensitizing, low voltage activated component was observed. Subsequent addition of the non-selective Ca^{2+} channel blocker CdCl_2 (10 μM), further suppressed all but $16.5 \pm 5.9\%$ of the whole currents (Fig. 1A, B). Blockade by NIM and Cd^{2+} did not alter the current/voltage relationship or activation kinetics of the residual currents (Fig. 1C). The residual current component remaining after Cd^{2+} may be attributed to TTX-resistant sodium currents or non-selective cation currents (Fadulova et al., 1991; Ma et al., 1997; Shkryl et al., 1999). Washout of Cd^{2+} partially reversed the blockade by this cation (to $60.9 \pm 8.8\%$ of baseline, $n=5$). Gradual rundown of HVA currents over the time of recording was sometimes observed, but most cells had stable maximal currents, and those with $> 20\%$ rundown over the course of the experiment were excluded from the analysis.

3.2 Effects of hypoxia on HVA Ca^{2+} currents in rat cortical neurons

Representative Ca^{2+} current traces elicited in cortical neurons within 2 h after 4 h exposure to 1% O_2 (Fig. 2A, Hypox.4/Rec.0) were approximately 1.5-fold larger than those from control cells maintained at atmospheric O_2 (20.9%, Normox). The atmospheric O_2 concentration at sea level is relatively hyperoxic compared to the 2.5 – 5.3% (19–40 mm Hg) found in cerebral cortex *in vivo* (Erecinska and Silver, 2001) but is routinely used for neuronal culture and thus considered “normoxic” for these studies. Peak current amplitudes at each activation voltage were normalized to cell capacitance to yield Ca^{2+} current densities and plotted across the activating voltage range studied (Fig. 2B). Current density was significantly increased immediately after 4 h hypoxia exposure. Maximal Ca^{2+} current density for both groups was observed at voltage steps to +5 mV, at which post-hypoxic neuron current density was significantly larger than that from normoxic controls (Normox: 14.1 ± 2.3 pA/pF, $n=10$; Hypox.4/Rec.0: 20.4 ± 1.8 pA/pF, $n=10$, $p<0.05$ by unpaired *t*-test). The changes in maximal current were not due to altered cell size, as membrane capacitance was not significantly different between normoxic and post-hypoxic groups (Normox: 21.4 ± 0.5 pF, $n=10$; Hypox.4/Rec.0: 21.2 ± 1.0 pF, $n=10$, $p>0.05$).

3.3 Voltage-dependence of Ca^{2+} current activation and inactivation were unchanged

Current amplitudes elicited from individual neurons by voltage steps were transformed into normalized conductances, plotted against activation voltage and then averaged for each voltage step to yield activation curves (Fig. 2C). There was no significant change in activation between hypoxia-exposed and normoxic control neurons. The voltages of half-

maximal activation (V_h) were not significantly different (Normox: 0.8 ± 2.0 mV, $n=10$; Hypox.4/Rec.0: -1.9 ± 3.7 mV, $n=10$; $p>0.05$), nor was there a significant difference in the Boltzmann slope (k) of the activation curves for post-hypoxic cells and matched normoxic controls (Fig 2C. Normox: 12.3 ± 1.6 , $n=10$; Hypox.4/Rec.0: 13.2 ± 1.8 , $n=10$, $p>0.05$).

Steady-state inactivation was assessed using test pulses of 200 ms duration at +10 mV evoked immediately following 1500 ms conditioning pre-pulses at voltages ranging from -80 mV to +10 mV, in 5 mV increments. Boltzmann inactivation curves derived from individual fits of Ca^{2+} currents elicited from control and post-hypoxic neurons are shown in Fig. 2C. There was no significant shift in the inactivation curve after 4 h exposure to 1% O_2 compared to matched normoxic control neurons. The voltage of half-maximal inactivation (V_h) was not significantly different (Normox: -40.3 ± 1.6 mV, $n=11$; Hypox.4/Rec.0: -37.6 ± 1.3 mV, $n=11$, $p>0.05$), nor was there a significant difference in the slope (k) of the inactivation curves (Normox: 12.7 ± 0.6 , $n=11$; Hypox.4/Rec.0: 13.8 ± 1.6 , $n=11$, $p>0.05$).

3.4 HVA Ca^{2+} currents after 48 h recovery from 4 h hypoxia

We previously reported that maximal GABA current in NT2-N neuronal cells was increased immediately after 8 h exposure to 1% O_2 but reduced after 48 h normoxic recovery (Gao et al., 2004), while in cortical neurons, 4 h hypoxic treatment reduced GABA_A receptor currents both immediately and 48 h after exposure (Wang and Greenfield, Jr., 2009). For cortical neurons, both phases were blocked by L-type VGCC antagonists. Since the 48 h time point was critical for post-hypoxic GABA_A receptor changes in two neuronal cell types, we hypothesized that post-hypoxic HVA Ca^{2+} current changes might also remain affected after 48 h normoxic recovery. In contrast to the time course for GABA_A receptor regulation, HVA Ca^{2+} currents recorded after 4 h hypoxia exposure followed by 48 h normoxic recovery (95% air/5% CO_2 , 37°C) showed no significant difference in current-voltage relationship compared to normoxic controls (Fig. 3A). Maximal Ca^{2+} current density (Fig. 3B) was also unchanged in post-hypoxic neurons after 48 h recovery (Hypox4/Rec.48: 14.6 ± 3.3 pA/pF, $n=7$) compared to matched controls (Normox: 14.7 ± 0.7 pA/pF, $n=7$, $p>0.05$), indicating that the augmentation of maximal Ca^{2+} current density immediately after hypoxic exposure was transient and reverted to baseline within 48 h of normoxic recovery. There were no changes in the voltage dependence of Ca^{2+} current activation (Normox: -3.4 ± 0.5 mV, $n=7$; Hypox.4/Rec.48: -2.5 ± 0.6 mV, $n=7$, $p>0.05$, data not shown) or steady-state inactivation (Normox: -42.3 ± 0.6 mV, $n=6$; Hypox.4/Rec.48: -39.9 ± 0.2 mV, $n=6$, $p>0.05$, data not shown).

3.5 Hypoxia selectively enhanced L-type VGCC Ca^{2+} currents

To evaluate whether hypoxia selectively regulates L-VGCC function, we used nimodipine (NIM, 10 μM) to identify the L-VGCC Ca^{2+} current component. The nimodipine-resistant residual Ca^{2+} current, presumed to be a mixture of N, P/Q and R-types as well as TTX-resistant sodium currents and nonspecific cation currents, was considered the non-L-type current component. The NIM-dependent Ca^{2+} current was calculated as the difference between total HVA current and residual current. Total HVA Ca^{2+} current, L-type Ca^{2+} current and non-L-type current were measured in post-hypoxic and normoxic control neurons within 2 hours of treatment. As previously noted, Ca^{2+} current density was significantly greater immediately after 4 h hypoxia compared with normoxic controls (at +5 mV, Normox: 15.9 ± 1.4 pA/pF, $n=8$; Hypox.4/Rec.0: 21.8 ± 2.1 pA/pF, $n=8$, $p<0.05$ by unpaired t -test). There was also a significant difference in the L-type (NIM-sensitive) Ca^{2+} current component (Fig. 4A; Normox-L: 6.3 ± 0.7 pA/pF, $n=8$; Hypox-L: 11.0 ± 2.0 pA/pF, $n=8$, $p<0.05$). There was no significant difference in non-L-type (nimodipine-insensitive) current after hypoxia (Normox-NonL: 9.6 ± 1.0 pA/pF, $n=8$; Hypox-NonL: 10.8 ± 1.0 pA/pF, $n=8$, $p<0.05$). Thus, the enhancement of total HVA Ca^{2+} current after hypoxia appears

to have been due primarily to increased L-type current. In normoxic control neurons, the percentage of L-type component was significantly lower than the non-L-type component (Fig 4B. Normox-L: $39.4 \pm 3.5\%$, $n=8$ vs Normox-NonL: $60.6 \pm 3.4\%$, $n=8$, $*p<0.05$). However, after acute hypoxia exposure, there was no difference in the percentage of L-type and non-L-type current (Hypox-L: $48.7 \pm 4.5\%$, $n=8$; Hypox-NonL: $51.3 \pm 4.5\%$, $n=8$, $p > 0.05$), consistent with a change in the proportion of functional L-type channels. Moreover, the magnitude of the post-hypoxic increase in L-type current density (about 4.7 pA/pF) is nearly sufficient to account for the increase in total HVA Ca^{2+} current density (about 5.9 pA/pF). Collectively, these data suggested that acute hypoxia selectively enhanced L-VGCC current in rat primary cortical neurons.

3.6 FK506 reversed HVA Ca^{2+} current enhancement after hypoxia

Based on the growing evidence of the involvement of the Ca^{2+} /calmodulin-dependent protein phosphatase, calcineurin, in VGCC regulation (Norris et al., 2002; Norris et al., 2008; Oliveria et al., 2007) and post-hypoxic signaling (Liu et al., 2007; Marunaka et al., 1998), we hypothesized that the increase in HVA Ca^{2+} current was related to activation of calcineurin. To determine whether calcineurin was required for upregulation of Ca^{2+} currents after hypoxia, we included the calcineurin inhibitor, FK-506, in the recording pipette at a concentration (5 μM) previously shown to suppress HVA Ba^{2+} currents in cultured hippocampal neurons (Norris et al., 2002). Representative HVA Ca^{2+} current traces (Fig. 5A) from post-hypoxic and normoxic control neurons demonstrate that FK-506 prevented the increase of HVA Ca^{2+} current following 4 h hypoxic exposure without affecting the current density in normoxic control neurons (maximal current density: Normox-FK506: 12.5 ± 3.1 pA/pF, $n=8$; Hypox-FK506: 13.0 ± 1.0 pA/pF, $n=7$, $p>0.05$). FK-506 also had no significant effect on the current-voltage relationship for HVA Ca^{2+} currents (Fig. 5B) or the voltage dependence of VGCC activation (Fig. 5C, Normox-FK506 $V_{h\text{act}}$: -7.3 ± 0.2 mV, $n=8$; Hypox-FK506 $V_{h\text{act}}$: -4.8 ± 0.2 mV, $n=7$, $p>0.05$). Steady-state inactivation was likewise unchanged by FK-506 (Fig. 5C, Normox-FK506 $V_{h\text{inact}}$: -43.0 ± 0.2 mV, $n=8$; Hypox-FK506 $V_{h\text{inact}}$: -39.1 ± 0.2 mV, $n=7$, $p>0.05$). Thus, FK-506 prevented the enhancement of HVA Ca^{2+} current density in cortical neurons after hypoxia exposure without altering voltage-dependent channel kinetics.

To evaluate whether this effect of FK-506 was specific to the post-hypoxia augmentation of L-type VGCC currents, we included 5 μM FK-506 in the whole-cell recording pipette and measured total and nimodipine-resistant HVA Ca^{2+} currents in neurons acutely treated with normoxia or hypoxia. As previously observed, FK-506 reversed the post-hypoxic increase in HVA maximal Ca^{2+} current density without significantly affecting the current density in normoxic controls (Normox-FK506: 13.4 ± 1.3 pA/pF, $n=8$; Hypox-FK506: 14.3 ± 0.7 pA/pF, $n=8$, $p>0.05$). FK-506 also prevented the post-hypoxic increase in NIM-sensitive L-type Ca^{2+} current density (Normox-FK506-L: 4.5 ± 0.9 pA/pF, $n=8$; HypoxFK506-L: 5.5 ± 0.9 pA/pF, $n=8$, $p>0.05$, Fig. 5D). The non-L-type Ca^{2+} current density was unchanged by inclusion of FK506 (Normox-FK506-NonL: 9.0 ± 1.0 pA/pF, $n=8$; Hypox-FK506-NonL: 8.7 ± 0.6 pA/pF, $n=8$, $p>0.05$).

FK-506 also prevented the post-hypoxic change in relative percentage of L-type and non-L-type components in total HVA Ca^{2+} current. In the absence of FK-506, hypoxia increased the percentage of L-type component such that the significant difference between L-type and non-L-type currents was abolished (Fig. 4B). With FK-506 in the recording pipette, the significantly higher proportion of non-L-type current in control cells (Normox-FK506-L: $32.6 \pm 5.0\%$, $n=8$; Normox-FK506-NonL: $67.4 \pm 5.0\%$, $n=8$, $p<0.05$) was still present in post-hypoxic neurons (Hypox-FK506-L: $37.9 \pm 5.2\%$, $n=8$ vs. Hypox-FK506-NonL: $62.1 \pm 5.2\%$, $n=8$, $p<0.05$, Fig. 5E). Prevention of the post-hypoxic increase in L-type Ca^{2+} current by FK-506 thus suggests that calcineurin-dependent enhancement of HVA Ca^{2+} current may

be specific for L-type VGCCs. Indeed, even in normoxic control neurons, there was a slight (but not significant) decrease in the L-type VGCC component in neurons recorded in the presence of FK-506 compared to non-FK506 normoxic control neurons (Normox-L: $39.4 \pm 3.5\%$, $n=8$; Normox-FK506-L: $32.6 \pm 5.0\%$, $n=8$, $p>0.05$). This is consistent with studies demonstrating that FK-506 selectively inhibited L-type VGCC current in hippocampal neurons (Norris et al., 2002) and again supports a role for calcineurin-dependent regulation of L-type VGCCs.

3.7 FK-506 block of post-hypoxic L-VGCC augmentation requires calcineurin inhibition

To evaluate the specificity of the FK-506 effect, we also tested the structurally distinct calcineurin inhibitor, cyclosporine A (CsA), which binds to the immunophilin, cyclophilin A (Hamawy, 2003; Snyder et al., 1998). In this case, cortical neurons were co-incubated during the 4 h hypoxic exposure with $20 \mu\text{M}$ CsA, a concentration shown to maximally inhibit calcineurin in lymphocytes (Kung and Halloran, 2000), which was washed out prior to recording. CsA was previously reported to reduce VGCC current density in cultured hippocampal neurons without altering the I–V relationship (Norris et al., 2002). CsA co-incubation during hypoxia also prevented the hypoxia-induced enhancement of HVA Ca^{2+} current density in cortical neurons compared to matched normoxic controls (Fig. 6A–C; Normox-CsA: $10.4 \pm 1.5 \text{ pA/pF}$, $n=8$; Hypox-CsA: $9.1 \pm 0.8 \text{ pA/pF}$, $n=8$, $p>0.05$). Of note, CsA incubation not only occluded the post-hypoxic increase in HVA Ca^{2+} current, but also reduced the current density in normoxic neurons by 51.4% relative to controls without CsA (Normox-CsA: $10.4 \pm 1.5 \text{ pA/pF}$, $n=8$; Normox: $21.4 \pm 0.5 \text{ pF}$, $n=10$, $p<0.05$), indicating that constitutive calcineurin activity likely regulates basal VGCC function. CsA did not change the voltage- dependence of VGCC activation in either normoxic or post-hypoxic neurons (Fig. 6C, Normox-CsA $V_{h\text{act}}$: $-2.1 \pm 0.1 \text{ mV}$, $n=8$; Hypox-CsA $V_{h\text{act}}$: $-1.4 \pm 0.2 \text{ mV}$, $n=8$, $p>0.05$). The steady-state inactivation was also unaltered by CsA (Fig. 6C, Normox-CsA $V_{h\text{inact}}$: $-40.2 \pm 1.4 \text{ mV}$, $n=10$; Hypox-CsA $V_{h\text{inact}}$: $-36.5 \pm 2.7 \text{ mV}$, $n=8$, $p>0.05$). Thus, co-incubation with CsA during hypoxia prevented the post-hypoxic augmentation of HVA Ca^{2+} current without altering channel activation or inactivation kinetics.

3.8 Rapamycin did not reverse HVA Ca^{2+} current enhancement after hypoxia

Although FK-506 specifically inhibits calcineurin activity, it also exhibits non-calcineurin-mediated activity through binding to FKBP-12. In addition, FKBP-12 itself can also modulate intracellular Ca^{2+} homeostasis and calcium-induced calcium release (Loughrey et al., 2004; Xiao et al., 1997) and thus could potentially influence VGCC activity through negative feedback. Similarly, CsA can reduce Ca^{2+} levels after hypoxia by preventing the opening of the mitochondrial permeability transition pore (MPTP; Halestrap, 2006; Halestrap, 2009). Therefore, to confirm that reversal of post-hypoxic HVA Ca^{2+} current enhancement by FK-506 and prevention of enhancement by CsA were specific to inhibition of calcineurin activity, we performed additional control experiments to rule out alternative mechanisms. Rapamycin (RAP) is structurally similar to FK-506 and competes with FK-506 for binding to FKBP-12, but does not inhibit calcineurin activity (Snyder et al., 1998). Inclusion of RAP ($5 \mu\text{M}$) in the whole-cell recording pipette did not prevent the post-hypoxic increase in HVA Ca^{2+} currents in hypoxia-treated neurons compared to matched normoxic controls (see Fig. 6D–F). Maximal current density was significantly increased after hypoxia (Normox-RAP: $11.0 \pm 1.0 \text{ pA/pF}$, $n=9$; Hypox-RAP: $17.5 \pm 1.2 \text{ pA/pF}$, $n=9$, $p<0.05$), but did not alter the voltage dependence of VGCC activation (Fig. 6F, Normox-RAP $V_{h\text{act}}$: $-2.5 \pm 0.2 \text{ mV}$, $n=9$; Hypox-RAP $V_{h\text{act}}$: $-2.4 \pm 0.1 \text{ mV}$, $n=9$, $p>0.05$) or steady-state inactivation (Normox-RAP $V_{h\text{inact}}$: $-39.3 \pm 5.1 \text{ mV}$, $n=7$; Hypox-RAP $V_{h\text{inact}}$: $-40.6 \pm 3.2 \text{ mV}$, $n=10$, $p>0.05$). Unlike FK-506, RAP did not reverse the post-

hypoxic enhancement of HVA Ca^{2+} current density, supporting the selective effect of FK-506 to inhibit calcineurin-dependent upregulation of VGCC activity after acute hypoxia.

3.9 Okadaic acid did not prevent post-hypoxic enhancement of HVA Ca^{2+} current

To determine whether other protein phosphatases might be involved in post-hypoxic enhancement of Ca^{2+} currents, we used okadaic acid to inhibit the serine/threonine phosphatases protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A). These phosphatases have been shown to regulate cardiac and neuronal L-type Ca^{2+} channels (Davare et al., 2000; Ono and Fozzard, 1993). Calcineurin disinhibits PP1 in the intracellular Ca^{2+} signaling cascade (Budde et al., 2002; Guerini, 1997). The effects of calcineurin on HVA Ca^{2+} current after hypoxia could thus also involve PP1 and PP2A. To test this hypothesis, we co-incubated cortical neurons with 150 nM okadaic acid (OKA) during 4h hypoxia exposure. OKA is a relatively specific inhibitor of PP1 (IC_{50} of 10–15 nM) and PP2A (IC_{50} of 100 pM) (Bialojan and Takai, 1988), and only inhibits calcineurin at high concentrations ($> 1 \mu\text{M}$, $\text{IC}_{50} \sim 5 \mu\text{M}$) (Bialojan and Takai, 1988; Cohen, 1991). Inclusion of OKA (150 nM) in the medium during hypoxic exposure did not prevent the post-hypoxic increase in HVA maximal Ca^{2+} current density compared to matched normoxic controls (Fig. 6G–I; Normox-OKA: $12.0 \pm 1.1 \text{ pA/pF}$, $n=6$; Hypoxia-OKA: $18.4 \pm 2.2 \text{ pA/pF}$, $n=7$, $p<0.05$). OKA did not change the voltage dependence of VGCC activation (Fig. 6I, Normox-OKA $V_{h\text{act}}$: $0.2 \pm 0.2 \text{ mV}$, $n=6$; Hypoxia-OKA $V_{h\text{act}}$: $-5.2 \pm 0.2 \text{ mV}$, $n=7$, $p>0.05$.) or steady-state inactivation (Fig. 6I, Normox-OKA $V_{h\text{inact}}$: $-37.4 \pm 3.0 \text{ mV}$, $n=8$; Hypoxia-OKA: $-35.6 \pm 3.3 \text{ mV}$, $n=8$, $p>0.05$). The inability of OKA to prevent the post-hypoxic enhancement of HVA Ca^{2+} current density again supports the involvement of calcineurin in post-hypoxic regulation of VGCC function.

3.10 Nimodipine during hypoxia prevented HVA Ca^{2+} current enhancement

A remaining question was the mechanism of calcineurin activation during hypoxia. Concurrent binding of Ca^{2+} to the regulatory calcineurin B subunit and Ca^{2+} /calmodulin (CaM) to the catalytic calcineurin A subunit is required for the full activation of calcineurin (Groth et al., 2003). One likely source of Ca^{2+} is influx through the L-type VGCC itself, which could activate calcineurin co-localized with the channel via protein kinase A anchoring protein (AKAP) 79/150 (Oliveria et al., 2007). To test this hypothesis, cortical neurons were exposed to 4 h normoxia or hypoxia in the presence of NIM (10 μM). Cultures were subsequently washed multiple times and replaced with fresh aerated SMEM/HS culture medium. To further eliminate any residual NIM that might have inhibited L-type Ca^{2+} current, a modification of the “multipuffer” drug-delivery system (Greenfield, Jr. and Macdonald, 1996) was used to wash the selected neuron by delivering external recording solution for at least 1 min prior to patch-clamp recording. There was no significant difference in the amplitude of HVA Ca^{2+} currents following NIM incubation compared to control medium (see below), suggesting the successful wash-out of residual NIM.

As previously observed, maximal HVA Ca^{2+} current density increased after hypoxia (Normox: $12.7 \pm 1.3 \text{ pA/pF}$, $n=7$; Hypox.4/Rec.0: $22.2 \pm 1.4 \text{ pA/pF}$, $n=9$, $p<0.05$). Inclusion of NIM (10 μM) did not change maximal Ca^{2+} current density in normoxic control neurons (Normox-NIM: $11.8 \pm 1.0 \text{ pA/pF}$, $n=7$), but completely prevented the post-hypoxic Ca^{2+} current increase (Hypox-NIM: $10.7 \pm 1.3 \text{ pA/pF}$, $n=9$, $p>0.05$, Fig. 7). Since blockade of L-VGCCs with NIM during hypoxia prevented the enhancement of HVA Ca^{2+} current, L-VGCCs likely represent the source of Ca^{2+} required to activate calcineurin, which suggests a positive feedback mechanism in which hypoxic activation of L-VGCCs mediates Ca^{2+} entry, activation of calcineurin and subsequent enhancement of L-VGCC current.

4. Discussion

In cultured rat cortical neurons, exposure to 1% O₂ for 4 hours resulted in Ca²⁺ entry through L-type VGCCs, activation of calcineurin and a transient increase in L-type VGCC currents that returned to baseline within 48 h after exposure. Blockade of L-type channels during hypoxia prevented the increase in HVA Ca²⁺ currents, suggesting a critical role for L-VGCCs in their own potentiation. Inhibition of calcineurin either by CsA during hypoxia or by FK-506 in the recording pipette after hypoxia prevented the VGCC current increase. The increase in HVA Ca²⁺ current was specific to L-type channels, as there was no post-hypoxic increase in the residual current after blockade of L-VGCCs with NIM. These findings suggest a positive feedback loop reinforcing neuronal calcium signaling in the immediate post-hypoxic period.

To our knowledge, this is the first demonstration of L-VGCC enhancement by transient hypoxia in cortical neurons, which could contribute to post-hypoxic cortical hyperexcitability (Yechikhov et al., 2002), seizures or status epilepticus (Bladin et al., 2000; Krumholz et al., 1988). Increased intracellular Ca²⁺ might activate calcium-dependent processes leading to necrotic or apoptotic cell death (Wasterlain et al., 1993), or alternatively, L-VGCC upregulation and calcium entry might facilitate post-hypoxic dephosphorylation and translocation of the Ca²⁺-dependent potassium channel Kv2.1, which suppresses neuronal excitability, and thus have a compensatory or protective effect (Misonou et al., 2005).

4.1 Post-hypoxic modulation of VGCCs

The mechanism responsible for L-VGCC activation by hypoxia is unclear. Hypoxia may have led to depolarization, either by downregulation of O₂-sensitive K⁺ ion channels (Lopez-Barneo et al., 2004), "anoxic depolarization" due to ATP depletion (Allen et al., 2005), or a direct effect of hypoxia on L-VGCCs. L-type VGCCs, which are expressed at high density in the somata of cerebral cortex and hippocampal neurons (Ricci et al., 2002), have a slow rate of inactivation that allows Ca²⁺ entry even with gradual depolarization, facilitating Ca²⁺ signaling in response to relatively mild depolarizing stimuli.

Our studies demonstrated enhancement of L-VGCC currents immediately after 4 h exposure to 1% O₂, consistent with prior reports showing that brief hypoxia (15–30 mm Hg, equivalent to 2–4% O₂) potentiated high-voltage-activated (HVA) Ca²⁺ currents in hippocampal CA1 neurons by 26–94% (Lukyanetz et al., 2003; Shkryl et al., 1999; Shkryl et al., 2001). More prolonged (24 h) moderate hypoxia (2.5 – 6% O₂) enhanced Ca²⁺ currents in PC12 cells, cerebellar granule neurons and wild-type HEK 293 cells expressing recombinant L-type α_{1c} (Cav1.2) subunits (Brown et al., 2005; Peers et al., 2005) by a trafficking mechanism involving the formation of amyloid beta (A β) peptide by γ -secretase and regulated by reactive oxygen species (Peers et al., 2005). It is unclear whether such a mechanism might be involved in our findings, though the lack of a change in voltage-dependent kinetics is consistent with altered trafficking rather than a direct effect on channel function.

4.2 Calcineurin regulation of VGCCs

In our experiments, the increase in HVA current was dependent on the action of the calcium-dependent phosphatase, calcineurin. Calcineurin-dependent upregulation of VGCC activity has been observed previously in hippocampal CA1 neurons. Inhibition of calcineurin by FK506, CsA or calcineurin autoinhibitory peptide reduced whole-cell HVA Ca²⁺ and Ba²⁺ currents through L-type Ca²⁺ channels both in culture (Norris et al., 2002) and in hippocampal "zipper" slices (Norris et al., 2008). In contrast, activation of calcineurin

has also been associated with reduced L-VGCC function in cultured hippocampal pyramidal neurons. Neuronal L-type Cav1.2 activity was enhanced by PKA-mediated phosphorylation through interaction with A-kinase activating protein (AKAP) 79/150, which binds both PKA and calcineurin (Oliveria et al., 2007). Local calcium entry through L-VGCCs activated calcineurin which dominantly suppressed PKA enhancement of calcium currents, possibly via Ca^{2+} -dependent inactivation of L-type channels (Sather et al., 2009), though an earlier study found no calcineurin dependence (Zeilhofer et al., 2000). Calcineurin-dependent downregulation of L-VGCCs was also observed in prefrontal cortical neurons, and was specific to Cav1.2 as it was not observed in Cav1.3 knockout animals (Day et al., 2002). These results differ from the present findings, in which calcineurin activation was essential for enhancement of Ca^{2+} currents, possibly suggesting that Cav1.3 is the primary L-VGCC alpha subunit involved. The lower activation threshold of Cav1.3 channels (Lipscombe et al., 2004) makes them suitable for upregulation by modest hypoxia-mediated depolarization, but altered kinetics might not be detected in a mixed channel population. Other studies have implicated protein phosphatase 2A (PP2A) in L-VGCC regulation, which binds directly to Cav1.2 and reverses PKA phosphorylation of serine 1928 (Davare et al., 2000). Since okadaic acid did not block hypoxia-induced potentiation of Ca^{2+} currents in our system, PP2A is not likely involved. The increase in L-VGCC current with no change in activation or inactivation parameters suggests a possible increase in channel number rather than a change in conductance or gating. The site of calcineurin action and the detailed mechanisms underlying increased L-VGCC current are important questions for future study.

4.3 Role of calcineurin in epilepsy and brain injury

Calcineurin activity (but not protein expression) was significantly increased in the rat hippocampus after pilocarpine-induced status epilepticus (Kurz et al., 2001) and was specifically enhanced in synaptic fractions and in the dendrites of hippocampal pyramidal neurons (Kurz et al., 2003). Similar increases were also seen in a fluid percussion model of brain injury (Kurz et al., 2005a; Kurz et al., 2005b). In the pilocarpine model of status epilepticus, calcineurin-dependent dephosphorylation and subsequent activation of the actin depolymerization factor, cofilin, led to loss of dendritic spines on hippocampal pyramidal neurons, possibly associated with epileptogenesis (Kurz et al., 2008). Indeed, calcineurin inhibitors prevented neuronal death and blocked epileptogenesis in a kainic acid model of status epilepticus (Moriwaki et al., 1998), inhibited progression of kindled seizures (Moia et al., 1994) and prevented axonal sprouting in a kindling model of epilepsy (Moriwaki et al., 1996), though recent studies did not confirm the latter finding (Ingram et al., 2009). Enhanced L-VGCC function could provide a mechanism for facilitation of calcineurin activity after status epilepticus and other post-hypoxic conditions associated with altered neuronal excitability.

4.4 Calcineurin and neuronal inhibition

One of the downstream effects of L-VGCC activation by hypoxia is regulation of neuronal inhibition. Blockade of L-VGCCs during hypoxia prevented the hypoxia-induced reduction of maximal GABA_{A} current both immediately and 48 h after 4h hypoxia (Wang and Greenfield, Jr., 2009), suggesting that L-VGCCs are activated during hypoxia and that the reductions in GABA_{A} current are Ca^{2+} -mediated. The early component of this regulation was calcineurin-dependent and prevented by calcineurin inhibitors, while the late component (48 h after hypoxia) was not (Wang and Greenfield, 2008). These findings may have clinical significance, as hypoxia-induced reduction of GABA_{A} function in hippocampal CA1 pyramidal neurons and subsequent seizures in P10 rat pups were associated with calcineurin activation via Ca^{2+} -permeable AMPA receptors, and both seizures and the reduction of miniature GABA_{A} currents were prevented by FK-506 (Sanchez et al., 2005). In this important study, hypoxia/seizure-induced Ca^{2+} entry for activation of calcineurin was

through calcium-permeable AMPARs rather than L-VGCCs as observed here. This discrepancy is likely related to the marked differences in model systems between *in vivo* hypoxia followed by hippocampal slice recording vs. dissociated cell culture in which synaptic activity is minimal. The finding that calcineurin-induced downregulation of GABA_AR function (Wang and Greenfield, Jr., 2009) was observed in both settings suggests that this may be a “final common pathway” for GABA_AR downregulation via dephosphorylation. In Sanchez et al. (2005), dephosphorylation of the GABA_AR β 2 and/or β 3 subunit was observed. However, calcineurin also interacts directly with the γ 2 subunit of the GABA_AR to mediate long-term depression of GABAergic neurotransmission (Wang et al., 2003).

Post-hypoxic enhancement of neuronal L-type VGCC currents, increased intracellular Ca²⁺ and augmented calcineurin activity may trigger downstream modulation of other ion channels as well, contributing to hyperexcitability and seizure generation or epileptogenesis. The downstream mechanisms underlying such long-term changes remain to be explored.

Acknowledgments

Grants

This work was supported in part by R01-NS049389 and a research grant from the Myoclonus Research Foundation, Ft. Lee, N.J. to L.J.G.

References

- Allen NJ, Karadottir R, Attwell D. A preferential role for glycolysis in preventing the anoxic depolarization of rat hippocampal area CA1 pyramidal cells. *J Neurosci.* 2005; 25:848–859. [PubMed: 15673665]
- Bergamasco B, Benna P, Ferrero P, Gavinelli R. Neonatal hypoxia and epileptic risk: a clinical prospective study. *Epilepsia.* 1984; 25:131–136. [PubMed: 6538479]
- Berridge MJ. Neuronal calcium signaling. *Neuron.* 1998; 21:13–26. [PubMed: 9697848]
- Bialojan C, Takai A. Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. Specificity and kinetics. *Biochem J.* 1988; 256:283–290. [PubMed: 2851982]
- Bladin CF, Alexandrov AV, Bellavance A, Bornstein N, Chambers B, Cote R, Lebrun L, Pirisi A, Norris JW. Seizures after stroke. *Arch Neurol.* 2000; 57:1617–1622. [PubMed: 11074794]
- Brown ST, Scragg JL, Boyle JP, Hudasek K, Peers C, Fearon IM. Hypoxic augmentation of Ca²⁺ channel currents requires a functional electron transport chain. *J Biol Chem.* 2005; 280:21706–21712. [PubMed: 15824110]
- Budde T, Meuth S, Pape HC. Calcium-dependent inactivation of neuronal calcium channels. *Nat Rev Neurosci.* 2002; 3:873–883. [PubMed: 12415295]
- Butcher SP, Henshall DC, Teramura Y, Iwasaki K, Sharkey J. Neuroprotective actions of FK506 in experimental stroke: in vivo evidence against an antiexcitotoxic mechanism. *J Neurosci.* 1997; 17:6939–6946. [PubMed: 9278529]
- Catterall WA. Structure and regulation of voltage-gated Ca²⁺ channels. *Annu Rev Cell Dev Biol.* 2000; 16:521–555. [PubMed: 11031246]
- Cohen P. Classification of protein-serine/threonine phosphatases: identification and quantitation in cell extracts. *Methods Enzymol.* 1991; 201:389–398. [PubMed: 1658556]
- Davare MA, Horne MC, Hell JW. Protein phosphatase 2A is associated with class C L-type calcium channels (Cav1.2) and antagonizes channel phosphorylation by cAMP-dependent protein kinase. *J Biol Chem.* 2000; 275:39710–39717. [PubMed: 10984483]
- Day M, Olson PA, Platzer J, Striessnig J, Surmeier DJ. Stimulation of 5-HT(2) receptors in prefrontal pyramidal neurons inhibits Ca(v)1.2 L type Ca(2+) currents via a PLCbeta/IP3/calcineurin signaling cascade. *J Neurophysiol.* 2002; 87:2490–2504. [PubMed: 11976386]

- Erecinska M, Silver IA. Tissue oxygen tension and brain sensitivity to hypoxia. *Resp Physiol*. 2001; 128:263.
- Erin N, Bronson SK, Billingsley ML. Calcium-dependent interaction of calcineurin with Bcl-2 in neuronal tissue. *Neuroscience*. 2003; 117:541–555. [PubMed: 12617961]
- Franco-Obregon A, Urena J, Lopez-Barneo J. Oxygen-sensitive calcium channels in vascular smooth muscle and their possible role in hypoxic arterial relaxation. *Proc Natl Acad Sci U S A*. 1995; 92:4715–4719. [PubMed: 7753871]
- Gao L, Lyons AR, Greenfield LJ Jr. Hypoxia alters GABAA receptor function and subunit expression in NT2-N neurons. *Neuropharmacology*. 2004; 46:318–330. [PubMed: 14975687]
- Greenfield LJ Jr, Macdonald RL. Whole-cell and single-channel $\alpha 1 \beta 1 \gamma 2S$ GABAA receptor currents elicited by a “multipuffer” drug application device. *Pflügers Arch*. 1996; 432:1080–1090. [PubMed: 8781204]
- Groth RD, Dunbar RL, Mermelstein PG. Calcineurin regulation of neuronal plasticity. *Biochem Biophys Res Commun*. 2003; 311:1159–1171. [PubMed: 14623302]
- Guerini D. Calcineurin: not just a simple protein phosphatase. *Biochem Biophys Res Commun*. 1997; 235:271–275. [PubMed: 9199180]
- Halestrap AP. Calcium, mitochondria and reperfusion injury: a pore way to die. *Biochem Soc Trans*. 2006; 34:232–237. [PubMed: 16545083]
- Halestrap AP. Mitochondria and reperfusion injury of the heart--a holey death but not beyond salvation. *J Bioenerg Biomembr*. 2009; 41:113–121. [PubMed: 19357938]
- Hamawy MM. Molecular actions of calcineurin inhibitors. *Drug News Perspect*. 2003; 16:277–282. [PubMed: 12942158]
- Hammarstrom AK, Gage PW. Inhibition of oxidative metabolism increases persistent sodium current in rat CA1 hippocampal neurons. *J Physiol*. 1998; 510 (Pt 3):735–741. [PubMed: 9660889]
- Ingram EA, Toyoda I, Wen X, Buckmaster PS. Prolonged infusion of inhibitors of calcineurin or L-type calcium channels does not block mossy fiber sprouting in a model of temporal lobe epilepsy. *Epilepsia*. 2009; 50:56–64. [PubMed: 18616558]
- Jensen FE. The role of glutamate receptor maturation in perinatal seizures and brain injury. *Int J Dev Neurosci*. 2002; 20:339–347. [PubMed: 12175872]
- Jiang C, Sigworth FJ, Haddad GG. Oxygen deprivation activates an ATP-inhibitable K^+ channel in substantia nigra neurons. *J Neurosci*. 1994; 14:5590–5602. [PubMed: 8083755]
- Jiang RG, Eyzaguirre C. Calcium channels of cultured rat glomus cells in normoxia and acute hypoxia. *Brain Res*. 2005; 1031:56–66. [PubMed: 15621012]
- Kahlert S, Reiser G. Requirement of glycolytic and mitochondrial energy supply for loading of Ca^{2+} stores and $InsP(3)$ -mediated Ca^{2+} signaling in rat hippocampus astrocytes. *J Neurosci Res*. 2000; 61:409–420. [PubMed: 10931527]
- Krnjevic K. Electrophysiology of cerebral ischemia. *Neuropharmacology*. 2008; 55:319–333. [PubMed: 18314146]
- Krumholz A, Stern BJ, Weiss HD. Outcome from coma after cardiopulmonary resuscitation: relation to seizures and myoclonus. *Neurology*. 1988; 38:401–405. [PubMed: 3347343]
- Kung L, Halloran PF. Immunophilins may limit calcineurin inhibition by cyclosporine and tacrolimus at high drug concentrations. *Transplantation*. 2000; 70:327–335. [PubMed: 10933159]
- Kurz JE, Hamm RJ, Singleton RH, Povlishock JT, Churn SB. A persistent change in subcellular distribution of calcineurin following fluid percussion injury in the rat. *Brain Res*. 2005a; 1048:153–160. [PubMed: 15919062]
- Kurz JE, Moore BJ, Henderson SC, Campbell JN, Churn SB. A cellular mechanism for dendritic spine loss in the pilocarpine model of status epilepticus. *Epilepsia*. 2008; 49:1696–1710. [PubMed: 18479390]
- Kurz JE, Parsons JT, Rana A, Gibson CJ, Hamm RJ, Churn SB. A significant increase in both basal and maximal calcineurin activity following fluid percussion injury in the rat. *J Neurotrauma*. 2005b; 22:476–490. [PubMed: 15853464]

- Kurz JE, Rana A, Parsons JT, Churn SB. Status epilepticus-induced changes in the subcellular distribution and activity of calcineurin in rat forebrain. *Neurobiol Dis.* 2003; 14:483–493. [PubMed: 14678764]
- Kurz JE, Sheets D, Parsons JT, Rana A, DeLorenzo RJ, Churn SB. A significant increase in both basal and maximal calcineurin activity in the rat pilocarpine model of status epilepticus. *J Neurochem.* 2001; 78:304–315. [PubMed: 11461966]
- Li XM, Yang JM, Hu DH, Hou FQ, Zhao M, Zhu XH, Wang Y, Li JG, Hu P, Chen L, Qin LN, Gao TM. Contribution of downregulation of L-type calcium currents to delayed neuronal death in rat hippocampus after global cerebral ischemia and reperfusion. *J Neurosci.* 2007; 27:5249–5259. [PubMed: 17494711]
- Lipscombe D, Helton TD, Xu W. L-type calcium channels: the low down. *J Neurophysiol.* 2004; 92:2633–2641. [PubMed: 15486420]
- Liu YV, Hubbi ME, Pan F, McDonald KR, Mansharamani M, Cole RN, Liu JO, Semenza GL. Calcineurin promotes hypoxia-inducible factor 1 α expression by dephosphorylating RACK1 and blocking RACK1 dimerization. *J Biol Chem.* 2007; 282:37064–37073. [PubMed: 17965024]
- Lopez-Barneo J, Del Toro R, Levitsky KL, Chiara MD, Ortega-Saenz P. Regulation of oxygen sensing by ion channels. *J Appl Physiol.* 2004; 96:1187–1195. [PubMed: 14766769]
- Loughrey CM, Seidler T, Miller SL, Prestle J, MacEachern KE, Reynolds DF, Hasenfuss G, Smith GL. Over-expression of FK506-binding protein FKBP12.6 alters excitation-contraction coupling in adult rabbit cardiomyocytes. *J Physiol.* 2004; 556:919–934. [PubMed: 14966299]
- Lukyanetz EA, Shkryl VM, Kravchuk OV, Kostyuk PG. Action of hypoxia on different types of calcium channels in hippocampal neurons. *Biochim Biophys Acta.* 2003; 1618:33–38. [PubMed: 14643931]
- Marunaka Y, Niisato N, Shintani Y. Protein phosphatase 2B-dependent pathway of insulin action on single Cl[−] channel conductance in renal epithelium. *J Membr Biol.* 1998; 161:235–245. [PubMed: 9493129]
- Misonou H, Mohapatra DP, Menegola M, Trimmer JS. Calcium- and metabolic state-dependent modulation of the voltage-dependent Kv2.1 channel regulates neuronal excitability in response to ischemia. *J Neurosci.* 2005; 25:11184–11193. [PubMed: 16319318]
- Moia LJ, Matsui H, de Barros GA, Tomizawa K, Miyamoto K, Kuwata Y, Tokuda M, Itano T, Hatase O. Immunosuppressants and calcineurin inhibitors, cyclosporin A and FK506, reversibly inhibit epileptogenesis in amygdaloid kindled rat. *Brain Res.* 1994; 648:337–341. [PubMed: 7522929]
- Moriwaki A, Lu YF, Hayashi Y, Tomizawa K, Tokuda M, Itano T, Hatase O, Matsui H. Immunosuppressant FK506 prevents mossy fiber sprouting induced by kindling stimulation. *Neurosci Res.* 1996; 25:191–194. [PubMed: 8829156]
- Moriwaki A, Lu YF, Tomizawa K, Matsui H. An immunosuppressant, FK506, protects against neuronal dysfunction and death but has no effect on electrographic and behavioral activities induced by systemic kainate. *Neuroscience.* 1998; 86:855–865. [PubMed: 9692722]
- Norris CM, Blalock EM, Chen KC, Porter NM, Landfield PW. Calcineurin enhances L-type Ca(2+) channel activity in hippocampal neurons: increased effect with age in culture. *Neuroscience.* 2002; 110:213–225. [PubMed: 11958864]
- Norris CM, Blalock EM, Chen KC, Porter NM, Thibault O, Kraner SD, Landfield PW. Hippocampal ‘zipper’ slice studies reveal a necessary role for calcineurin in the increased activity of L-type Ca(2+) channels with aging. *Neurobiol Aging.* 2008
- Oliveria SF, Dell’Acqua ML, Sather WA. AKAP79/150 anchoring of calcineurin controls neuronal L-type Ca²⁺ channel activity and nuclear signaling. *Neuron.* 2007; 55:261–275. [PubMed: 17640527]
- Ono K, Fozzard HA. Two phosphatase sites on the Ca²⁺ channel affecting different kinetic functions. *J Physiol.* 1993; 470:73–84. [PubMed: 8308752]
- Peers C, Scragg JL, Boyle JP, Fearon IM, Taylor SC, Green KN, Webster NJ, Ramsden M, Pearson HA. A central role for ROS in the functional remodelling of L-type Ca²⁺ channels by hypoxia. *Philos Trans R Soc Lond B Biol Sci.* 2005; 360:2247–2254. [PubMed: 16321794]

- Porter NM, Thibault O, Thibault V, Chen KC, Landfield PW. Calcium channel density and hippocampal cell death with age in long-term culture. *J Neurosci*. 1997; 17:5629–5639. [PubMed: 9204944]
- Ricci A, Sabbatini M, Tomassoni D, Mignini F, Petrelli C, Amenta F. Neuronal populations of rat cerebral cortex and hippocampus expressed a higher density of L-type Ca²⁺ channel than corresponding cerebral vessels. *Clin Exp Hypertens*. 2002; 24:715–726. [PubMed: 12450246]
- Sanchez RM, Dai W, Levada RE, Lippman JJ, Jensen FE. AMPA/kainate receptor-mediated downregulation of GABAergic synaptic transmission by calcineurin after seizures in the developing rat brain. *J Neurosci*. 2005; 25:3442–3451. [PubMed: 15800199]
- Sather, WA.; Dell'Acqua, ML.; Oliveria, SF. Neuroscience Meeting Planner. Society for Neuroscience; 2009. Localized calcineurin in calcium-dependent inactivation of L-type calcium channels.
- Scheufler KM, Rohrborn HJ, Zentner J. Does tissue oxygen-tension reliably reflect cerebral oxygen delivery and consumption? *Anesth Analg*. 2002; 95:1042–8. table. [PubMed: 12351291]
- Sharkey J, Butcher SP. Immunophilins mediate the neuroprotective effects of FK506 in focal cerebral ischaemia. *Nature*. 1994; 371:336–339. [PubMed: 7522303]
- Shkryl VM, Kostyuk PG, Lukyanetz EA. Dual action of cytosolic calcium on calcium channel activity during hypoxia in hippocampal neurones. *Neuroreport*. 2001; 12:4035–4039. [PubMed: 11742234]
- Shkryl VM, Nikolaenko LM, Kostyuk PG, Lukyanetz EA. High-threshold calcium channel activity in rat hippocampal neurones during hypoxia. *Brain Res*. 1999; 833:319–328. [PubMed: 10375713]
- Snyder SH, Lai MM, Burnett PE. Immunophilins in the nervous system. *Neuron*. 1998; 21:283–294. [PubMed: 9728910]
- Sochivko D, Chen J, Becker A, Beck H. Blocker-resistant Ca²⁺ currents in rat CA1 hippocampal pyramidal neurons. *Neuroscience*. 2003; 116:629–638. [PubMed: 12573706]
- Tai KK, Truong DD. NMDA receptor-mediated excitotoxicity contributes to the cerebral hypoxic injury of a rat model of posthypoxic myoclonus. *Brain Res*. 2007; 1133:209–215. [PubMed: 17196560]
- Vannucci RC, Vannucci SJ. Glucose metabolism in the developing brain. *Semin Perinatol*. 2000; 24:107–115. [PubMed: 10805166]
- Vannucci RC, Vannucci SJ. Hypoglycemic brain injury. *Semin Neonatol*. 2001; 6:147–155. [PubMed: 11483020]
- Wang HG, Pathan N, Ethell IM, Krajewski S, Yamaguchi Y, Shibasaki F, McKeon F, Bobo T, Franke TF, Reed JC. Ca²⁺-induced apoptosis through calcineurin dephosphorylation of BAD. *Science*. 1999; 284:339–343. [PubMed: 10195903]
- Wang J, Liu S, Haditsch U, Tu W, Cochrane K, Ahmadian G, Tran L, Paw J, Wang Y, Mansuy I, Salter MM, Lu YM. Interaction of calcineurin and type-A GABA receptor gamma 2 subunits produces long-term depression at CA1 inhibitory synapses. *J Neurosci*. 2003; 23:826–836. [PubMed: 12574411]
- Wang L, Greenfield LJ Jr. Post-hypoxic changes in rat cortical neuron GABA_A receptor function require L-type voltage-gated calcium channel activation. *Epilepsia*. 2008; 49(suppl 7):335. Prog. IW.69.
- Wang L, Greenfield LJ Jr. Post-hypoxic changes in rat cortical neuron GABA A receptor function require L-type voltage-gated calcium channel activation. *Neuropharmacology*. 2009; 56:198–207. [PubMed: 18674547]
- Wasterlain CG, Fujikawa DG, Penix L, Sankar R. Pathophysiological mechanisms of brain damage from status epilepticus. *Epilepsia*. 1993; 34(Suppl 1):S37–S53. [PubMed: 8385002]
- Xiao RP, Valdivia HH, Bogdanov K, Valdivia C, Lakatta EG, Cheng H. The immunophilin FK506-binding protein modulates Ca²⁺ release channel closure in rat heart. *J Physiol*. 1997; 500 (Pt 2): 343–354. [PubMed: 9147322]
- Yakel JL. Calcineurin regulation of synaptic function: from ion channels to transmitter release and gene transcription. *Trends Pharmacol Sci*. 1997; 18:124–134. [PubMed: 9149541]
- Yechikhov S, Shchipakina T, Savina T, Kalemenev S, Levin S, Godukhin O. The role of Ca²⁺/calmodulin-dependent protein kinase II in mechanisms underlying neuronal hyperexcitability

induced by repeated, brief exposure to hypoxia or high K⁺ in rat hippocampal slices. *Neurosci Lett.* 2002; 335:21–24. [PubMed: 12457733]

Zeilhofer HU, Blank NM, Neuhuber WL, Swandulla D. Calcium-dependent inactivation of neuronal calcium channel currents is independent of calcineurin. *Neuroscience.* 2000; 95:235–241. [PubMed: 10619480]

Zhu Y, Yakel JL. Calcineurin modulates G protein-mediated inhibition of N-type calcium channels in rat sympathetic neurons. *J Neurophysiol.* 1997; 78:1161–1165. [PubMed: 9307144]

Figure 1.

A. Sample HVA Ca^{2+} current traces evoked by voltage steps to +10 mV in normal extracellular solution (Control) followed by traces evoked during superfusion with nimodipine (NIM, 10 μM), CdCl_2 (10 μM) and washout with normal solution. B. Sample plot of peak Ca^{2+} currents evoked in a single cell over time during an experiment as described in A. Washout partially reversed blockade by Cd^{2+} , though some current rundown was observed over the course of the experiment. C. Current density vs. voltage I-V plot for control (closed circles, $n=5$), NIM (open circles, $n=5$), CdCl_2 (open triangles, $n=5$) and washout (Recovery, closed triangles, $n=5$). D. Maximal current density for HVA currents evoked at +10 mV, demonstrating blockade of non-L-type channels with addition of Cd^{2+} and partial washout of Cd^{2+} inhibition. $N=5$ each condition, ** $p<0.01$, *** $p<0.001$ by t-test with Bonferroni correction after ANOVA.

Figure 2.

A. Sample HVA Ca^{2+} current traces elicited by step depolarizations in a control neuron (Normox) and a neuron recorded immediately after 4 h at 1% O_2 (Hypox.4/Rec.0). B. IV curve of peak currents vs. voltage, showing significantly enhanced Ca^{2+} current after hypoxia ($n=10$, $*p<0.05$). C. Activation kinetics in neurons recorded immediately after hypoxia (Hypox.4/Rec.0) did not differ from control (Normox, $n=10$ each condition, $p>0.05$). Inactivation voltage parameters were also unchanged in cells recorded within 2 h after 4 h hypoxia ($n=11$ for each condition, $p>0.05$).

Figure 3.

A. Current density IV plot from control neurons (Normox, closed circles, $n=7$) and after 4 h hypoxia followed by 48 h normoxic recovery (Hypox.4/Rec.48, open squares, $n=7$). B. In contrast to recordings made immediately after hypoxia (Normox vs. Hypox.4/Rec.0, $n=10$ each condition, $p<0.05$), there was no significant difference in maximal Ca^{2+} current density after 48 h recovery ($n=7$ each condition, $p>0.05$). There were also no changes in the voltage dependence of activation or steady-state inactivation (data not shown).

Figure 4.

Nimodipine (NIM, 10 μ M) was used to quantify L-type VGCC Ca^{2+} current, measured as the difference between total and NIM-resistant, non-L-type currents at +5 mV. A. Both total and L-type VGCC current density increased immediately after hypoxia (Normox vs. Hypox. 4/Rec.0, n=8 each condition, $p < 0.05$), while non-L type current density did not change. B. The fraction of total VGCC current sensitive to nimodipine increased slightly after hypoxia, such that the difference between L-type and non-L-type current was no longer significant (n=8 each condition, * $p < 0.05$ by *t*-test with Bonferroni correction after ANOVA).

Figure 5.

Sample HVA Ca^{2+} current traces (A), I-V curves (B) and activation/inactivation plots (C) from normoxic (Normox-FK506, $n=8$) and post-hypoxic neurons (Hypox-FK506, $n=7$), recorded with the selective calcineurin inhibitor, FK506 ($5 \mu\text{M}$), in the whole-cell recording pipette. FK506 prevented the hypoxia-induced enhancement, without altering the kinetics, IV relationship, or activation/inactivation properties. Gray line without symbols in part B represents control IV plot in absence of FK506. D. FK506 ($5 \mu\text{M}$ in the recording pipette) prevented enhancement of total and L-type (NIM-sensitive) VGCC currents after hypoxia.. E. The increase in L-type current relative to non-L-type current was also prevented by FK506, such that the difference between L and non-L current remained significant ($n=8$ each condition, ** $p<0.01$, *** $p<.001$ by t-test with Bonferroni correction after ANOVA).

Figure 6.

A–C. Like FK506, cyclosporin A (CsA, 20 μM) present during 4 h hypoxia prevented hypoxia-induced augmentation of HVA Ca^{2+} current traces (A) without altering the I–V curve (B) or voltage activation/inactivation parameters (C, Normox–CsA vs. Hypox–CsA, $n=8$ each condition). CsA also suppressed control HVA Ca^{2+} currents, suggesting a regulatory role for calcineurin in normoxia as well as hypoxia. Gray line without symbols in part B represents control IV plot in absence of CsA. Rapamycin (RAP, 5 μM in the recording pipette), unlike FK506, did not prevent the post-hypoxic increase in VGCC current. Sample HVA Ca^{2+} current traces (D) and current density vs. voltage IV curves (E) demonstrated a significant difference between normoxic cells (Normox–RAP, $n=9$) and post-hypoxic cells (Hypox–RAP, $n=9$) in the presence of RAP. No significant differences were observed in voltage activation or inactivation parameters (F, $n=9$ each condition). G–I: Okadaic acid (OKA, 150 nM) during hypoxia did not prevent hypoxia-induced enhancement of HVA Ca^{2+} currents. Sample HVA Ca^{2+} current traces (G) and current density vs. voltage IV curves (H) demonstrate a significant difference between normoxic cells (Normox–OKA, $n=6$) and post-hypoxic cells (Hypox–OKA, $n=7$) exposed to hypoxia in the presence of OKA. No significant differences were observed in voltage activation or inactivation parameters (I, Normox–OKA $n=6$, hypoxia–OKA $n=7$, $p>0.05$).

Figure 7.

Prevention of hypoxia-induced increase in HVA Ca^{2+} current by NIM incubation during hypoxia. A. NIM ($10 \mu\text{M}$) did not alter the current density/voltage IV curve in normoxic cells (Normox-NIM, closed squares, $n=7$) compared to normoxic controls without nimodipine (Normox, closed circles, $n=7$, $p>0.05$). Current density in neurons exposed to hypoxia in the presence of NIM (Hypoxia-NIM, open squares, $n=9$) was not significantly different from control and markedly smaller than in neurons exposed to hypoxia without NIM (Hypox.4/Rec.0, open circles, $n=8$). B. Peak HVA Ca^{2+} current density at +5 mV for post-hypoxic neurons (Hypox, $n=8$) significantly differed from normoxic control neurons (Normox, $n=7$, $p<0.05$), while post-hypoxic neurons exposed in the presence of NIM ($n=9$) did not differ from normoxic controls treated 4 h with NIM ($n=7$, $p>0.05$).