Scavenging ROS dramatically increases NMDA receptor whole cell currents in painted turtle cortical neurons

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ABSTRACT

Oxygen deprivation triggers excitotoxic cell death in mammal neurons through excessive calcium loading via over-activation of N-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. This does not occur in the western painted turtle which overwinters for months without oxygen. Neurological damage is avoided through anoxia-mediated decreases in NMDA and AMPA receptor currents that are dependent upon a modest rise in intracellular Ca$^{2+}$ concentrations ([Ca$^{2+}]_{i}$) originating from mitochondria. Anoxia also blocks mitochondrial reactive oxygen species (ROS) generation which is another potential signaling mechanism to regulate glutamate receptors. To assess the effects of decreased intracellular [ROS] on NMDA and AMPA receptor currents we scavenged ROS with N-2-mercaptopropionylglycine (MPG) or N-acetylcysteine (NAC). Unlike anoxia, ROS scavengers increased NMDA receptor whole-cell currents by 100% while hydrogen peroxide decreased currents. AMPA receptor currents and [Ca$^{2+}]_{i}$ concentrations were unaffected by ROS manipulation. Since decreases in [ROS] increased NMDA receptor currents we next asked if mitochondrial Ca$^{2+}$ release prevents receptor potentiation during anoxia. Normoxic activation of mitochondrial ATP-sensitive potassium (mK$_{ATP}$) channels with diazoxide decreased NMDA receptor currents and was unaffected by subsequent ROS scavenging. Diazoxide application following ROS scavenging did not rescue scavenger-mediated increases in NMDA receptor currents. Fluorescent measurement of [Ca$^{2+}]_{i}$ and ROS levels demonstrated that [Ca$^{2+}]_{i}$ increases before ROS decreases. We conclude that decreases in ROS concentration are not linked to anoxia-mediated decreases in NMDA / AMPA receptor currents but are rather associated with an increase in NMDA receptor currents that is prevented during anoxia by mitochondrial Ca$^{2+}$ release.
INTRODUCTION

Aerobic organisms use diatomic oxygen (O$_2$) as the terminal electron acceptor of the mitochondrial electron transport chain (ETC). As a result of inconsistencies in electron flux a portion of all oxygen consumed (~ 3%) is left partially reduced as the superoxide anion (O$_2^{-}$) (Chen et al., 2003; Liu et al., 2002). This highly reactive molecule reacts rapidly with water leading to the formation of other reactive oxygen species (ROS), the most prevalent and stable of which is hydrogen peroxide (H$_2$O$_2$) (Chandel and Schumacker, 2000). Generated ROS diffuse out of the mitochondria and into the intracellular and extracellular environment where they can oxidize various cellular components (Henzler and Steudle, 2000; Ottaviano et al., 2008). ROS generated from non-mitochondrial sources, including nitric oxide (NO) from intracellular nitric oxide synthase (nNOS) and H$_2$O$_2$ from extracellular xanthine oxidase (XO), also contribute to baseline ROS concentrations and rates of oxidation (Ottaviano et al., 2008). ROS concentrations are managed by a series of antioxidant proteins including: superoxide dismutase (SOD), catalase and glutathione (GSH)/glutathione peroxidase (GPx). This antioxidant defense system maintains intracellular ROS concentrations ([ROS]$_i$) within non-toxic ranges and reverses ROS-mediated protein oxidation (Ottaviano et al., 2008; Sies, 1993). However, despite mechanisms to control changes in ROS levels, significant variations in intracellular ([ROS]$_i$) and extracellular ROS concentrations can occur (Starkov, 2008). Recently, changes in ROS levels have been identified to play roles in feedback systems and cellular signalling processes through reversible oxidation of critical cysteine (Cys) residues on target proteins that can alter protein conformation and levels of activity (Cross and Templeton, 2006; D’Autreaux and Toledano, 2007; Rhee et al., 2003).

In the absence of O$_2$, or anoxia, ROS production ceases and it is not known what effects this may have on cellular metabolism or health. For the most part it is a non-issue since most vertebrate species are unable to survive under anoxic conditions and are deleteriously affected by more than a few minutes of oxygen deprivation. Damage is most rapidly incurred within the central nervous system where the loss of oxidative phosphorylation reduces adenosine triphosphate (ATP) production to levels that cannot sustain the high energetic demands of neural tissue. Na$^+$ / K$^+$-ATPase activity decreases and membrane ion gradients are lost leading to membrane potential (V$_m$) depolarization, increased action potential (AP) firing and a rise in excitatory
amino acid release. Excessive glutamate release over-activates postsynaptic alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors increasing membrane permeability to $\text{Na}^+$ and resulting in $V_m$ depolarization and removal of the magnesium ($\text{Mg}^{2+}$) block from the N-methyl-D-aspartate (NMDA) receptors. Subsequently, NMDA receptor over-activation results in excessive calcium ($\text{Ca}^{2+}$) influx and eventual excitotoxic cell death (ECD) (Bosley et al., 1983; Choi, 1992). This sequence of events does not occur in the western painted turtle. It overwinters at the bottom of ice covered lakes and ponds for up to 4 months and is naturally anoxia-tolerant (Jackson, 2000; Jackson and Ultsch, 1982). Its ability to withstand extended periods of anoxia is in part due to an increase in inhibitory signaling. In the cerebrocortex, the onset of anoxia results in a large increase in the concentration of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA; Nilsson and Lutz, 1991). The consequent increase in GABA receptor activity serves to counteract excitatory inputs by effectively “clamping” the cell near its resting membrane potential, at the reversal potential ($E_{\text{GABA}}$) for the $\text{GABA}_A$ receptor (approximately -80 mV). This results in a decrease in action potential (AP) frequency (75–95%), and a reduction in ATP consumption to a rate met through glycolytic fermentation alone (Pamenter et al., 2011; Perez-Pinzon et al., 1992).

In turtle cerebrocortex, pyramidal neurons account for approximately 80-90\% the neuronal population (Ulinski, 2007). Their response to anoxia is of particular interest due to their glutamatergic nature and their role in excitotoxic cell death in mammals. Anoxia induces a 50\% reduction in NMDA and AMPA receptor evoked currents in pyramidal neurons, which protects against excessive receptor activation, $\text{Ca}^{2+}$ influx, and ECD (Bickler, 1998; Pamenter et al., 2008b; Shin and Buck, 2003). The anoxic down-regulation of NMDA and AMPA receptor currents is the result of a mitochondrial based $\text{Ca}^{2+}$ signalling cascade that is initiated by the activation of the mitochondrial ATP-sensitive potassium (mK$_{\text{ATP}}$) channel. Although the mechanism through which anoxia activates mK$_{\text{ATP}}$ channels has yet to be established, its’ activation initiates a decrease in mitochondrial membrane potential ($\Psi_m$) and triggers subsequent mitochondrial $\text{Ca}^{2+}$ release (Hawrysh and Buck, 2013; Pamenter et al., 2008a; Zivkovic and Buck, 2010). The effect of changing [ROS] on NMDA / AMPA receptor activity or [Ca$^{2+}$]$_i$ in turtle cortical pyramidal neurons has not been explored, although studies in other vertebrate species demonstrate that NMDA receptor activity is significantly increased by a decrease in ROS levels (Aizenman et al., 1989; Bodhinathan et al., 2010; Choi and Lipton, 2000). However, since
ROS levels naturally decrease in anoxic turtle brain we propose that it will trigger an increase in 
$[\text{Ca}^{2+}]_i$, and a decrease in NMDA and AMPA receptor whole cell currents (Pamenter et al., 2007). 
The aims of this study were to determine: 1) if decreasing $[\text{ROS}]_i$ decrease whole-cell evoked NMDA and AMPA receptor currents in turtle pyramidal neurons, 2) if mitochondrial $\text{Ca}^{2+}$ release is induced by ROS scavenging, and 3) if these responses are dependent on mitochondrial ROS production by using a complex I inhibitor.

RESULTS

Pharmacological ROS scavengers alter $[\text{ROS}]_i$

To investigate a role for $[\text{ROS}]_i$ in modulating NMDA / AMPA receptor currents we first confirmed that $[\text{ROS}]_i$ were eliminated by our anoxic experimental protocol. Using the chloromethyl derivative of 2',7'-dichlorodihydrofluorescein diacetate (CM-H$_2$DCF), a ROS-sensitive dye, we measured changes in fluorescence in cortical brain sheets during normoxia and anoxia, and with and without pharmacological ROS modulation. Fluorescence did not change significantly during one hour of normoxic perfusion (0.46 ± 0.5%; n = 8; Figure 1A & Ci), indicating maintenance of cellular redox homeostasis. A 30 min anoxic treatment significantly decreased fluorescence (-7.2 ± 1.2%; n = 8; p ≤ 0.001; Figure 1A & Cii) compared to normoxic control. A 30 min normoxic perfusion with N-2-mercaptopropionylglycine (MPG) or N-acetylcysteine (NAC) (0.5 mM each) significantly decreased fluorescence (-9.1 ± 1.5 and -7.3 ± 0.5% respectively; n = 5 each; p ≤ 0.001 for both; Figure 1A, Ciii), compared to normoxic controls. Conversely, the addition of MPG or NAC during anoxia did not significantly decrease fluorescence beyond the effects of anoxia alone or of normoxia plus ROS scavengers (-9.2 ± 0.9 and -8.7 ± 0.5% respectively; n = 5 each; Figure 1A, Civ). The mitochondrial ETC is the primary source of ROS generation in the cell, making it a potentially important component of any ROS-mediated signaling cascade. To investigate the connection between decreased mitochondrial ROS production and NMDA / AMPA receptor function we added rotenone (25 µM), a complex I (NADH dehydrogenase) inhibitor, to the perfusate. A 30 min perfusion of normoxic aCSF plus rotenone significantly decreased CM-H$_2$DCF fluorescence by -8.6 ± 1.5% (n = 4; p ≤ 0.001; Figure 1A, Cv), while addition of rotenone during anoxia had no additional effect (-9.1 ± 0.6%; n = 4; Figure 1A, Cvii). To demonstrate that ROS levels could be experimentally increased we used H$_2$O$_2$ and first determined an appropriate physiological concentration of H$_2$O$_2$ to apply. Drip
application of H$_2$O$_2$ increased CM-H$_2$DCF fluorescence in a dose-dependent manner (n = 4 - 8 each; Figure 1B), with 50 µM [H$_2$O$_2$] being the lowest concentration in which we could detect a significant change in fluorescence. This finding is in agreement with normoxic measurements of H$_2$O$_2$ from the media of cultured turtle neurons and is near the reported physiological range of mammalian neuronal [H$_2$O$_2$]$_c$ (1-20 µM) (Hoyt et al., 1997; Lei et al., 1998; Milton et al., 2007). In addition, this concentration did not affect baseline electrophysiological properties of pyramidal neurons, such as membrane potential, whole-cell conductance, and action potential threshold indicating it did not induce oxidative damage (Table 1). A 5 min application of H$_2$O$_2$ significantly increased fluorescence during both normoxia and anoxia (23.6 ± 1.1 and 25.4 ± 1.3% respectively; n = 5 each; P ≤ 0.001 for both; Figure 1A).

**Pharmacological ROS manipulation modifies NMDA receptor activity**

Evoked NMDA receptor current amplitudes did not change significantly after 90 min of normoxic perfusion (n = 8; Figure 2A, Bii). Anoxic perfusion decreased evoked NMDA receptor currents after 20 min to 61.9 ± 8.6% of normoxic control (n = 7; p = 0.004) and to 58.6 ± 9.0%; (n = 7; p = 0.008) after 40 min, and were reversed after 20 (82.7 ± 12.2%; n = 6) and 40 (98.2 ± 4.2%; n = 5) min of normoxic washout (Figure 2 A, Bii) (Note: 20 and 40 min treatment values were not significantly different therefore only the 20 min data are presented in summary graphs). ROS scavenging during normoxia increased evoked NMDA receptor currents after 20 to 201.4 ± 7.1% (n = 6; p = 0.001) of normoxic control and to 195.1 ± 16.3% (n = 4; p ≤ 0.001) after 40 min of MPG application (Figure 2A, Biii) or after 20 (192.5 ± 19.9%; n = 6; p = 0.046) and 40 (208.9 ± 37.3%; n = 4; p = 0.035) min of NAC application (Figure 2A, Biv). The effects of these increases resulted in hyperactivity, depolarization in all patches and the loss of the patch in ~50% of recordings. In situations where the patch was maintained, normoxic washout failed to reverse the effects of MPG after 20 and 40 min (182.8 ± 16.3 and 186.7 ± 5.3% respectively; n = 3 each; p < 0.001 for both) but a trend towards recovery was observed after 20 and 40 min of NAC washout (168.4 ± 26.1 and 139.6 ± 24.7%; n = 3 each) (Figure 2A). H$_2$O$_2$ addition during normoxia decreased evoked NMDA receptor currents to 80.2 ± 3.1% (n = 9, p = 0.023) of control after 20 and to 78.4 ± 4.4% (n = 5; p = 0.036) following 40 min of treatment, which was reversed after 20 and 40 min of normoxic washout (93.2 ± 7.1 and 99.3 ± 7.8% respectively; n = 4 each) (Figure 2A, Bv).
In order to evaluate the role of mitochondrial produced ROS in regulation of NMDA receptor activity, the complex I inhibitor rotenone was administered under normoxic conditions to prevent mitochondrial ROS formation. Rotenone increased evoked NMDA receptor currents after 20 to 216.6 ± 30.1% (n = 5; p = 0.017) of control and to 232.3 ± 28.5% (n = 4; p = 0.01) following 40 min of treatment, which was reversed after 20 and 40 min of normoxic washout (101.1 ± 2.9 and 104.6 ± 3.4% respectively; n = 3 each) (Figure 2A, Bvi). To determine that this was not a result of the chloroform used to solubilise rotenone, neurons were exposed to aCSF solution containing 0.05% chloroform and this did not affect NMDA receptor currents after 20 (106.9 ± 3.9%; n=3) or 40 (109.5 ± 0.18%; n=3) min of treatment (data not shown).

Pharmacological ROS manipulation does not modify AMPA receptor activity

Evoked AMPA receptor current amplitudes did not change significantly after 90 min of normoxic perfusions (n = 6 each; Figure 3A, Bi). Anoxic perfusion decreased evoked AMPA receptor currents to 69.95 ± 5.69% (n = 5; p = 0.006) of control at 20 min and to 55.16 ± 6.04% (n = 5; p = 0.001) after 40 min, and were reversed after 20 and 40 min of normoxic washout (98.8 ± 4.9 and 101.3 ± 5.2%; n = 4 each) (Figure 3A, Bii) (Note: 20 and 40 min treatment values were not significantly different therefore only the 20 min data are presented in summary graphs). ROS scavenging during normoxia had no effect on evoked AMPA receptor currents relative to control after 20 (105.4 ± 4.6%; n = 6) and 40 (94.7 ± 7.3%; n = 4) min of MPG application (Figure 3A, Biii) or after 20 (100.5 ± 3.2%; n = 7) and 40 (103.5 ± 2.8%; n = 6) min of NAC application (Figure 3 A, Biv). Currents remained unchanged through 20 and 40 min of normoxic washout following MPG (95.26 ± 3.29 and 97.81 ± 12.60% respectively; n = 4 and n = 3 respectively) and NAC (98.62 ± 2.81 and 97.04 ± 3.86% respectively; n = 4 and n = 3 respectively) applications (Figure 3A). \( \text{H}_2\text{O}_2 \) addition during normoxia had no effect on evoked AMPA receptor currents relative to control after 20 (100.0± 3.4%; n = 11) and 40 (99.2 ± 2.4%; n = 7) min of treatment, and remained unchanged through 20 and 40 min of normoxic washout (106.33 ± 3.91 and 100.00 ± 2.95% respectively; n = 5 and n = 3 respectively) (Figure 3A, Bv).

Pharmacological manipulation of ROS has no effect on \([\text{Ca}^{2+}]_i\)
To investigate if [ROS] modulates Ca\(^{2+}\) signaling we next assessed the effect of pharmacological ROS scavenging on [Ca\(^{2+}\)]. Using the Ca\(^{2+}\) sensitive dye Oregon Green we measured changes in fluorescence in cortical brain sheets with and without pharmacological ROS modulation, and fluorescence did not change significantly from baseline following 20 min of normoxic perfusion (0.6 ± 0.8%; n = 6) (Figure 4A, Bi). Anoxic perfusion resulted in a significant increase in fluorescence (16.9 ± 3.3%; n = 6; p = 0.001; Figure 4A, Bii) while ROS scavenging with MPG (0.6 ± 0.6%; n = 6; Figure 4A, Biii) or NAC (1.0 ± 1.3%; n = 6; Figure 4A, Biv) or addition of H\(\text{H}_2\)O\(_2\) (0.84 ± 1.39%; n = 6; Figure 4A, Bv) did not significantly change fluorescence.

To demonstrate that ROS scavenging did not affect NMDA receptor currents through a change in intracellular Ca\(^{2+}\), BAPTA (5 mM) was included in the patch pipette. The Ca\(^{2+}\) chelator did not prevent MPG-mediated increases in NMDA receptor currents after 20 min to 205.37 ± 23.58% (n = 4; p = 0.020) or 40 min to 222.65 ± 33.24% of control (n = 4; p = 0.012). However, exposure to MPG or NAC beyond the 40 min treatment period resulted in a slow increase in fluorescence (11.4 ± 0.9 % over 10 min; n = 4; data not shown). Drip perfusion of amino-5-phosphonovaleric acid (APV selective NMDA receptor blocker) reduced the rate of fluorescent increase (5.1 ± 0.5 % over 10 min; n = 4; data not shown) indicating Ca\(^{2+}\) influx through NMDA receptors is partly responsible for the increase. This supports our findings that long-term exposure to ROS scavengers is toxic to neurons due to activation of NMDA receptors and agrees with our findings that neurons treated with ROS scavengers for long periods are difficult to recover.

Anoxia-mediated changes in NMDA receptor activity are unaffected by H\(\text{H}_2\)O\(_2\) application

To determine if anoxic down regulation of NMDA receptor current amplitudes were affected by increases in ROS, H\(\text{H}_2\)O\(_2\) was applied following the transition to anoxia and changes in NMDA receptor currents were measured. Anoxic perfusion alone decreased evoked NMDA receptor currents after 20 min to 69.6 ± 3.5% (n = 5; p = 0.031) and after 40 min to 66.6 ± 6.0% (n = 5; p = 0.013) of control. Subsequent anoxic H\(\text{H}_2\)O\(_2\) administration did not reverse the decrease in NMDA receptor current amplitudes after 20 to 58.6 ± 6.73% (n = 5; p = 0.001) and after 40 min to 54.36 ± 5.57% (n = 5; p < 0.001) of control, changes were not significantly different from anoxia alone. Normoxic washout did not reverse the anoxic effects after 20 min (59.00 ±
Mitochondrial Ca$^{2+}$ release prevents ROS scavenger-induced increases in NMDA receptor currents

Anoxia-mediated activation of mitochondrial ATP-sensitive potassium (mK$_{ATP}$) channels leads to mitochondrial Ca$^{2+}$ release and prevents excessive NMDA receptor currents (Hawrysh and Buck, 2013; Pamenter et al., 2008a; Zivkovic and Buck, 2010). However, this occurs at the same time as decreasing [ROS]$_i$ which we found to increase NMDA receptor currents. To better understand the interaction of anoxic mitochondrial Ca$^{2+}$ release and decreasing [ROS]$_i$ on NMDA receptor currents we pharmacologically modulated both signals and assessed the effect on whole-cell NMDA receptor currents. First we tested the effect of pharmacologically increasing mitochondrial Ca$^{2+}$ release prior to ROS scavenging. To achieve a small increase in [Ca$^{2+}]_i$, and generate reductions in NMDA receptor currents, the mitochondrial ATP-sensitive K$^+$ channel activator diazoxide was administered (Pamenter et al., 2008a; Zivkovic and Buck, 2010). Diazoxide administration decreased evoked NMDA receptor currents after 20 min to 66.9 ± 3.9% (n = 5; p = 0.049) and after 40 min to 62.6 ± 5.7% (n = 5; p = 0.022) relative to control (Figure 5A, Bii). Note that none of the 40 min treatment data where significantly different from the 20 min treatment data and are therefore not shown in the figures. MPG application following diazoxide treatment did not produce increases in NMDA receptor current amplitudes relative to diazoxide alone after 20 (57.3 ± 5.5%; n = 5; p = 0.008) and 40 (62.8 ± 8.8%; n = 4; p = 0.036) min (Figure 5A, Bii). The effects were reversed after 20 min of normoxic washout (83.7 ± 21.2 and 89.8 ± 22.6% respectively; n = 4 each) (Figure 5A). To determine if the effects of ROS scavenging are reversed by an increase in [Ca$^{2+}]_i$, diazoxide was applied after the addition of MPG. MPG application was limited to 20 min in order to try and prevent the loss of the patch encountered in previous ROS scavenging experiments and attributed to NMDA receptor over-activation. MPG administration increased evoked NMDA receptor currents after 20 min to 198.9 ± 21.2% (n = 4; p = 0.001) relative to normoxic control (Figure 5A, Biii) and subsequent diazoxide application did not reverse the MPG effects significantly after 20 (180.4 ± 11.8%; n = 4; p = 0.003) or 40 (175.4 ± 19.8%; n = 4; p = 0.006) min (Figure 5A, Biii). The effects were
reversed after 20 min of normoxic washout (101.6 ± 11.4 and 104.2 ± 8.0%; n = 4 each) (Figure 5A).

Increases in \([\text{Ca}^{2+}]_i\) and decreases in \(\Psi_m\) occur prior to \([\text{ROS}]_i\); decreases during anoxia

To understand the sequence of events leading to anoxic NMDA and AMPA receptor inhibition we next assessed the timeline of changes in \(\text{PO}_2\), \(\Psi_m\), \([\text{Ca}^{2+}]_i\), and \([\text{ROS}]_i\). First we assessed the changes in bath chamber \(\text{PO}_2\) using a fluorescent \(\text{O}_2\) probe to measure changes in \(\text{PO}_2\) during a 60 min normoxic to anoxic transition and normoxic recovery. Chamber \(\text{PO}_2\) significantly decreased from a normoxic value of 147.32 ± 2.8 to 0.52 ± 0.3 mmHg during a 30 min anoxic treatment (\(n = 5; p \leq 0.001; \)Figure 6A). Reperfusion with normoxic aCSF returned chamber \(\text{PO}_2\) levels to the pre-anoxic values (146.4 ± 2.7 mmHg; \(n = 5\)). Following the switch to anoxic perfusion the onset of the decrease in chamber \(\text{PO}_2\) occurred in 6.0 ± 0.7 seconds and reached a steady state level by 607 ± 63.7 sec (\(n = 5\)). The \(\text{Ca}^{2+}\) signal responsible for modulating NMDA and AMPA receptor currents during anoxia originates from the mitochondria and is the result of depolarization of \(\Psi_m\). To investigate the timeline of the \(\text{Ca}^{2+}\) signal we assessed the onset and steady state changes in \(\Psi_m\) using the fluorescent dye rhodamine-123 and changes in \([\text{Ca}^{2+}]_i\) with Oregon-Green. The onset of increase in rhodamine fluorescence (\(\Psi_m\) depolarization) occurred 70.6 ± 5.1 seconds after switch to anoxia and reached a steady state in 355 ± 25.1 sec after the switch (\(n = 11; \)Figure 6B). The onset of Oregon-Green fluorescence increase (elevated \([\text{Ca}^{2+}]_i\)) began at 70.4 ± 5.6 sec after the switch to anoxia and reached steady state 196 ± 19.9 after switch to anoxia (\(n = 5; \)Figure 6C). The rate of ROS generation started to decrease 120.2 ± 7.6 sec after anoxic switch and reached a plateau indicating no new ROS formation 597 ± 62.8 sec after the anoxic switch (\(n = 8; \)Figure 6D).

DISCUSSION

In this study we explored the effects of scavenging ROS on glutamatergic signalling in pyramidal neurons of the anoxia-tolerant western painted turtle. To our knowledge this is the first investigation into the effects of oxidizing/reducing agents on glutamate receptor activity in reptiles. Using pharmacological ROS scavengers we demonstrate that decreases in \([\text{ROS}]_i\) do not initiate an increase in \([\text{Ca}^{2+}]_i\) or reductions in NMDA and AMPA receptor currents during anoxia (Pamenter et al., 2008b; Zivkovic and Buck, 2010). Under normoxic conditions, neither the
application of ROS scavenging agents (MPG or NAC) nor H$_2$O$_2$ had an effect on [Ca$^{2+}$]; or AMPA receptor whole cell currents (Figure 3 and 4). The application of ROS scavenging agents resulted in approximately a 100% increase in NMDA receptor whole-cell currents while exogenous application of H$_2$O$_2$ decreased whole-cell currents by ~ 20% (Figure 2). Furthermore, the re-introduction of ROS via H$_2$O$_2$ application under anoxic conditions did not reverse anoxia-mediated decreases in NMDA receptor currents.

Our findings of a change in NMDA receptor currents and no effect on AMPA receptor currents in response to ROS scavenging is consistent with results from other vertebrate species in which application of oxidizing agents (e.g., 5,5'-dithiobis-2-nitrobenzoic acid (DNTB) or glutathione disulfide (GSSG)) decrease and reducing agents (e.g., dithiothreitol (DTT)) increase NMDA receptor currents and neither treatment affects AMPA receptor currents (Aizenman et al., 1989; Bodhinathan et al., 2010; Choi et al., 2001; Choi and Lipton, 2000; Gozlan et al., 1995; Janáky et al., 1993). The increase in NMDA receptor currents resulting from application of reducing agents is generally 6-11 times greater than decreases initiated by oxidizing agents, a ratio comparable to the 5:1 we found for ROS scavenging or H$_2$O$_2$ application in turtle pyramidal neurons (Aizenman et al., 1989; Bodhinathan et al., 2010; Choi and Lipton, 2000). The large increase in receptor currents triggered by ROS scavenging may permit excessive Ca$^{2+}$ influx into patched pyramidal cells during NMDA application, explaining why NAC/MPG application often resulted in cell depolarization, and death. The redox sensitivity of NMDA receptors has been attributed to the existence of extracellular cysteine (Cys) residues located on specific NMDA receptor subunits (GluN1, GluN2A and GluN2B), collectively termed NMDA receptor redox modulatory sites (Choi et al., 2001; Gozlan and Ben-Ari, 1995; Kim et al., 1999; Sullivan et al., 1994). The high sensitivity of NMDA receptors to reducing agents, compared to oxidizers, is thought to be the result of extracellular redox sites being maintained in a predominantly oxidized state since extracellular antioxidants are produced within the cell and are slow to diffuse out (Jones et al., 2000; Ottaviano et al., 2008). This may also explain why the effects of H$_2$O$_2$ were reversed by reperfusion but the effects of MPG and NAC were not, similar to other studies in which the effects of oxidizing but not reducing agents were reversed by washout (Bodhinathan et al., 2010; Köhr et al., 1994). Because cellular ROS production is slow, if extracellular levels are eliminated it may take some time before baseline concentrations are re-established, and scavenging agents are degraded or cleared (Ottaviano et al., 2008).
Blocking mitochondrial ROS production using the complex I inhibitor rotenone resulted in increases in NMDA receptor activity to a similar degree as that of ROS scavenging, demonstrating that changes in mitochondrial ROS production can directly modulate NMDA receptor activity. Full recovery from rotenone treatment was achieved within the reperfusion period, supporting the finding that the lack of recovery seen during ROS scavenging is the result of delays in oxidation/degradation/clearance of the scavenging agents used. NMDA receptors are highly expressed in the post synaptic densities of excitatory synapses, as are mitochondria which provide the necessary ATP for synaptic activities (Danysz and Parsons, 1998; Ly and Verstreken, 2006). H$_2$O$_2$ produced from these mitochondria can freely diffuse across the cellular membrane, or move through ion channels, in order to oxidize extracellular NMDA receptor redox modulatory sites (Mollajew et al., 2010; Ottaviano et al., 2008). The function of NMDA receptor redox control or the connection to the mitochondria has yet to be established; however, we propose that ROS changes may be involved in a negative feedback system between mitochondria, a primary site for intracellular Ca$^{2+}$ storage/regulation, and NMDA receptor-mediated Ca$^{2+}$ entry. Mitochondrial ROS generation is increased by NMDA receptor activation unless blocked by the removal of extracellular Ca$^{2+}$, mitochondrial uncouplers, or Ca$^{2+}$ uniporter inhibitors (Duan et al., 2007; Dugan et al., 1995). A feedback loop between Ca$^{2+}$ influx and storage could be utilized to prevent excessive uptake leading to cell death.

The mechanism by which Ca$^{2+}$ release from the mitochondria is triggered has yet to be established. We have proposed that it is the result of mK$_{ATP}$ activation and subsequent formation of low conductance mitochondrial permeability transition pores (mPTP) (Pamenter et al., 2008a; Zivkovic and Buck, 2010, Hawrysh and Buck, 2013). mK$_{ATP}$ channels remain closed under normoxic conditions when ATP levels are high and are activated during anoxia as mitochondrial ATP production decreases. Opening of the channel permits an influx of K$^+$ into the mitochondria that triggers mPTP formation and Ca$^{2+}$ release (Murchison and Griffith, 2000). In the western painted turtle addition of the mPTP activator atractyloside decreases NMDA receptor currents and increases [Ca$^{2+}$]$_i$ to levels comparable to those induced by anoxia and the addition of the Ca$^{2+}$ chelator 1,2-bis (o-aminophenoxy) ethane-N, N, N', N'-tetraacetic acid blocks the decrease in NMDA receptor currents (Hawrysh and Buck, 2013). The mechanism through which an increase in [Ca$^{2+}$]$_i$ brings about a decrease in NMDA and AMPA receptor currents has previously been attributed to increases in the activity of the Ca$^{2+}$-binding messenger protein
calmodulin (Ca$^{2+}$/calmodulin) and subsequent activation of the Ca$^{2+}$/calmodulin-dependent phosphatase PP2B (calcineurin) (Shin et al., 2005). Calmodulin binding and dephosphorylation of NMDA receptor subunits during anoxia may cause changes in protein conformation that block / inhibit NMDA receptor redox sites and decrease NMDA receptor activity.

We have shown that decreases in [ROS]$_i$ increase NMDA receptor activity; however, receptor currents decrease during anoxia and indicate that a secondary mechanism overrides modulatory redox control and initiates receptor inhibition. Replicating the anoxia-mediated increase in mitochondrial Ca$^{2+}$ release with the mitochondrial K$_{ATP}$ channel agonist diazoxide produced a decrease in NMDA receptor currents comparable to anoxia and was unaffected by MPG application indicating that Ca$^{2+}$ release overrides redox control. It is important to note that application of diazoxide after MPG addition was not successful in reversing the large increases in NMDA receptor currents brought about by ROS scavenging, suggesting that if increases in [Ca$^{2+}]_i$ do not occur before ROS decreases, NMDA receptor activity will rise and may lead to ECD. This effect was also seen when the anoxia-mediated increase in [Ca$^{2+}]_i$ was blocked with a Ca$^{2+}$ chelator, which prevented the anoxia-mediated down regulation of the NMDA receptor and also produced significant increases in NMDA receptor currents, potentially as a result of ROS decreases (Shin et al., 2005). For mitochondrial Ca$^{2+}$ release to prevent redox induced NMDA receptor potentiation activation of mK$_{ATP}$ channels, mPTP formation and mitochondrial Ca$^{2+}$ release must all occur before anoxic decreases in ROS. To assess the timing of intracellular anoxia-mediated signals we compared changes in Ψ$_m$, [Ca$^{2+}]_i$ and [ROS]$_i$. The onset of the increase in fluorescence of both rhodamine-123 and Oregon Green dyes occurred at ~70 seconds after the switch to anoxia. Since Rhodamine-123 has a response time in the sec - min range (Plásek and Sigler, 1996) and Oregon Green in the msec range (Canepari and Mammano, 1999), it is likely that Ψ$_m$ depolarization occurred prior to Ca$^{2+}$. This finding further supports the hypothesis that anoxia-mediated depolarization of Ψ$_m$ is the signal to induce mitochondrial Ca$^{2+}$ release. The onset of the anoxia-mediated Ca$^{2+}$ signal occurs ~ 40 seconds before [ROS]$_i$ begin to decrease and reaches steady state ~ 400 seconds before [ROS]$_i$ reaches steady state. We propose that this provides sufficient time to inhibit NMDA receptors before redox modulation could occur. Interestingly, changes in Ψ$_m$ and [Ca$^{2+}]_i$ occurred before bath chamber pO$_2$ reached ~ 0 mmHg indicating that depolarization of Ψ$_m$ occurs before tissue pO$_2$ reaches 0 mmHg. When
considering the aforementioned information collectively, we conclude that the time-dependency of Ca\textsuperscript{2+} release is critical with respect to changes in ROS and this order of events may have been selected for in the turtle brain.

In summary, we have demonstrated that decreases in [ROS]\textsubscript{i} during anoxia within cortical pyramidal neurons of the western painted turtle are not responsible for triggering down regulation of NMDA and AMPA receptors. Instead, we have provided evidence that NMDA receptors in turtle pyramidal neurons respond to oxidative/reductive challenges in a similar manner as in other vertebrate species. Our findings indicate that during the transition to anoxia mitochondrial Ca\textsuperscript{2+} release prior to depletion of ROS levels is essential for overriding mechanisms of redox control and the down regulation NMDA and AMPA receptor activity.

MATERIALS AND METHODS

Animals

This study was approved by the University of Toronto Animal Care Committee and conforms to the relevant guidelines issued by the Canadian Council on Animal Care regarding the care and use of experimental animals. Adult female turtles (carapace length ~ 15 cm, 200-300g) were purchased from Niles Biological Inc. (Sacramento, CA, USA). The animals were housed in large indoor ponds (2 m x 4 m x 1.5 m) equipped with basking platform, heating lamp and a flow-through dechlorinated fresh water system. The water temperature was maintained at ~ 18ºC and the air temperature at 20ºC. Turtles were given continuous access to food and kept on a12h light:12h dark photoperiod.

Cortical brain sheet preparation and experimental setup

Basic protocols for cortical sheet dissection and whole-cell patch clamp recordings under normoxic and anoxic conditions are described elsewhere (Shin and Buck, 2003). Briefly, turtles were decapitated and whole brain was rapidly excised from the cranium within 30 sec of decapitation. Six cortical sheets were isolated from whole brains and bathed in artificial cerebrospinal fluid (aCSF) in mM: 107 NaCl, 2.6 KCl, 1.2 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 2 NaH\textsubscript{2}PO\textsubscript{4} · 2H\textsubscript{2}O, 26.5 NaHCO\textsubscript{3}, 10 glucose, and 5 imidazole (pH 7.4; osmolarity 285–290 mOsm). Cortical sheets were placed in an RC-26 chamber with a P1 platform (Warner Instruments, Hamden, CT). The
The chamber was gravity perfused with aCSF at a rate of 2–3 mL•min⁻¹. Normoxic aCSF was gassed with air/5% CO₂ and anoxic aCSF with 95% N₂/5% CO₂. Preliminary experiments comparing the use of 95% O₂/5% CO₂ and air/5% CO₂ demonstrated that there were no differences in any of the results and of particular note – switching between 95% O₂ and air had no effect on ROS production. To maintain anoxic conditions in the bathing chamber perfusion tubes from the intravenous bottle were double jacketed and the outer jacket was gassed with 95% N₂/5% CO₂ and a plastic cover with a hole for the recording electrode was placed over the perfusion chamber, and the space between the fluid surface and cover was gently gassed with 95% N₂/5% CO₂. The anoxic aCSF reservoir was bubbled vigorously for 30 min prior to an experiment and gently throughout the experiment to maintain anoxic conditions. The partial pressure of oxygen (pO₂) in bath aCSF decreased to ~ 0 mmHg under these experimental conditions in ~ 10 min (i.e. bath pO₂ not different from reservoir pO₂; see Results and Figure 6A for timeline of pO₂ changes). Bath chamber pO₂ was measured using a fluorescent oxygen analyzer and Witrox-1 v1.6.0 software (Witrox 1; Loligo Systems; Denmark). A fast-step drug perfusion system (VC-6 model perfusion valve controller and SF-77B fast-step perfusion system; Warner Instruments) was used to deliver pharmacological modifiers directly above the cortical sheet (see details below). Fast-step perfusion syringes were also bubbled and jacketed in the same manner as above to maintain anoxic conditions. All experiments were performed at a room temperature of 22°C.

Whole-cell patch clamp electrophysiology

Whole-cell recordings from pyramidal neurons located in the dorsomedial area of the dorsal cortex were obtained using fire-polished 4-6 MΩ borosilicate glass pipettes (Harvard Apparatus LTD, Holliston, MA). Pipette solutions contained (in mM): 8 NaCl, 0.0001 CaCl₂, 10 Na HEPES, 110 K gluconate, 1 MgCl₂, 0.3 NaGTP, 2 NaATP (pH 7.4; osmolarity 295–300 mOsM). An Ag-AgCl electrode connected to a CV-7B headstage and MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA) was inserted into pipettes and a motorized patch-clamp micromanipulator (Burleigh, PCS-6000 series, Thorlabs, Newton, NJ) was used to position them within the tissue. Cell-attached 5-10 GΩ seals were obtained using blind-patch techniques described elsewhere (Blanton et al., 1989). Upon seal formation, negative pressure was applied to achieve the whole-cell patch clamp-configuration. Following whole-cell capacitance
compensation, typical whole-cell access resistance \( (R_a) \) was 20-25 MΩ. \( R_a \) was determined before each measurement and recordings were discarded if \( R_a \) changed by more than 20% or whole-cell leak currents changed more than 30 pA during the course of the experiment. Prior to the commencement of experiments, a step protocol to identify cell type was performed as described elsewhere (Shin and Buck, 2003), and patches from non-pyramidal cells were discarded. All data was collected at 5-10 kHz using an Axopatch-1D amplifier, a CV-4 head stage, and a Digidata 1200 interface and analyzed using Clampex 10 software (Molecular Devices, Sunnyvale, CA, USA).

Evoked NMDA receptor and AMPA receptor current recordings

Following membrane rupture and formation of the whole-cell patch a 5 min period was allowed for patch stabilization prior to commencement of recordings. Control evoked NMDA / AMPA receptor currents were recorded at the start of the experiment at \( (t=0 \text{ min}) \) and following 10 min of normoxic perfusion. The initial current recording was set to a value of 100% and all subsequent recordings were normalized to that first control value. The second control value \( (t=10 \text{ min}) \) was used to confirm consistency within the normoxic recordings and for future statistical analysis. Cells were next perfused with experimental bulk aCSF treatments and/or drip perfusions. Experimental conditions were maintained for 40-80 min and evoked current recordings were taken at 20 min intervals. The tissue was reperfused following the experimental treatment period with control normoxic aCSF for 40 min and current recordings were taken at 20 min intervals.

Fluorescence measurements

In all fluorescence experiments cortical sheets were placed in a flow-through bath chamber of an upright microscope (Olympus BX51WI) equipped with an Olympus 0.8 N.A., 40X water immersion objective. Dyes were imaged using a FITC filter set (Semrock, Rochester, NY) and a monochromonator (Photon Technology international), controlled by Easy Ratio Pro imaging software (PTI, London, ON). Fluorescence emissions were detected with an EMCCD camera (Rolera-MGi; Q imaging; Burnaby, BC). Neurons were excited for 0.5 sec every 10 sec to prevent bleaching of the dye and permit experiments of up to an hour in length. To assess if endogenous fluorescence of cortical sheets affects fluorescence measurements, control cortical
sheets were exposed to each treatment in the absence of fluorophores. The background fluorescence was minimal and remained constant with each treatment and, therefore, background fluorescence was not subtracted from fluorescent data. For statistical analysis, 10 neurons per cortical sheet were chosen at random and the average change in regions of interest (ROI) from the center of the cell body was used as a single replicate. Brightly fluorescing cells were avoided. Sample traces were smoothed using Easy Ratio Pro imaging software to reduce noise and simplify interpretation. Oregon-Green and rhodamine traces were drift corrected to a linear regression line fit to the 10 min normoxic portion of the trace to enable comparison and produce average traces (Figure 4).

**CM-H$_2$DCF fluorescence measurements for [ROS]$_i$**

Changes in [ROS]$_i$ were assessed using the membrane-permeable ROS sensitive fluorescent indicator 5-(and-6)-chloromethyl-2',7'-dichlorodihydro-fluorescein diacetate, acetyl ester (CM-H$_2$DCFDA; Invitrogen, Burlington, ON). Cortical sheets were incubated in aCSF containing 5 μM CM-H$_2$DCFDA (from a 1 mM stock solution in dimethylsulfoxide, (DMSO)) for 30 min (4°C) followed by a 30 min wash in aCSF (22°C). During loading the acetate groups on CM-H$_2$DCFHDA are removed by intracellular esterases preventing dye leakage. CM-H$_2$DCF was excited with a wavelength of 495 nm and fluorescence emission was detected at wavelength of 520 nm. Cortical sheets were exposed to treatment aCSF for 30 min then reperfused with control aCSF for 20 min or treated with 50 μM H$_2$O$_2$ for 5 min. Steady state normoxic generation of ROS results in oxidation of the CM-H$_2$DCFH to CM-H$_2$DCF and a subsequent increase in fluorescence. Cessation of ROS generation results in no change in CM-H$_2$DCF fluorescence. To assess treatment effects on ROS generation the fluorescence at treatment steady state was compared to a linear regression line fit to the 10 min normoxic portion of the trace. Data are presented as percent change expressed relative to that fitted normoxic regression line (Crowe et al., 1995).

**Oregon Green fluorescence measurements for [Ca$^{2+}$]$_i$**

Changes in [Ca$^{2+}$]$_i$ were assessed using the membrane-permeable Ca$^{2+}$-sensitive fluorescent indicator Oregon-Green 488 BAPTA-1 AM (Invitrogen, Burlington, ON). Oregon-Green was selected due to its high Ca$^{2+}$ affinity (Kd ~ 170 nM) and previous use in turtle cortical tissue
(Hawrysh and Buck, 2013). Cortical sheets were incubated in aCSF containing 5 μM Oregon-Green (from a 1 mM stock solution in DMSO for two consecutive 1 hr periods at 4°C followed by a 30 min wash in aCSF (22°C). The double loading period resulted in elevated baseline fluorescence levels indicating sufficient dye uptake. Oregon-Green was excited with a wavelength of 488 and fluorescence emissions were detected at a wavelength of 520 nm. Cortical sheets were exposed to treatment aCSF for 20-40 min then reperfused with control aCSF for at least 10 min to allow fluorescence to return to baseline. Once each experiment was completed, aCSF flow was halted and tissues were incubated in ionomycin (2 μmol) for 5 min, followed by application of MnCl₂ (2 mM), to quench the Ca²⁺ fluorescence signal and obtain a value for background fluorescence. This value was subtracted from all recordings during analysis to isolate the fluorescence attributed to changes in [Ca²⁺]. The protocol and concentrations used were based on previous investigations (Hawrysh and Buck, 2013).

**Rhodamine-123 fluorescence measurements for mitochondrial membrane potential (Ψₘ)**

Cortical neurons were loaded with the membrane-permeable mitochondrial-membrane-potential-sensitive dye rhodamine-123 (Invitrogen, Burlington, ON) for 50 min (4°C). Rhodamine-123 was dissolved in DMSO to a stock concentration of 25 mM and then diluted to 50 μM in aCSF. Following dye loading cortical sheets were washed in aCSF (22°C) for 20 min. Rhodamine-123 was excited at a wavelength of 495 nm and fluorescence emissions was detected at a wavelength of 520 nm. Cortical sheets were exposed to treatment aCSF for 30 min then reperfused with control aCSF for 30 min to allow fluorescence to return to baseline. The protocol and concentrations used were based on previous investigations (Hawrysh and Buck, 2013).

**Pharmacology and drug administration**

Decreases in [ROS]ᵢ was achieved through the separate application of two cell permeable ROS scavengers: MPG (0.5 mM) and NAC (0.5 mM). Direct increases in ROS were induced through drip application of cell permeable H₂O₂ (50 μM) as it represented the primary mitochondrial ROS product (Chen et al., 2003; Ottaviano et al., 2008). Mitochondrial specific ROS production was halted using the complex I inhibitor rotenone (25 μM). Rotenone has been successfully used in other vertebrate tissues to decrease [ROS]ᵢ (Li and Trush, 1998; Liu et al., 2002; Liu et al., 1993). [Ca²⁺]ᵢ increases were replicated using the mKᵥ ATP channel agonist diazoxide (100 μM).
Diazoxide is a potent activator of mK<sub>ATP</sub> channels as demonstrated by K<sup>+</sup> flux in bovine heart mitochondria, which possesses a 2000-fold greater sensitivity to diazoxide than the sarcolemma (Garlid et al., 1997). Furthermore, diazoxide application to turtle cortical neurons results in a depolarization of Ψ<sub>m</sub>, release of mitochondrial [Ca<sup>2+</sup>], and a decrease in NMDA / AMPA receptor currents to levels comparable to those seen during anoxia (Hawrysh and Buck, 2013; Pamenter et al., 2008a; Zivkovic and Buck, 2010). Concentrations of rotenone and diazoxide were based on previous experiments on turtle cortical tissue (Pamenter et al., 2007). Diazoxide was initially solubilized in DMSO and used at a final concentration of less than 1%; rotenone was solubilized in 100% chloroform at 4°C and further diluted in aCSF to 0.05% at 22°C for use in an experiment. DMSO was not utilized to dissolve rotenone as the combination often caused the solution to become cloudy. All other pharmacological compounds were dissolved in aCSF. During whole-cell recordings both ROS scavengers and rotenone were drip and bulk perfused. Diazoxide and H<sub>2</sub>O<sub>2</sub> were administered through drip application only. In addition, H<sub>2</sub>O<sub>2</sub> was administered for only 5 min prior to TTX administration in order to limit potential toxicity. For experiments involving Ca<sup>2+</sup> chelation, BAPTA (5 mmol l<sup>−1</sup>) was included in the recording electrode solution. Tetrodotoxin was purchased from Tocris Bioscience (Ellisville, MO, USA) and all other chemicals were obtained from Sigma-Aldrich (Oakville, ON, Canada).

**Statistical Analysis**

Data were analyzed using SigmaPlot software version 11.0 (Systat Software, Inc., San Jose, CA). Fluorescence and pO<sub>2</sub> data were analyzed by a one-way analysis of variance (ANOVA) followed by a Tukey’s post hoc test to identify differences between treatment and control groups while NMDA and AMPA receptor whole-cell peak current amplitude data were analyzed using a one-way repeated measure ANOVA. Data was divided by a factor of 1000 and transformed using an arcsine transformation to normally distribute the data prior to statistical analysis. An ANOVA was used to compare the means of normoxic controls and treatments within treatment groups. Significance for all data was determined at p < 0.05. Results are expressed as means ± standard error of the means (S.E.M.).

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REFERENCES


Table 1. Effect of 50 µM H$_2$O$_2$ on electrophysiological parameters of cortical pyramidal neuron.

<table>
<thead>
<tr>
<th></th>
<th>Normoxia</th>
<th>H$_2$O$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane potential (mV)</td>
<td>-86.3 ± 0.6</td>
<td>-80.3 ± 3.1</td>
</tr>
<tr>
<td>Whole-cell conductance (nS)</td>
<td>4.3 ± 0.6</td>
<td>4.4 ± 0.5</td>
</tr>
<tr>
<td>Action potential threshold (mV)</td>
<td>-43.5 ± 1.4</td>
<td>-39.3 ± 1.2</td>
</tr>
</tbody>
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Data shown represent the means ± S.E.M. (n = 4 per treatment). A paired t-test comparing normoxia to H$_2$O$_2$ within each measured parameter found the data to not be significantly different.

Figure Legends

Figure 1. Anoxia and ROS scavenging decrease [ROS]. (A) Summary of treatment induced changes in CM-H$_2$DCF fluorescence. (B) Dose-response relationship of [H$_2$O$_2$] versus ΔCM-H$_2$DCF fluorescence. (C) Sample CM-H$_2$DCF fluorescence recordings from (A), neurons treated as indicated. Black bars represent duration of treatment. Arrow indicates the onset of a 5 min application of H$_2$O$_2$ (Note: horizontal linear portion of the trace indicates no new ROS generation). Treatments: normoxia (95% O$_2$/5% CO$_2$ bubbled aCSF), anoxia (95% N$_2$/5% CO$_2$ bubbled aCSF), 0.5 mM N-2-mercaptopropionyl glycine (MPG), 0.5 mM n-acetylcysteine (NAC), 25 µM rotenone, 50 µM H$_2$O$_2$. Data are expressed as means ± S.E.M. Asterisks (*) indicate significant difference from normoxic controls. Pound sign (#) indicates significant difference from anoxic controls (p < 0.05).

Figure 2. NMDA receptor currents are modulated by ROS. (A) Whole-cell NMDA receptor peak current amplitudes following 20 min of treatment and 20 min of washout. Continuous line represents normoxic controls, dashed line represents anoxic controls. Data are expressed as means ± S.E.M. Asterisks (*) indicate data significantly different from the paired normoxic control (p < 0.05). (B) Sample paired NMDA receptor current recordings of the normoxic baseline current and following 20 min of the indicated treatment. Abbreviations: hydrogen peroxide (H$_2$O$_2$), N-2-mercaptopropionyl glycine (MPG), n-acetylcysteine (NAC).
Figure 3. AMPA receptor currents are unaffected by pharmacological ROS manipulation. (A) Whole-cell AMPA receptor peak current amplitudes after 20 min of treatment and 20 min of washout. Continuous line represents normoxic controls, dashed line represents anoxic controls. Data are expressed as means ± S.E.M. Asterisks (*) indicate data significantly different from the paired normoxic control (p < 0.05). (B) Paired sample AMPA receptor current recordings of the normoxic baseline and following 20 min of the indicated treatment. Abbreviations: hydrogen peroxide (H₂O₂), N-2-mercaptopropionyl glycine (MPG), n-acetylcysteine (NAC).

Figure 4. Manipulation of [ROS]ₖ has no effect on [Ca²⁺]ᵢ. (A) Summary of treatment induced changes in Oregon Green fluorescence. Data are expressed as means ± S.E.M. Asterisks (*) indicate data significantly different from the paired normoxic control (p ≤ 0.001). (B) Drift corrected sample traces from (A), neurons treated as indicated. Black bars represent duration of treatment. Changes in fluorescence were calculated between two steady state parallel tangents, as illustrated in B ii. Abbreviations: arbitrary fluorescence units (AFU), N-2-mercaptopropionyl glycine (MPG), n-acetylcysteine (NAC).

Figure 5. Anoxia or mitochondrial [Ca²⁺] release mediated reductions in NMDA receptor currents are unaffected by ROS manipulation. (A) Whole-cell NMDA receptor peak current amplitudes after 20 min of treatment as indicated (open bars), 20 min of a combined treatments as indicated (black bars), and 20 min of washout (grey bars). Continuous line represents normoxic controls, whereas dashed line represents anoxic controls. Data are expressed as means ± S.E.M. Asterisks (*) indicate data significantly different from the paired normoxic control (p < 0.05). (B) Paired sample whole-cell NMDA receptor current recordings of the normoxic baseline, following 20 min of the indicated initial treatment and after 20 min of the indicated combination treatment. Abbreviations: hydrogen peroxide (H₂O₂), N-2-mercaptopropionyl glycine (MPG), n-acetylcysteine (NAC).

Figure 6. Timeline of anoxia induced changes in PO₂, Ψₚ, [Ca²⁺]ᵢ, and [ROS]ₖ. (A) Sample trace of bath chamber PO₂ during anoxic treatment protocol. (B) Rhodamine fluorescence trace demonstrating the timing of Ψₚ depolarization with anoxic treatment. (C) Oregon Green fluorescence trace showing changes in [Ca²⁺]ᵢ with anoxia. (D) CM-H₂DCF trace outlining changes in [ROS]ₖ with anoxia. Time to the onset of the response following the switch to anoxic perfusion and the time to steady state levels are shown in each panel. Each trace in panels B-D represents a trace averaged from 10 ROI’s per replicate. The onset and recovery portions of the traces were fitted with a non-linear four parameter curve to reduce noise and highlight the timeline of events. Data in Panel B and C were drift corrected and panels B-D were artificially set to a baseline fluorescence of zero to simplify interpretation of the figure.
Figure 1
Figure 2
Figure 3

A

- AMPA receptor currents (% normalized)

- Treatment and Recovery

- Normoxia, Anoxia, MPG, NAC, H$_2$O$_2$

B

- i
- ii
- iii
- iv
- v

- Control, Normoxia, Anoxia, MPG, NAC, H$_2$O$_2$

Figure 3
Figure 4
Figure 5

A

NMDA receptor currents (% normalized)

B

i

ii

iii

Control
Anoxia
Anoxia + H₂O₂
Diazoxide
Diazoxide + MPG
MGP
MGP + Diazoxide
Recovery
Recovery

0
50
100
150
200
250

* * *** **

Figure 5
Figure 6

Onset: 6 sec
Steady state: 607 sec

Onset: 70 sec
Steady state: 355 sec

Onset: 70 sec
Steady state: 196 sec

Onset: 120 sec
Steady state: 597 sec