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H₂O₂ INDUCES DELAYED HYPEREXCITABILITY IN NUCLEUS TRACTUS SOLITARII NEURONS

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Abstract

Hydrogen peroxide (H₂O₂) is a stable reactive oxygen species and potent neuromodulator of cellular and synaptic activity. Centrally, endogenous H₂O₂ is elevated during bouts of hypoxia-reoxygenation, a variety of disease states, and aging. The nucleus tractus solitarii (nTS) is the central termination site of visceral afferents for homeostatic reflexes and contributes to reflex alterations during these conditions. We determined the extent to which H₂O₂ modulates synaptic and membrane properties in nTS neurons in rat brainstem slices. Stimulation of the tractus solitarii (which contains the sensory afferent fibers) evoked synaptic currents that were not altered by 10 – 500 μ M H₂O₂. However, 500 μ M H₂O₂ modulated several intrinsic membrane properties of nTS neurons, including a decrease in input resistance, hyperpolarization of resting membrane potential (RMP) and action potential (AP) threshold (THR), and an initial reduction in AP discharge to depolarizing current. H₂O₂ increased conductance of barium-sensitive potassium currents, and block of these currents ablated H₂O₂-induced changes in RMP, input resistance and AP discharge. Following washout of H₂O₂ AP discharge was enhanced due to depolarization of RMP and a partially maintained hyperpolarization of THR. Hyperexcitability persisted with repeated H₂O₂ exposure. H₂O₂ effects on RMP and THR were ablated by intracellular administration of the antioxidant catalase, which was immunohistochemically identified in neurons throughout the nTS. Thus, H₂O₂ initially reduces excitability of nTS neurons that is followed by sustained hyperexcitability, which may play a profound role in cardiorespiratory reflexes.

Keywords

ROS; autonomic nervous system; synaptic transmission

The nucleus tractus solitarii (nTS) is vital to the cardiorespiratory reflex response during low arterial oxygen, blood pressure fluctuations, and other visceral stimuli (Andresen and

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T.D.O. and D.D.K. designed the study. T.D.O. performed the experiments and analyzed the data. T.D.O. prepared figures and drafted the manuscript. T.D.O., D.D.K., E.M.H. and C.M.H. interpreted the data, edited the manuscript and approved final version.

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Kunze, 1994; Kline *et al.*, 2010). The nTS is the first central termination site in the brainstem for sensory afferents. nTS processing and integration of visceral sensory information is essential for an effective response to a given challenge, including chemoreflex augmentation of ventilation and sympathetic nerve activity in response to hypoxia (Andresen and Paton, 2011). Glutamate is the primary neurotransmitter released from afferent terminals, and numerous neuromodulators influence its release or postsynaptic receptor function (Kline *et al.*, 2009; Sekizawa *et al.*, 2009; Fawley *et al.*, 2011), in addition to altering neuronal activity independent of neurotransmission. It is increasingly recognized that reactive oxygen species (ROS) are signaling neuromodulators in the cardiorespiratory system. ROS are implicated in the augmentation of sympathetic nerve activity in hypertension and heart failure (Ito *et al.*, 2001; Zucker, 2006; Hirooka, 2008), in part due to actions within the nTS (Schultz *et al.*, 2007; Wang *et al.*, 2008).

Two major ROS are superoxide anion ($O_2^{\cdot-}$) and the $O_2^{\cdot-}$ -derived, more stable hydrogen peroxide (H_2O_2 ; (Forman *et al.*, 2010)). Due to its stability and ability to freely cross the plasma membrane, H_2O_2 has been suggested to be an important signaling molecule in synaptic and neuronal activity. H_2O_2 alters neurotransmission (Chen *et al.*, 2001), synaptic long-term depression (Kamsler and Segal, 2003), ion channel function (Lebuffe *et al.*, 2003; Avshalumov *et al.*, 2005) and cellular activity (Avshalumov *et al.*, 2005; Pouokam *et al.*, 2009) in several central nuclei. The production and function of H_2O_2 is counter-balanced by cellular defense mechanisms (antioxidants), including catalase, which reduces H_2O_2 to H_2O and O_2 (Hou *et al.*, 2010). H_2O_2 , and its balance with antioxidants within the cardiorespiratory neuroaxis, modulates neuronal activity or function. For instance, in the rostral ventrolateral medulla of spontaneously hypertensive rats (SHR), hypertension is associated with elevated H_2O_2 and reduced catalase expression. Gene transfer of catalase into the rostral ventrolateral medulla reduces blood pressure in SHR (Chan *et al.*, 2006). H_2O_2 in the hypothalamic paraventricular nucleus modulates regulation of sympathetic nerve activity (Cardoso *et al.*, 2012). In the pre-Bötzinger complex, a region essential for respiratory rhythmogenesis, H_2O_2 initially depresses then augments action potential (AP) discharge and fictive breathing (Garcia *et al.*, 2011). H_2O_2 in the nTS modulates blood pressure and heart rate control (Cardoso *et al.*, 2009), and augments L-type calcium currents in isolated vagal-afferent labeled nTS cells (Wang *et al.*, 2006). However, the function of H_2O_2 in modulating synaptic, neuronal or network activity in the nTS remains largely unknown.

We used electrophysiology in brainstem slices to determine the neurophysiological effect(s) of H_2O_2 on second-order nTS cells. Results demonstrate H_2O_2 hyperpolarized resting membrane potential (RMP) and initially reduced cellular excitability, but did not alter afferent-evoked synaptic transmission. The former effects are attributed to H_2O_2 -mediated changes in membrane properties that are prevented by intracellular application of the antioxidant catalase. H_2O_2 also augmented conductance of barium-sensitive potassium currents, which were responsible for RMP hyperpolarization. Importantly, washout was associated with sustained hyperexcitability that persisted during subsequent application of H_2O_2 . Last, immunohistochemistry identified catalase in neurons throughout the nTS. Altogether, these data demonstrate H_2O_2 is an important signaling molecule in the nTS.

Experimental Procedures

Ethical approval

The Animal Care and Use Committee of the University of Missouri approved all experimental protocols in accordance with NIH guidelines (“Guide for the Care and Use of Laboratory Animals”).

Animals

Male Sprague-Dawley rats (Harlan; $n = 69$, aged 4 – 6 weeks) were maintained in the AAALAC accredited vivarium of the Dalton Cardiovascular Research Center. The animals had water and food available *ad libitum* and were held at 22°C and 40% humidity on a 12 hour day/night cycle.

In Vitro Brainstem Preparation

As detailed previously (Kline *et al.*, 2010), the brainstem of Isoflurane (VetOne) anesthetized rats was rapidly removed and placed in ice-cold low calcium-high magnesium aCSF (in mM: 124 NaCl, 3 KCl, 1.2 NaH₂PO₄, 1.2 MgSO₄, 25 NaHCO₃, 11 D-glucose, 0.4 L-Ascorbic Acid, 1 CaCl₂ and 2 MgCl₂, saturated with 95% O₂ - 5% CO₂, pH 7.4, ~300 mOsm). Horizontal slices (~280 μ m) with lengthy segments of the tractus solitarius (TS) were cut using a vibrating microtome (VT 1000S, Leica). Tissue sections were placed in a superfusion chamber, secured via nylon mesh and superfused at ~3 mL/min with standard recording aCSF (in mM: 124 NaCl, 3 KCl, 1.2 NaH₂PO₄, 1.2 MgSO₄, 25 NaHCO₃, 11 D-glucose and 2 CaCl₂, saturated with 95% O₂ - 5% CO₂, pH 7.4, ~300 mOsm) at 31 – 33°C.

Electrophysiological recording

We utilized the patch clamp technique to record from nTS cell somas in the caudal portion of the medial nTS, a region that receives cardiorespiratory and gastrointestinal afferent information (Chitravanshi and Sapru, 1995; Guyenet, 2000; Travagli *et al.*, 2006). Electrodes (King Precision Glass, type 8250) were pulled with a Flaming/Brown micropipette puller (Sutter Instruments, Model P-97) and had resistances of 3.0 – 4.5 M Ω when filled with standard recording solution (in mM: 10 NaCl, 130 K⁺ Gluconate, 11 EGTA, 1 CaCl₂, 10 HEPES, 1 MgCl₂, 2 MgATP, 0.2 NaGTP, pH 7.3, ~280 mOsm) (Kline *et al.*, 2010). The recording pipette was guided with a piezoelectric micromanipulator (Burleigh, PCS-6000) and recordings were performed under voltage- and current-clamp. Neurons were rejected if the membrane potential was more positive than –45 mV upon initial rupture or if series resistance changed more than 20% throughout the experiment. In some experiments the series resistance was compensated. Liquid junction potential (approximately +13.2 mV) was not compensated. Data were recorded using a Multiclamp700B amplifier (Molecular Devices), filtered at 2 kHz and sampled at 20 kHz.

Protocols

Voltage clamp protocols—All neurons were clamped at –60 mV. Membrane conductance (i.e., input resistance, R_i) was determined from a 10 ms step-hyperpolarization to –65 mV. TS evoked EPSCs (TS-EPSCs) were generated with an isolated stimulator

(World Precision Instruments, A310 Accupulser) and a concentric bipolar stimulating electrode (F. Haer) placed on the afferent containing TS. Stimulation intensity was increased until a TS-EPSC was evoked after which stimulation intensity was set at 1.5x TS-EPSC threshold. Properties of single TS-EPSCs at 0.5 Hz stimulation, or multiple (20) TS-EPSCs at 40 Hz stimulation [stimulus train; to mimic increased afferent discharge (Andresen and Kunze, 1994)] were measured in the absence and presence of H_2O_2 (10 – 500 μM). Initial studies using 1 mM H_2O_2 resulted in cellular and slice death, thus this concentration was not studied further. Miniature (m)EPSCs were recorded without external stimulation with 1 μM TTX (Tocris) and 10 μM bicuculline methobromide (Tocris) added to the aCSF. The lack of AP discharge to current ramps (see below) at the beginning of the mEPSC protocol ensured events were not due to action potential-driven network activity. While methyl derivatives of bicuculline have been shown to block Ca^{2+} -activated K^+ channels (Seutin *et al.*, 1997) and alter AP-dependent neurotransmitter release, bicuculline methobromide used in the current experiments should not influence our mEPSC and current ramp protocols due to the presence of the sodium channel blocker TTX and our negative holding currents (–100 to –50 mV), which are outside the range over which these currents are observed in the nTS (Moak and Kunze, 1993). Because H_2O_2 has been shown to target IRK and $\text{K}_{2\text{P}}$ channels in other tissue (Lebuffe *et al.*, 2003; Avshalumov *et al.*, 2005; Kim *et al.*, 2007), we identified possible changes in K^+ conductance through these channels by membrane voltage ramps (–100 mV to –50 mV, 1 sec.) and steps (–100 mV to –50 mV, 500 ms, 10 mV per step) (Bayliss *et al.*, 1997; Austgen *et al.*, 2012). Slope conductance was measured on ramps between –95 to –70 mV and with steps between –90 to –70 mV. Slope conductance during aCSF (pre- H_2O_2), barium chloride (BaCl_2 , Sigma; 100 μM), H_2O_2 and H_2O_2 + BaCl_2 was examined. The subtracted H_2O_2 - and Ba^{2+} -sensitive currents were identified by subtracting the obtained current (e.g., H_2O_2) from the treatment immediately before (e.g., barium or aCSF). In a different set of experiments, we evoked K^+ currents between –80 and +80 mV (20 mV per step, 400 ms) to measure the current amplitude of A-type potassium channels and other voltage-sensitive K^+ channels in response to H_2O_2 . Transient (A-type) K^+ current under both conditions was examined by determining the difference between the peak and steady state current (Kline *et al.*, 2010).

Current clamp protocols—AP discharge was evoked by either step-depolarization (0 to +90 pA, +10 pA steps, 100 ms duration) or ramp-depolarization (–20 pA to +50, +100 or +200 pA; 1 s ramp). Maximum ramp current used varied depending on the excitability of the particular cell, since some cells only responded with AP discharge to currents greater than 50 pA from RMP ($I=0$). AP threshold was determined from the AP differential and identified as the onset of the steepest slope. R_i was determined under current clamp conditions from a –20 pA current step while holding the cell at approximately –60 mV. The delay in AP discharge (i.e. delayed excitation, DE) was tested in a subset of cells by depolarizing current (+50, +100 or +200 pA, depending on the excitability of the cell; 800 ms; holding potential –60 to –65 mV) that was preceded by step hyperpolarizations (0 to –50 pA, –10 pA steps, 800 ms) to relieve A-type K^+ channels from inactivation (Schild *et al.*, 1993). RMP was measured under $I=0$ condition prior to current injection.

Physiologically relevant concentrations of H₂O₂ (10, 100, 300 and 500 μ M H₂O₂ (Schroder and Eaton, 2008)) were freshly prepared from stock (Certified ACS 30%, Fisher Scientific) and added to the aCSF immediately before use. We typically recorded neurons 1–2 cell layers deep and perfused H₂O₂ for 5 minutes to allow penetration into the slice and to compensate for dead space in the perfusion tubing. To exclude potential pH changes of the bath solution by H₂O₂, we determined the pH of the aCSF-H₂O₂ solution up to 1 M H₂O₂. The highest concentration used in this study (500 μ M H₂O₂) did not alter the pH of aCSF (pH change 0.002 ± 0.011 ; $n = 5$; $p = 0.86$, t -test). The first significant change of aCSF pH was measured at a H₂O₂ concentration 200-fold greater than used in the present study. For some experiments the antioxidant catalase (500 Units/mL; bovine liver, Sigma) was added to the recording solution within the recording pipette.

Immunohistochemistry

Immunohistochemistry was performed as in our previous manuscripts (Austgen *et al.*, 2009; Kline *et al.*, 2010). Briefly, deeply Isoflurane anesthetized rats were transcardially perfused using 0.1 M PBS followed by 4% paraformaldehyde (Sigma). The brainstem was removed and cut in 30 μ m coronal brain sections with a vibratome (VT 1000S, Leica). The sections were rinsed in PBS, followed by heat-induced epitope retrieval in a decloaking chamber (Biocare Medical) at 80 °C (30 min) and then blocked by 10% normal donkey serum (Millipore) in 0.3 % Triton-PBS. Tissue sections were incubated with primary antibodies against the antioxidant catalase (rabbit, 1:300, ab1877, Abcam) and Neuronal nuclear antigen (NeuN; neuronal marker; mouse, 1:500, MAB377, Millipore) for 24 hours. Subsequently sections were rinsed and incubated in 0.3 % Triton-PBS including the following secondary antibodies: Cy2-conjugated donkey anti-rabbit IgG (1:200; 711-225-152, Jackson Immuno) and Cy5-conjugated donkey anti-mouse IgG (1:200; 715-175-151, Jackson Immuno). Sections were mounted on gelatin coated slides, air dried and coverslipped with ProLong Gold with 4',6-diamidino-2-phenylindole (DAPI, nuclear marker; Invitrogen). Incubation of one section without primary antibody served as negative control (no fluorescent staining visible). Specificity of the antibody for catalase and NeuN has been confirmed previously [catalase, (Clarke *et al.*, 2009); NeuN (Al-Khater *et al.*, 2008)]. In this study, specificity for catalase antibody was also confirmed in nTS tissue via western blots (see below).

Immunoreactivity (-IR) was visualized with a conventional epifluorescent microscope (BX51, Olympus), a digital monochrome camera (ORCA-ER, Hamamatsu) and a spinning disc confocal unit (Olympus). Appropriate filter sets and excitation wavelength were used to visualize the different fluorophores. Z-stacks (0.5 μ m) were taken (same focal planes) for each fluorophore used. Images were post-processed using ImageJ (Version 1.45r, NIH) by adjusting contrast and brightness for clarity.

Western blot

Western blots were performed as described previously (Kline *et al.*, 2007). Frozen nTS tissue from 3 rats was pooled, homogenized in RIPA buffer (1% NP- 40, 0.5% DOC, 0.1% SDS, 0.15M NaCl, 50mM Tris/HCl and 2.5mM EDTA), and complemented with protease inhibitors (Complete, mini-EDTA-free tablets; Roche). Samples were incubated on ice for 2

hours and then centrifuged at $14,000 \times G$ for 30 min at $4^{\circ}C$. Protein concentration of the supernatant was measured by the Micro BCA method (Pierce, Rockford, IL). Twenty micrograms of protein were separated on 4 – 20% Tris-Glycine gel (BioRad) and transferred to a PVDF membrane. Primary antibody anti-catalase (1:300; rabbit polyclonal, Abcam) was used to immunoblot.

Data Analysis

Electrophysiological data were analyzed with pClamp10 (Molecular Devices), MiniAnalysis (Synaptosoft) and Microsoft Excel software. Statistical analyses were performed using SigmaPlot 12.0 (Systat Software). Only second-order nTS cells, those that directly receive input from TS-afferents, were analyzed for this study. A direct connection was verified based on a low variability (jitter) of TS-EPSC onset ($< 300 \mu\text{sec}$; SD of 30 TS-EPSC latencies from shock artifact, 20 kHz acquisition) (Doyle and Andresen, 2001; Kline *et al.*, 2002; Hisadome *et al.*, 2010; Accorsi-Mendonca *et al.*, 2011). Each data point for a given TS-EPSC was an average of 30 events. Spontaneous postsynaptic current (sPSC and mEPSC) detection was set at 5x the root-mean square noise level and events were manually confirmed. Within an individual cell, Kolmogorov-Smirnov two sample tests were used to examine the distribution of spontaneous current amplitude and interevent intervals between pre- H_2O_2 and H_2O_2 . Group sPSC and mEPSC data are reported as the sample average for amplitude and interevent frequency. To distinguish asynchronous (a)EPSCs from sEPSCs, the ratio of the number of EPSCs after and before the 40 Hz stimulus train was generated (after/before; ten 500 ms segments each). Generally, “baseline” refers to the aCSF condition prior to any H_2O_2 perfusion, unless noted otherwise (e.g. barium-baseline). The following statistical analyses were performed, and their p-values are reported in the text. The effect of H_2O_2 on EPSC amplitude, AP discharge threshold, AP delay, amplitude, rise slope, and decay slope was compared within treatments using paired *t*-tests. Synaptic and neuronal properties among multiple treatments were compared to their baseline response by one-way repeated measures (RM) ANOVA (with Student-Newman-Keuls post hoc test). Multiple treatments were compared by 2-way RM ANOVA (with Student-Newman-Keuls post hoc or Fishers LSD test). Statistical analyses were performed on the total sample size, unless otherwise noted. In addition the percentage of cells that responded to H_2O_2 by a change of at least 10% is provided in the text. H_2O_2 responses were also normalized and expressed relative to their individual baseline responses (defined as “1”). Results are considered significantly different at *p* values ≤ 0.05 . Data are presented as means $\pm$ SEM.

RESULTS

In this study only cells monosynaptically connected to the tractus solitarius were evaluated. Across the cells tested, TS stimulation evoked invariant EPSCs with a mean amplitude of $131.74 \pm 11.87 \text{ pA}$, latency of $4.63 \pm 0.17 \text{ ms}$ and jitter of $158.61 \pm 5.82 \mu\text{sec}$ ($n = 114$). This is consistent with recording from nTS neurons that are monosynaptically connected to TS afferents (Kline *et al.*, 2002; Hisadome *et al.*, 2010; Accorsi-Mendonca *et al.*, 2011).

H₂O₂ does not alter afferent-induced neurotransmission

As shown in the representative example in Figure 1A, stimulating the TS (0.5 Hz, average of 30 sweeps) elicited TS-EPSCs in nTS cells. The TS-EPSC amplitude was not altered during perfusion of the nTS with 500 μ M H₂O₂. Across the cells tested, TS-EPSC amplitudes during H₂O₂ (10, 100, 300, 500 μ M) were similar to vehicle or the baseline response ("1"; Figure 1B). For example, TS-EPSC amplitude was comparable during baseline (104.1 ± 17.5 pA) and 500 μ M H₂O₂ (102.7 ± 16.8 pA; $n = 8$; $p = 0.81$). Also, TS-EPSC decay time ($\tau_{90-10\%}$) was not altered with any concentration of H₂O₂ (e.g. 500 μ M: baseline, 3.31 ± 1.00 ms vs. H₂O₂, 2.39 ± 0.60 ms; $n = 5$; $p = 0.24$). Following exposure to H₂O₂, in a subset of cells 10 μ M CNQX (non-NMDA antagonist) was applied. CNQX reduced the TS-EPSC amplitude by 86%, indicating that such TS-evoked currents are primarily mediated by glutamatergic non-NMDA receptors (baseline, 117.5 ± 15.4 vs. CNQX, 15.3 ± 4.8 ; $n = 7$; $p < 0.001$).

Increasing TS stimulation frequency to 40 Hz (20 pulses, 10 sweeps) mimics a brief increase of afferent activity and provides insight into synaptic release properties. As typical of the first nTS synapse, high frequency stimulation progressively decreased the amplitude of TS-EPSCs following the first event (use-dependent depression; see example recording in Figure 1C inset). Consistent with the above results, neither H₂O₂ (10, 100, 300 and 500 μ M) nor vehicle altered the amplitude of the TS-EPSCs compared to the baseline response, or the magnitude of change from the first event. Figure 1C contains the averaged results for 500 μ M H₂O₂ ($n = 8$). The paired pulse ratio (PPR; ratio of TS-EPSC2/TS-EPSC1; box in the inset of Figure 1C), which differentiates alterations in presynaptic release or postsynaptic receptor properties, was also not altered by H₂O₂ (500 μ M: baseline, 0.47 ± 0.06 vs. H₂O₂, 0.54 ± 0.07 ; $n = 9$; $p = 0.35$) or vehicle.

Asynchronous (a)EPSCs occur directly after TS stimulation and are another indicator of presynaptic alterations in transmitter release (Kline *et al.*, 2007; Peters *et al.*, 2010). To examine the role of H₂O₂ on aEPSCs, we examined the number of spontaneous EPSCs after the stimulus train and compared it to that occurring before the train. Subsequently, the EPSC after/before ratio was generated. A ratio greater than 1 indicates aEPSCs after the stimulus train. aEPSCs were observed following the stimulus train with all H₂O₂ concentrations tested, however, they were not altered compared to baseline (e.g. baseline ratio, 1.74 ± 0.32 vs. 500 μ M H₂O₂, 2.19 ± 0.50 ; $n = 9$; $p = 0.11$). Taken together, these data suggest that H₂O₂ does not alter TS-afferent evoked EPSCs.

H₂O₂ decreases nTS network activity without altering miniature (m)EPSCs

Spontaneous postsynaptic currents (sPSCs) represent network activity within the nTS circuitry of the slice (Fortin and Champagnat, 1993). In response to 10, 100, 300, and 500 μ M H₂O₂, the amplitude of sPSCs was not altered compared to baseline in any of the cells tested (e.g. for 500 μ M: baseline, 21.63 ± 3.82 pA vs. H₂O₂, 24.01 ± 5.11 pA; $n = 9$; $p = 0.39$). H₂O₂ at 10 – 300 μ M did not alter sPSC frequency. However, sPSC frequency decreased with 500 μ M H₂O₂ (baseline, 26.81 ± 3.87 Hz vs. 500 μ M H₂O₂, 20.67 ± 3.88 Hz; $p < 0.01$; a significant decrease to 0.74 ± 0.08 normalized to baseline; $n = 9$; 89 % of the 9 cells decreased 10 %). This decrease in sPSC frequency with H₂O₂ was independent of the

cell's baseline sPSC frequency ($R^2 = 0.173$; $n = 9$; $p = 0.27$, Pearson correlation). In the present study, sPSCs are likely glutamatergic excitatory currents due to their recording at a holding potential (-60 mV) near the calculated reversal potential of chloride (-59 mV). In addition, CNQX reduced or eliminated sPSCs in 7 cells by $93.2 \pm 5.6\%$ ($p < 0.001$), in agreement with our previous studies (Kline *et al.*, 2010).

To further identify potential effects of H_2O_2 on synaptic transmission, we examined mEPSCs in the presence of TTX ($1\text{ }\mu\text{M}$; a voltage-gated Na^+ -channel blocker) and bicuculline ($10\text{ }\mu\text{M}$; a $GABA_A$ -receptor antagonist). Given that lower concentrations of H_2O_2 did not alter synaptic parameters, we examined $500\text{ }\mu\text{M}$ H_2O_2 exclusively. Compared to baseline, $500\text{ }\mu\text{M}$ H_2O_2 did not change mEPSC amplitude (baseline, $35.58 \pm 5.4\text{ pA}$ vs. $500\text{ }\mu\text{M}$ H_2O_2 , $36.32 \pm 5.2\text{ pA}$; $n = 7$; $p = 0.77$) or frequency (baseline, $10.56 \pm 4.08\text{ Hz}$ vs. $500\text{ }\mu\text{M}$ H_2O_2 , $12.69 \pm 5.28\text{ Hz}$; $n = 7$; $p = 0.23$). Thus, H_2O_2 does not alter mEPSC amplitude or frequency in the absence of network activity.

H_2O_2 hyperpolarizes resting membrane potential (RMP) and action potential threshold (THR)

We tested the extent to which H_2O_2 alters cell membrane properties. Changes in input resistance (R_i) are indicative of an alteration in channel conductance. Perfusions with vehicle and 10, 100 and $300\text{ }\mu\text{M}$ H_2O_2 did not alter R_i . By contrast, $500\text{ }\mu\text{M}$ H_2O_2 significantly and reversibly decreased R_i , from $485.35 \pm 46.00\text{ M}\Omega$ (baseline) to $367.42 \pm 31.36\text{ M}\Omega$ ($p < 0.05$, $n = 9$; 78 % of the 9 cells decreased by 10 % or more). During washout, R_i was $509.09 \pm 95.91\text{ M}\Omega$ ($n = 5$). nTS cells had a mean RMP of $-56.75 \pm 0.87\text{ mV}$ ($n = 122$) across all cells tested. Relative to baseline and vehicle, H_2O_2 at 10 and $100\text{ }\mu\text{M}$ did not alter RMP, yet 300 and $500\text{ }\mu\text{M}$ H_2O_2 significantly hyperpolarized RMP (Figure 2A). The raw values for RMP and their changes with H_2O_2 are shown in Table 1. This effect typically occurred between 1 – 3 minutes after the start of H_2O_2 perfusion. THR of all nTS cells tested was $-28.34 \pm 0.58\text{ mV}$ ($n = 93$). Relative to baseline and vehicle, 10 – $300\text{ }\mu\text{M}$ H_2O_2 did not alter THR but $500\text{ }\mu\text{M}$ H_2O_2 significantly hyperpolarized THR (Figure 2B). The raw values for THR and their changes with H_2O_2 are shown in Table 1. Since only $500\text{ }\mu\text{M}$ H_2O_2 significantly altered R_i , RMP and THR, we chose this concentration for the remainder of this study to examine the effects of H_2O_2 .

When effects of $500\text{ }\mu\text{M}$ H_2O_2 on RMP and THR were examined in a larger dataset (Table 2), there also was a significant hyperpolarization of RMP (62 % of cells hyperpolarized 10 %) and THR (74 % of cells hyperpolarized 10 %). The magnitude of response for RMP and THR to $500\text{ }\mu\text{M}$ H_2O_2 was similar between the cells examined in the initial dose-response ($n = 9$) and this population ($p = 0.35$ [RMP] and $p = 0.48$ [THR]). With washout, RMP returned to baseline whereas THR remained significantly hyperpolarized. The difference between THR and RMP ([THR-RMP]), which is the voltage that must be exceeded by a depolarizing stimulus to initiate AP discharge, was not affected during H_2O_2 perfusion, but decreased significantly at washout. Rheobase significantly increased from baseline to H_2O_2 , and decreased significantly during wash. Other membrane properties of the first induced AP during baseline, H_2O_2 and wash are contained in Table 2.

H₂O₂ may alter RMP and THR by modifying proteins through extracellular or intracellular domains. To determine if H₂O₂ was acting intracellularly, membrane-impermeable catalase was included within the pipette solution and effects of H₂O₂ were reevaluated. Catalase is a primary antioxidant of the cell that specifically breaks down H₂O₂ into H₂O and O₂. Adding 500 Units/mL catalase into the recording pipette antagonized the hyperpolarizing effects of 500 μ M H₂O₂ on RMP and THR. The lack of change in RMP and THR during 500 μ M H₂O₂ + 500 Units/mL catalase from its baseline as well as compared to other H₂O₂ concentrations is shown in Figure 2A, B and Table 1. These data indicate an intracellular site of modulation by H₂O₂ and suggest that the changes in RMP and THR are mediated specifically by H₂O₂.

H₂O₂ modulates the firing rate (AP discharge) of nTS neurons

Figure 3A shows a representative example of the effects of 500 μ M H₂O₂ on the neuronal properties of an nTS cell. Step current depolarization (0 to +40 pA, +10 pA steps, 100 ms duration) evoked AP discharge under baseline conditions. As described above, H₂O₂ induced membrane hyperpolarization and upon current depolarization, there was a decrease in AP discharge and overshoot. In addition, the THR voltage for evoking an AP was shifted in the hyperpolarized direction. With five minutes of washout, RMP returned to baseline level and upon current depolarization, AP discharge again occurred. Furthermore, compared to the baseline response, discharge was greater following washout.

We quantified the input-output relationship of AP discharge in a subset of 13 cells, for which also the responses of R_i , RMP and THR to 500 μ M H₂O₂ are shown in Figure 3. As in the previous experiments, H₂O₂ decreased R_i (Figure 3B; 92 % of these cells decreased by 10 %). With wash, R_i partially returned to baseline and was significantly different from H₂O₂. H₂O₂ significantly hyperpolarized RMP (Figure 3C *bottom*) and THR (Figure 3C *middle*) as observed in the large dataset above. Upon wash, in this subpopulation RMP returned again to baseline, but THR remained hyperpolarized, which significantly reduced [THR-RMP] (Figure 3C *top*). Quantifying the AP discharge in response to step depolarizations (0 to +90 pA, +10 pA steps, 100 ms duration, corresponding to our observed TS-EPSC amplitudes), there was a progressively greater discharge during baseline (Figure 3D, with the maximal discharge for each individual cell plotted as 1). The prominent decrease in R_i during H₂O₂ significantly increased rheobase from 31.5 ± 4.5 pA (baseline) to 67.7 ± 16.9 pA (H₂O₂, $n = 13$, $p = 0.05$). This significantly reduced AP discharge evoked by depolarizing currents ~ 50 pA when compared to the baseline response. At wash, rheobase significantly decreased to 32.3 ± 6.8 pA ($n = 13$, $p = 0.05$). This returned AP discharge to baseline values that even significantly exceeded baseline discharge upon depolarization ~ 60 pA. Taken together, these results suggest H₂O₂ initially decreases AP discharge, but induces hyperexcitability upon washout when compared to the baseline response.

To further examine and correlate H₂O₂-induced alterations in R_i , RMP and THR to neuronal discharge, we monitored these properties in continuous current clamp during repetitive H₂O₂ applications. Membrane potential during current ramps in response to repetitive exposure to vehicle (aCSF) or H₂O₂ (two treatments with 500 μ M H₂O₂, 10 min. each) are shown in Figure 4A & B. Vehicle did not alter AP discharge over the 40 minute recording

time ($T_{5\min} - T_{40\min}$). In contrast to vehicle and similar to above, a single bout of H_2O_2 decreased AP discharge relative to its baseline response. Washout increased discharge above baseline values that persisted even following a subsequent H_2O_2 perfusion and washout. Quantitatively, AP discharge to current ramps was significantly higher subsequent to the first H_2O_2 exposure when compared to vehicle or the baseline response (Figure 4C). THR, RMP, [THR-RMP], R_i and rheobase during repetitive H_2O_2 and vehicle are quantified in Table 3. While THR tended to hyperpolarize over time during vehicle ($T_{5\min} - T_{40\min}$), it was not significantly different from baseline. Likewise R_i , RMP, [THR-RMP] and rheobase were unaltered in vehicle. On the contrary to vehicle and similar to the protocol with one bout, in this sample H_2O_2 initially ($T_{10\min}$) significantly hyperpolarized RMP, tended to decrease R_i , did not change THR (note, only 4 cells that continued to respond), and tended to increase rheobase. During and following the first washout, there is a significant reduction in [THR-RMP] and rheobase, correlating with the increased AP discharge shown in Figure 4C. After 40 minutes of recording (wash/ $T_{40\min}$) data for H_2O_2 treatment shows a significantly depolarized RMP and hyperpolarized THR, explaining the observed decreases in [THR-RMP] and rheobase, and likely resulting in the increase in AP discharge. Thus, although the protocol was designed to evaluate the effects of repeated H_2O_2 application, the excitatory effects of the initial washout persisted throughout the second application of H_2O_2 .

H_2O_2 modulates potassium currents

H_2O_2 initially hyperpolarizes RMP and reduces R_i suggesting the involvement of one or more K^+ -currents that operate at RMP. We postulated the involvement of IRK and K_{2p} currents which have been shown in other systems to control membrane potential and be altered by H_2O_2 . Here we compared holding currents during voltage ramps (-100 mV to -50 mV, 1 sec.) within the conducting range of IRK and K_{2p} channels (Bayliss *et al.*, 1997; Day *et al.*, 2005), under baseline and H_2O_2 perfusion. Prior to application of the voltage ramp, H_2O_2 produced a general positive (outward) shift in holding current consistent with hyperpolarization of RMP (example from one cell shown in Figure 5A). The voltage ramp during baseline elicited an inward holding current with an average slope conductance of 2.222 ± 0.522 pA/mV (nS, measured between -95 to -70 mV; $n = 6$). The addition of H_2O_2 significantly increased slope conductance to 2.716 ± 0.625 nS ($n = 6$; $p < 0.05$; an increase to 1.27 ± 0.12 when normalized to the baseline response), indicating an increase of IRK/ K_{2p} channel conductance. The subtracted H_2O_2 -sensitive current reversed at -75.4 ± 5.1 mV ($n = 6$; Figure 5B), towards our calculated reversal potential of potassium (-98.7 mV). In five cells, adding 100 μ M barium chloride ($BaCl_2$), a concentration which blocks IRK and K_{2p} channels (Day *et al.*, 2005), to the H_2O_2 perfusion solution significantly reduced slope conductance from 2.716 ± 0.625 nS (during H_2O_2) to 0.800 ± 0.073 nS ($p < 0.05$). This Ba^{2+} -sensitive current reversed at -81.2 ± 1.5 mV, which again is towards the calculated equilibrium potential for K^+ in our recording conditions and not significantly different than the H_2O_2 sensitive current. These data show that H_2O_2 increases K^+ conductance, which is subsequently antagonized by barium. The H_2O_2 -effect on increased slope conductance during voltage ramps was additionally confirmed by a conventional voltage step protocol between -100 and -50 mV. Similarly, addition of H_2O_2 significantly increased the IRK/ K_{2p} channel conductance (slope measured between -90 to -70 mV steps; baseline, 1.785 ± 0.347 nS vs. H_2O_2 , 3.098 ± 0.412 nS; $n = 6$; $p = 0.01$; an increase to 1.95 ± 0.34 when

normalized to the baseline response). To eliminate potential effects of tonic background currents from ionotropic receptors on our responses, we repeated H₂O₂ perfusion during the voltage step protocol in the presence of the non-NMDA receptor antagonist CNQX (10 μ M) and bicuculline methobromide (10 μ M). During H₂O₂, cells demonstrated the typical increase in holding current (i.e. hyperpolarization of RMP) and decrease of R_i , and increased conductance through IRK/K_{2p} channels (slope measured between -90 to -70 mV steps; CNQX + bicuculline-baseline, 1.728 ± 0.271 nS vs. H₂O₂, 2.307 ± 0.159 nS; $n = 5$; $p = 0.05$). An example is shown in Figure 5C & D.

Next we determined whether pre-block of Ba²⁺-sensitive currents would prevent the increase in conductance by H₂O₂. Barium alone produced a negative shift in holding current under voltage clamp condition (baseline, -18.23 ± 8.76 pA vs. BaCl₂, -23.28 ± 9.40 pA; $n = 6$; $p < 0.05$, Figure 5E), consistent with depolarization of RMP (see below). In the presence of BaCl₂, H₂O₂ perfusion had no effect on holding current (-23.01 ± 9.19 pA; $n = 6$; $p = 0.89$). As expected, slope conductance during voltage ramps significantly decreased from baseline to barium (baseline, 1.827 ± 0.478 nS vs. BaCl₂, 1.316 ± 0.374 nS; $n = 6$; $p < 0.05$), indicating blockade of IRK/K_{2p} channel conductance. The subtracted Ba²⁺-sensitive current reversed at -79.7 ± 2.3 mV (example in Figure 5F), similar to the above results. Barium prevented the H₂O₂-induced increase in slope conductance (1.242 ± 0.334 nS; $n = 6$; $p = 0.30$). The elimination of the subtracted H₂O₂-sensitive current is shown in Figure 5F. These results have also been affirmed in voltage step protocols where barium prevented increases in conductance due to H₂O₂ (BaCl₂, 1.084 ± 0.170 nS vs. H₂O₂ and BaCl₂, 1.014 ± 0.185 nS; $n = 6$; $p = 0.48$). Altogether, these results confirm that H₂O₂ alters potassium conductance, likely through of IRK/K_{2p} channels.

H₂O₂-sensitive potassium currents modulate RMP, R_i and AP discharge

To examine whether H₂O₂-sensitive IRK/K_{2p} channels are responsible for membrane hyperpolarization, we monitored RMP during H₂O₂ and BaCl₂ (Figure 6). RMP significantly depolarized with Ba²⁺, and H₂O₂ produced no additional change in the presence of Ba²⁺ (Figure 6A). Thus, barium (presumably by blocking IRK and K_{2p} channels) eliminated the effect of H₂O₂ on RMP. THR, on the other hand, remained stable with Ba²⁺ perfusion but significantly hyperpolarized with the addition of H₂O₂. The barium-dependent depolarization of RMP significantly decreased [RMP-THR], which however remained stable during the H₂O₂-dependent hyperpolarization of THR. Barium also blocked the H₂O₂-dependent reduction in R_i and AP discharge, as well as the increase in rheobase (Figure 6B–D). Overall, these data suggest that changes in RMP and R_i , and the resulting decrease in AP discharge by H₂O₂ are due to increases in IRK and K_{2p} conductance.

H₂O₂ and delayed excitation (DE) of nTS neurons

nTS neurons exhibit heterogeneity in their firing properties, namely “phasic”, “tonic” and “delayed excitation” cells (Paton *et al.*, 1993; Kline *et al.*, 2010). We examined the extent to which H₂O₂ may alter one or more of these firing phenotypes in a subset of cells. Cellular phenotype was classified by discharge properties in response to a depolarizing current that was preceded by hyperpolarization (0 to -50 pA, -10 pA steps, 800 ms duration; inset Figure 7A). Out of 12 nTS neurons examined for their firing properties, 8 neurons were DE

cells, 3 neurons were tonic firing cells and 1 cell exhibited phasic firing. In the small subset of tonically or phasic firing cells, discharge delay was not altered by either hyperpolarizing pre-pulses or H_2O_2 , and thus these cells were not studied further.

A representative example and the role of H_2O_2 on a DE cell is shown in Figure 7A. As typical of a DE nTS cell, during aCSF baseline, onset of AP discharge to a depolarizing pulse was delayed (black arrow denotes initial spike). Augmenting hyperpolarization increased the delay in discharge upon depolarization (grey arrow denotes initial spike, Figure 7A). Such delay reflects hyperpolarization-induced relief of inactivation of A-type K^+ -currents that enables their activation upon depolarization (Schild *et al.*, 1993). Preventing the hyperpolarization of RMP by H_2O_2 via bias current (-65 mV; Figure 7A *right*) decreased the initial delay in AP discharge in response to the depolarizing pulse (black arrow). Hyperpolarization in the presence of H_2O_2 induced AP delay (DE, grey arrow) when compared to no prior hyperpolarization (black arrow).

The group data illustrating the effect of H_2O_2 on DE in nTS neurons are shown in Figure 7B. As shown in our representative example (Figure 7A), there was an initial delay in depolarization-induced AP discharge, and increasing hyperpolarization prior to depolarization significantly prolonged the delay of AP discharge under baseline and H_2O_2 (Figure 7B; $p < 0.001$). Due to the H_2O_2 -induced decrease in R_i , negative current injection did not hyperpolarize the membrane to the same potential as during the baseline response. Thus, we compared AP delay between baseline and H_2O_2 only under similar hyperpolarizing pre-pulse potentials (i.e. depolarized more than -90 mV). During H_2O_2 , the overall delay in discharge was reduced compared to baseline (average delay for all pre-pulse potentials > -90 mV; baseline, 189.15 ± 41.83 ms vs. H_2O_2 , 123.13 ± 36.82 ms; $n = 8$; $p < 0.05$). However, there was a similar progression of the delay (based on the regression) during baseline and H_2O_2 (average slope of regression > -90 mV; baseline, -6.82 ± 1.52 vs. H_2O_2 , -6.04 ± 2.11 ; $n = 8$; $p = 0.71$). Such results may suggest A-type K^+ -current amplitude is unaltered by H_2O_2 . This was confirmed by examining voltage-activated outward K^+ -currents in response to voltage steps (-80 to $+80$ mV; 400 ms; 20 mV steps). There was a small but non-significant decrease in transient K^+ currents by H_2O_2 at voltages greater than 0 mV ($n = 9$; $p = 0.31$).

An important part of this protocol held the initial RMP constant (-60 to -65 mV without a hyperpolarizing pre-pulse) during baseline and H_2O_2 with bias current. This was done to examine the influence of H_2O_2 on AP discharge in the absence of membrane hyperpolarization. Under these conditions, H_2O_2 increased overall AP discharge in response to current depolarization. Elevated discharge was observed without pre-hyperpolarization steps (i.e., black trace in Figure 7A *right*) in 5 out of 8 cells (baseline, 9.2 ± 3.1 vs. $500 \mu\text{M}$ H_2O_2 , 22.8 ± 4.1 AP/stimulus; $n = 5$; $p < 0.01$). In the remaining cells, AP discharge either decreased (1 cell) or was stable (2 cells). The data across this entire population did not reach statistical significance (baseline, 8.25 ± 2.01 vs. $500 \mu\text{M}$ H_2O_2 , 15.38 ± 4.40 AP/stimulus; $n = 8$; $p = 0.09$). In the presence of bias current, H_2O_2 significantly hyperpolarized THR across all DE neurons (THR at 0 pA hyperpolarization: baseline, -26.49 ± 1.96 mV vs. $500 \mu\text{M}$ H_2O_2 , -35.04 ± 3.67 mV; $n = 8$; $p < 0.01$).

Catalase in nTS neurons

H₂O₂ modulates cellular excitability which was antagonized by intracellular application of catalase. Thus, we sought to immunohistochemically determine the endogenous presence of the H₂O₂-reducing antioxidant enzyme catalase within nTS neurons. Specificity of the antibody for catalase in nTS tissue was verified, in part, via western blot analysis (Figure 8A). A single band for catalase was found as expected at ~65 kDa. Strong catalase-IR was found ubiquitously throughout the whole nTS with a few scattered cells exhibiting very strong IR for catalase (Figure 8B). In NeuN-identified neurons, catalase-IR was primarily localized surrounding DAPI-labeled nuclei (perinuclear) to a dense network that extended into the cellular processes (Figure 8C).

DISCUSSION

Our data demonstrate H₂O₂ alters intrinsic and firing properties of nTS neurons while not affecting synaptic transmission from visceral afferents. The H₂O₂-induced immediate postsynaptic changes included hyperpolarization of RMP and THR, increase in rheobase, and reduction of AP discharge. H₂O₂ increased slope conductance of barium-sensitive IRK and/or K₂P channels, and Ba²⁺ blocked the H₂O₂ changes in RMP, R_i and AP discharge. Intracellular application of the antioxidant enzyme catalase, which is ubiquitously expressed in nTS neurons, prevented H₂O₂-mediated effects on RMP and THR. The H₂O₂-effect on RMP, but not THR, was fully reversible and produced hyperexcitability in response to greater depolarization. Altogether, these data suggest H₂O₂ is an important modulator of neuronal activity in the nTS.

In the present study, we used short exposures of H₂O₂ at concentrations to approximate levels typically observed *in vivo*. Specifically, 0.01 – 1 mM exogenous H₂O₂ in the central nervous system has been shown to mimic the concentration of H₂O₂ released endogenously by cellular effectors [e.g., NADPH oxidase, mitochondria, and xanthine oxidase (Schroder and Eaton, 2008)], due in part to the 10-fold reduction of H₂O₂ concentration by spontaneous and antioxidant-mediated breakdown. Given these assumptions, the intracellular H₂O₂ concentrations achieved in our study are likely to range from 1 – 50 μ M. Furthermore, our concentrations are considerably less than previous studies which have illustrated an effect of millimolar concentrations of H₂O₂ on synaptic or neuronal function (Frantseva *et al.*, 1998; Avshalumov *et al.*, 2005; Pouokam *et al.*, 2009; Garcia *et al.*, 2011). H₂O₂ concentrations greater than 10 μ M and up to 1 mM have been shown to occur physiologically but are also associated with oxidative stress (Stone and Yang, 2006; Schroder and Eaton, 2008). Thus, our results may be directly correlated to a variety of conditions and provide mechanistic insight into the role of H₂O₂ in the nTS on the cardiorespiratory system.

Previous studies using similar concentrations to ours (up to 500 μ M for ~30 minutes) demonstrated that H₂O₂ alters synaptic transmission. For instance, H₂O₂ reduces hippocampal long-term potentiation (Auerbach and Segal, 1997) and EPSP amplitude (Kamsler and Segal, 2003), and increases GABA release (Takahashi *et al.*, 2007). In the current experiments, H₂O₂ did not alter postsynaptic CNQX-sensitive non-NMDA glutamate receptor properties at the TS-nTS synapse, as indicated by the lack of change in

evoked and miniature EPSC amplitude, and TS-EPSC decay time. Likewise, the lack of effect on PPR, use-dependent depression, as well as asynchronous and miniature EPSC frequency suggests that H_2O_2 did not alter glutamate release from TS terminals. Across all cells tested, H_2O_2 also did not alter TS-EPSCs regardless of initial current amplitude or failure rate (not shown), indicators of synapses containing myelinated (A-type) or unmyelinated (C-type) afferents (Andresen and Peters, 2008), suggesting the H_2O_2 response was not fiber type specific. Conversely, $500 \mu\text{M}$ H_2O_2 decreased sEPSC frequency, but not amplitude, in the neurons studied. Given that sEPSCs derive from network activity within the available nTS circuitry (Fortin and Champagnat, 1993), the decrease in sEPSC frequency may indicate that H_2O_2 reduced the activity of neurons which form a synapse with the recorded cell. This is further supported by the lack of change in mEPSC frequency during H_2O_2 .

In the present study, AP discharge decreased during the initial bout of H_2O_2 . This likely resulted from alteration in postsynaptic cell properties independent of glutamate receptors, including R_i , RMP and THR. Specifically, H_2O_2 hyperpolarized RMP and THR, and reduced R_i . These effects are likely inter-related. The H_2O_2 -induced decrease in R_i suggests opening, or increased conductance, of membrane channel(s) that contribute to RMP hyperpolarization and the decrease in discharge, as in cells from other central nuclei (Seutin *et al.*, 1995; Avshalumov *et al.*, 2005; Pouokam *et al.*, 2009; Garcia *et al.*, 2011). Overall, changes in R_i , RMP and THR are also responsible for the increase in rheobase during peroxide exposure. H_2O_2 hyperpolarized RMP toward our calculated K^+ equilibrium potential, suggesting an involvement of K^+ -channels. H_2O_2 activates ATP-sensitive K^+ -channels (Avshalumov *et al.*, 2005), an inward rectifying K^+ -channel that modulates RMP in nTS neurons during hypoxia (Duprat *et al.*, 2005; Kim *et al.*, 2007; Zhang *et al.*, 2008). Additional IRK and $\text{K}_{2\text{p}}$ channels have also been demonstrated within the nTS (Dunn-Meynell *et al.*, 1998; Gabriel *et al.*, 2002; Yamamoto *et al.*, 2008). To identify the effect of H_2O_2 on IRK and $\text{K}_{2\text{p}}$ channels in nTS neurons, we recorded holding currents in H_2O_2 -responsive cells during depolarizing ramps. Results demonstrated that H_2O_2 augmented slope conductance, which was blocked or prevented by barium at a concentration specific for IRK/ $\text{K}_{2\text{p}}$ currents (Bayliss *et al.*, 1997; Day *et al.*, 2005; Austgen *et al.*, 2012). Since the augmented H_2O_2 -sensitive current reversed towards the calculated K^+ equilibrium potential and was blocked by barium, these data suggest that H_2O_2 augments IRK/ $\text{K}_{2\text{p}}$ conductance. The holding current during H_2O_2 had an overall linear shape suggesting H_2O_2 has a main effect on $\text{K}_{2\text{p}}$ conductance, although alterations of IRK channel conductance cannot be excluded. Also, the positive shift in reversal potential may suggest that additional channels contribute. For instance, H_2O_2 also increases conductance of non-selective cation channels (Simon *et al.*, 2002; Sato *et al.*, 2009) and sodium channels (Meng and Nie, 2004). The potential increase of sodium influx by H_2O_2 through these channels is consistent with greater ease to reach THR in our current study, as demonstrated in our DE protocols. Regardless, the decrease in discharge during the initial bout of H_2O_2 is likely due to the reduction in R_i and the concurrent change in RMP, reducing the stimulus effect and increasing rheobase. Importantly, action potential discharge was reduced by H_2O_2 in response to current steps in the range of our TS-EPSC amplitudes. Thus it is likely that

sensory afferent or network evoked currents would also be less effective in the presence of H_2O_2 .

We observed that following washout of the initial bout of H_2O_2 , AP discharge increased above baseline, especially during greater depolarizing currents. This was observed whether AP discharge was monitored via step or ramp depolarization. This is likely due to the fact that during wash RMP readily returned to baseline with an increase of R_i (likely due to closing of K^+ channels), whereas THR remained hyperpolarized in comparison to baseline. This shift significantly reduced [THR-RMP] and returned rheobase to or below baseline values. Therefore, hyperexcitability and an enhancement of AP discharge were observed in response to a depolarizing stimulus. It is anticipated that network or afferent-driven increases in activity would also produce elevated AP discharge. R_i partially reversed, consistent with the closing of H_2O_2 -sensitive K^+ channels and the return of RMP to baseline. Because R_i does not fully reverse, it is possible that other channels, which may contribute to changes in THR, remain in a state of increased conductance. This would be consistent with a persistent hyperpolarization of THR. One should note that the majority of H_2O_2 -inhibitory effects occurred primarily in the initial responses, with subsequent exposures being less effective. Thus, AP discharge was significantly greater during the second H_2O_2 exposure compared to the first exposure and to baseline. The persistent increase in discharge may be due to protein modification of the ion channel(s) altered. This notion however requires further study. Of interest, sustained hyperexcitability with H_2O_2 (subsequent to a transient decrease of excitability) has also been reported in neurons of the pre-Bötzinger complex (Garcia *et al.*, 2011).

We also demonstrate that H_2O_2 modulates AP firing in DE neurons. The typical delay of discharge following a hyperpolarizing pre-pulse, a phenomenon attributed to the relief of A-type K^+ channel inactivation (Schild *et al.*, 1993), was observed during baseline and H_2O_2 . H_2O_2 decreased the overall delay of depolarization-induced firing (i.e., neurons fired earlier) across the hyperpolarizing pre-pulses, yet the slope of the relationship between pre-pulse potential and magnitude of delay was unchanged. Such changes suggest the magnitude of A-type channel currents are not altered by H_2O_2 . Furthermore, even though there was a tendency to decrease A-type currents during H_2O_2 , which would promote a decrease in delay and increase in firing, it was not significant in the 9 cells studied. However, we did not examine whether transient A-type channel kinetics were altered, including inactivation rate and voltage-dependence of activation and inactivation, which may substantially alter firing as they do in other central neurons, including the PVN (Sonner and Stern, 2007). Future studies in isolated nTS cells which lack many of the space constant and pharmacological constraints inherent in the slice preparation will further elucidate if H_2O_2 alters channel kinetics. Preventing the H_2O_2 -induced hyperpolarization of RMP with bias current increased AP discharge and decreased its delay in the majority of cells, due to a decrease in THR. Taken together, these results further suggest that the initial decrease in AP discharge by H_2O_2 is likely due to membrane hyperpolarization (and the interrelated increase in membrane conductance), and that the reduction in THR coupled with the depolarized RMP is responsible for the delayed hyperexcitability seen following washout.

H_2O_2 is endogenously eliminated by intracellular antioxidants, including catalase. We observed catalase-IR throughout the nTS and dorsal motor complex. Catalase-IR was primarily perinuclear and organized to a dense structure in nTS neurons. Catalase has been located in peroxisomes (Mavelli *et al.*, 1982) and mitochondria (Radi *et al.*, 1991), which can be closely associated with cisternae of the endoplasmic reticulum (Schrader and Fahimi, 2004). The importance of intracellular catalase in breaking down H_2O_2 is evidenced in our protocols in which we elevated intracellular catalase via its placement in the recording pipette. We reasoned that if H_2O_2 modulated RMP and THR via an intracellular mechanism, then supplemental catalase should reduce or eliminate its effects. Our results confirm this notion and indicate that H_2O_2 likely alters postsynaptic activity via intracellular rather than extracellular sites.

H_2O_2 may be converted to hydroxyl radical ($\cdot\text{OH}$), which itself may alter neuronal activity (Avshalumov *et al.*, 2000; Garcia *et al.*, 2011). While we cannot completely rule out that H_2O_2 exerts some of its action through $\cdot\text{OH}$, the ablation of H_2O_2 -induced changes on RMP and THR by supplemental catalase suggests that the contribution of $\cdot\text{OH}$ may be small. Particularly, if $\cdot\text{OH}$ mediates part or all of the actions of H_2O_2 , then $\cdot\text{OH}$ produced by exogenous H_2O_2 , either spontaneously in the bath or in nearby cells, would likely continue to alter RMP and THR even during elevated catalase. This did not occur. While the availability of free iron, which is needed to produce $\cdot\text{OH}$, in an isolated nTS slice is unknown, it is reasonable to suggest it may be limited, especially during repetitive cycles of H_2O_2 perfusion and washout. Thus, the observed reduction of cell excitability in nTS cells in the current experiments is likely mediated by H_2O_2 .

In summary, we demonstrate that H_2O_2 modulates nTS neuronal function. These studies provide insight into the functional consequences of H_2O_2 under normal conditions and disease states. The medial and commissural nTS are the primary afferent termination sites for multiple visceral reflexes, including the gastrointestinal, cardiovascular and respiratory systems (Andresen and Kunze, 1994; Travagli *et al.*, 2006; Dean and Putnam, 2010). H_2O_2 in the nTS may profoundly alter these reflexes individually as well as their coordination (Dean, 2011). While the effects of nTS H_2O_2 have not been examined for the gastrointestinal system, one can speculate that the prolonged hyperexcitability in the presence of H_2O_2 may augment the inhibition of gastric tone or motility that occurs through the vaso-vagal reflex (Travagli *et al.*, 2006). H_2O_2 may also alter the coordination between the respiratory and digestive system for pH regulation that has been proposed to occur in the nTS (Dean, 2011). In regards to the cardiovascular system, microinjection of H_2O_2 into the nTS evokes hypotension and bradycardia (Cardoso *et al.*, 2009), consistent with an excitatory role on cells in the baroreflex pathway. Likewise, ROS have been implicated in the disease manifestation following chronic intermittent hypoxia (CIH), an animal model for obstructive sleep apnea (OSA) (Sica *et al.*, 2000). Both CIH and OSA cause increased chemoreflex sensitivity, augmented sympathetic nerve activity and hypertension (Fletcher *et al.*, 1992; Somers and Abboud, 1993; Prabhakar *et al.*, 2011). While an increase in peripheral chemoreceptor activity contributes to CIH-induced sympathoexcitation (Peng and Prabhakar, 2004; Peng *et al.*, 2004), a substantial contribution is also ascribed to the central component (Ling *et al.*, 2001; Kline *et al.*, 2007). ROS are strongly implicated to mediate

these changes in the cardiorespiratory system (Kuo *et al.*, 2011; Prabhakar, 2011) when produced in excess during bouts of hypoxia-reoxygenation (Prabhakar, 2001). Thus, increased neuronal excitability due to H₂O₂ within the reflex neuroaxis (including arterial chemoreceptors, their afferents and central terminals, interneurons and projection nTS neurons, as well as other important cardiorespiratory nuclei) may occur during each hypoxic bout and account for increasingly augmented sympathetic nerve activity observed in CIH or OSA. Our results demonstrate a biphasic response to exogenous H₂O₂ that ultimately leads to cell hyperexcitability, which may contribute to elevated sympathetic nerve activity through downstream nTS projections. Moreover, given the potential therapeutic use of antioxidants, these results may provide insight into the mechanism and their site of action.

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Glossary

aEPSC	asynchronous EPSC
AP	action potential
DE	delayed excitation
H₂O₂	hydrogen peroxide
IR	immunoreactivity
IRK	inward rectifying K ⁺ channel
mEPSC	miniature EPSC
nTS	nucleus tractus solitarii
O₂^{•-}	superoxide anion
·OH	hydroxyl radical
K₂P	tandem-pore domain K ⁺ channel
PPR	paired pulse ratio
PSC	postsynaptic current
R_i	input resistance
RMP	resting membrane potential
ROS	reactive oxygen species
sEPSC	spontaneous EPSC
THR	action potential threshold
TS	tractus solitarii
TS-EPSCs	evoked EPSCs

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Highlights

- We studied effects of H_2O_2 on synaptic and intrinsic properties of nTS neurons.
- Catalase blocked effects of H_2O_2 to decrease membrane potential and AP threshold.
- Conductance through IRK/K_{2P} channels was increased with H_2O_2 .
- H_2O_2 initially depressed discharge, yet resulted in sustained hyperexcitability.
- The anti-oxidant enzyme catalase is present throughout the nTS.

Figure 1. Effects of H₂O₂ on synaptic transmission

A. Example of TS-EPSCs in the absence and presence of H₂O₂ (500 μM). Note H₂O₂ did not alter TS-EPSCs. Example shown is the average of 30 TS-EPSCs. **B.** H₂O₂ did not alter TS-EPSC amplitude compared to the baseline response or vehicle across multiple H₂O₂ concentrations. Data are normalized to each group's baseline, defined as 1. **C.** Group data showing that H₂O₂ did not alter the current amplitude across the 20 events (use-dependent depression; also see inset) or the time course of depression when mimicking increased sensory afferent activity by augmenting TS stimulation frequency (40 Hz). Box in the inset highlights the depression from the first to the second pulse used for calculating the paired

pulse ratio. All cells were held at -60 mV. Numbers of observations are depicted in parentheses.

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Figure 2. H_2O_2 alters neuronal membrane properties

RMP (A) and THR (B) relative to their baseline (1 = no change, >1 = hyperpolarization) for vehicle, 10 – 500 μM H_2O_2 and 500 μM H_2O_2 + 500 U/mL catalase in the pipette. H_2O_2 did not alter RMP or THR at 10 and 100 μM . However, 300 and 500 μM H_2O_2 significantly hyperpolarized RMP, and 500 μM H_2O_2 significantly hyperpolarized THR. Further, the changes mediated by 500 μM H_2O_2 in RMP and THR were eliminated by catalase in the recording pipette. Two-way RM ANOVA; * compared to baseline, ‡ compared to vehicle, 10 μM and 500 μM H_2O_2 + 500 U/mL catalase for THR, and additionally to 100 μM H_2O_2 for RMP; $p < 0.05$. Numbers of observations are indicated in parentheses. CAT = catalase.

Figure 4. Prolonged hyperexcitability due to H₂O₂

Representative traces of AP discharge during ramp depolarization at repetitive vehicle (**A**) or H₂O₂ perfusion and wash (**B**). The time points shown (T_{5min} – T_{40min}) during vehicle and H₂O₂ depict the time at which a ramp depolarization was performed, which was 5 minutes after a new treatment. Note the AP discharge to depolarization during baseline, which was eliminated with H₂O₂, but was augmented following washout and persisted during a second bout of H₂O₂. Vehicle did not alter AP discharge over the 40 minute period. **C**. Group data for AP discharge during ramp depolarization. Data are presented as the relative increase from each cell's baseline response (normalized to "1"). Note the significant hyperexcitability following the initial H₂O₂ perfusion, when compared to the baseline response or vehicle (time control). ^{*}#, $p < 0.05$, two-way RM ANOVA; ^{*} compared to baseline, [#] compared to vehicle. Numbers of observations are depicted in parentheses.

Figure 5. H_2O_2 increases the conductance through potassium channels

Examples of the holding currents recorded during voltage ramps (A–B, E–F) and voltage steps (C–D). **A.** H_2O_2 (dark grey trace) elicited a positive shift in holding current at -60 mV when compared to baseline (black trace), as shown in the segments at the beginning and end of each trace (dotted line denotes initial baseline holding potential). H_2O_2 also increased the slope conductance of holding currents during voltage ramps. Adding $100 \mu M$ $BaCl_2$ to H_2O_2 (light grey trace) reversed the H_2O_2 -induced increase in slope conductance. Note the breakthrough sodium spikes towards the end of the ramp, a region not included in our slope conductance measurements. *Inset*, Voltage ramp used to examine the holding currents from

–100 to –50 mV (1 sec. ramp). **B.** The subtracted H_2O_2 -sensitive current. **C.** Verification of the observed H_2O_2 -effects using voltage steps from –100 to –50 mV (10 mV steps) during perfusion of CNQX and bicuculline (Bic) to block potential tonic background and synaptic currents. H_2O_2 again elicited a positive shift in holding current (i.e. RMP hyperpolarization) illustrated by the initial segments before the step protocol. During H_2O_2 the conductance increased with each voltage step. **D.** Current-voltage relationship. H_2O_2 increased slope conductance, similar to panel A, in response to voltage steps. **E.** Barium (light grey trace) elicited a negative shift in holding current at –60 mV when compared to baseline (black trace). Barium decreased the slope conductance of holding currents to the voltage ramps. Adding H_2O_2 to barium chloride (dark grey trace) induced no additional changes. **F.** The subtracted barium-sensitive and H_2O_2 -sensitive current. Note that the H_2O_2 -sensitive current is completely eliminated in the presence of barium.

Figure 6. Potassium channel blockade with barium eliminates H₂O₂-induced changes in membrane properties

Membrane properties and AP discharge were measured before and during 1 sec ramp depolarizations. Note, barium blocked H₂O₂-dependent alterations in RMP (A) and R_i (B), and the resulting changes in [THR-RMP], rheobase (C) and AP discharge (D). Barium did not prevent H₂O₂-induced hyperpolarization of THR. One-way RM ANOVA, * compared to baseline response, † compared to barium; $p < 0.05$. Numbers of observations are depicted in parentheses.

Figure 7. H_2O_2 alters delayed excitation of nTS neurons

A. Representative traces of AP discharge of cells characterized by delayed excitation (DE). *Left.* During baseline, a hyperpolarizing pre-pulse (grey trace) increased AP discharge delay to a depolarizing stimulus, compared to no pre-pulse (black trace). Arrows depict the initial AP for each condition. See inset for stimulus. *Right.* During H_2O_2 , while maintaining the cell at baseline (bsl) membrane potential using bias current (i.e., preventing H_2O_2 -induced hyperpolarization), an increase in AP discharge to depolarization is observed (without pre-pulse; black trace). This is associated with the reduction in THR. A hyperpolarizing pre-pulse significantly delayed AP discharge (grey arrow), similar to the baseline response. **B.** Group data plotting pre-pulse potential (x-axis; elicited by hyperpolarizing current) vs. AP discharge delay (y-axis) under baseline (black line) and H_2O_2 (grey line) for cells exhibiting DE ($n = 5 - 8$, delayed spiking of some cells was greater than the duration of the depolarizing pulse when pre-hyperpolarization was -20 pA). Note the overall reduced delay in AP discharge with H_2O_2 during step hyperpolarization. Also note that increasing the hyperpolarizing pre-pulse increased the delay during both the baseline response and H_2O_2 , and this increase in delay was not altered by H_2O_2 . For comparison, regression lines correspond to only pre-pulse potential values more depolarized than -90 mV. *, averaged delay of multiple pre-pulse potentials, baseline vs. H_2O_2 , $p < 0.05$, t -test.

Figure 8. Distribution of catalase within the nTS

A. Analysis of antibody specificity via immunoblots for 20 μ g of protein from the nTS. A single band for catalase was confirmed at the appropriate size of ~ 65 kDa. **B** Catalase-IR (pseudocolored green) in a coronal brainstem section. Catalase-IR is present ubiquitously throughout the nTS (dashed line). Spinning disc confocal image. AP = area postrema, TS = tractus solitarius, CC = central canal. Scale bar, 200 μ m. **C** Higher magnification of catalase (green) with co-labeling of neuronal cells (NeuN, blue) and nuclei (DAPI, grey). Merged image is shown in the bottom right panel. Representative images are a maximal projection of a z-stack with 20 images 0.5 μ m apart. Catalase-IR was identified perinuclear in NeuN-identified neurons. Scale bar, 15 μ m.

Table 1**RMP and THR is altered by H₂O₂ and blocked by exogenous catalase**

Raw values of the dose-response relationship between different H₂O₂ concentrations and RMP and THR. Note, only 500 μ M H₂O₂ significantly altered both parameters, which was antagonized by increasing the intracellular antioxidant catalase.

Resting membrane potential (RMP) [mV]	baseline	treatment	n	p
vehicle	-57.00 $\pm$ 4.79	-56.58 $\pm$ 4.56	7	0.79
10 μ M H ₂ O ₂	-55.70 $\pm$ 3.34	-53.77 $\pm$ 3.93	11	0.32
100 μ M H ₂ O ₂	-53.50 $\pm$ 2.16	-52.77 $\pm$ 1.87	12	0.63
300 μ M H ₂ O ₂	-61.07 $\pm$ 2.76	-65.39 $\pm$ 2.43	9	0.07
500 μ M H ₂ O ₂	-64.83 $\pm$ 2.36	-71.10 $\pm$ 2.23	9	0.04
500 μ M H ₂ O ₂ + catalase	-66.88 $\pm$ 2.48	-66.55 $\pm$ 2.57	8	0.79

Action potential threshold (THR) [mV]	baseline	treatment	n	p
vehicle	-27.69 $\pm$ 1.14	-28.90 $\pm$ 1.73	9	0.18
10 μ M H ₂ O ₂	-30.82 $\pm$ 1.27	-30.78 $\pm$ 2.20	12	0.98
100 μ M H ₂ O ₂	-34.45 $\pm$ 2.40	37.36 $\pm$ 3.84	5	0.16
300 μ M H ₂ O ₂	-33.23 $\pm$ 2.50	-36.63 $\pm$ 2.01	5	0.04
500 μ M H ₂ O ₂	-28.70 $\pm$ 1.39	-34.70 $\pm$ 3.04	9	0.02
500 μ M H ₂ O ₂ + catalase	-28.69 $\pm$ 1.95	-29.57 $\pm$ 2.10	7	0.10

Statistical analysis using paired *t*-tests; *p* value shown on the right. "n" represents the number of observations.

Table 2

Membrane potential properties during baseline and H₂O₂

Properties of THR, RMP, [THR-RMP], rheobase and other parameters of the first AP generated by step depolarization during baseline, H₂O₂ and wash.

	baseline	H ₂ O ₂	wash	n (H ₂ O ₂ /wash)
RMP [mV]	-59.82 ±1.56	-69.61 ±2.36 *	-57.17 ±1.83 †	26/23
THR [mV]	-26.09 ±1.03	-33.35 ±2.15 *	-31.13 ±1.58 *	23/20
[THR-RMP] [mV]	33.33 ±1.95	35.62 ±1.83	25.74 ±1.86 *†	23/20
Rheobase [pA]	66.55 ±13.55	92.15 ±15.58 *	38.40 ±6.69 †	23/19
Overshoot [mV] (over 0 mV)	35.61 ±1.71	25.10 ±3.15 *	26.60 ±3.21 *	23/20
AP amplitude from RMP [mV]	95.03 ±2.20	94.07 ±2.72	83.47 ±3.61 *†	23/20
AP amplitude from THR [mV]	61.70 ±1.64	58.45 ±2.25 *	57.73 ±3.00	23/20
Half width [ms]	1.11 ±0.06	1.17 ±0.05	1.19 ±0.05	19/16
Rising slope [mV/ms]	195.39 ±11.47	178.70 ±13.31	158.08 ±13.80 *	20/17
Falling slope [mV/ms]	-93.16 ±6.05	-86.64 ±4.53	-72.49 ±4.21 *†	20/17
Afterhyperpolarisation from THR [mV]	19.94 ±3.74	18.72 ±4.79	15.51 ±4.14	23/20

One-way RM ANOVA;

* compared to baseline,

† compared to H₂O₂; *p* < 0.05. "n" represents the number of observations during H₂O₂ and wash.

Table 3

Membrane properties during repetitive exposure to H₂O₂

Membrane properties (raw and normalized to their baseline [defined as “1”]) during baseline, two bouts of H₂O₂ or vehicle and their respective wash. Top panel, vehicle; bottom panel, H₂O₂ (500 μ M). T_{5min} – T_{40min} represent the time points during H₂O₂ or vehicle in which cell properties were measured. Note THR, RMP, [THR-RMP], R_i and rheobase did not change during vehicle when compared to the respective baseline values. By contrast, in this smaller cell sample, the initial bout of H₂O₂ hyperpolarized RMP and reduced R_i in the same direction as our larger sample. H₂O₂ significantly altered RMP, THR, [THR-RMP], and rheobase when compared to baseline values at later time points.

VEHICLE		baseline/T _{5min}	treatment/T _{10min}	wash/T _{20min}	treatment/T _{30min}	wash/T _{40min}	n
THR	raw [mV]	-28.28 $\pm$ 2.65	-29.75 $\pm$ 3.01	-32.30 $\pm$ 2.89	-32.72 $\pm$ 3.22	-32.51 $\pm$ 3.49	6
	normalized	1.00 $\pm$ 0.00	1.05 $\pm$ 0.02	1.15 $\pm$ 0.03	1.16 $\pm$ 0.03	1.14 $\pm$ 0.04	
RMP	raw [mV]	-50.99 $\pm$ 4.03	-50.46 $\pm$ 3.25	-50.92 $\pm$ 2.97	-51.38 $\pm$ 2.94	-51.55 $\pm$ 2.95	6
	normalized	1.00 $\pm$ 0.00	1.00 $\pm$ 0.02	1.01 $\pm$ 0.04	1.02 $\pm$ 0.06	1.02 $\pm$ 0.05	
[THR-RMP]	raw [mV]	22.72 $\pm$ 4.09	20.71 $\pm$ 3.19	18.62 $\pm$ 3.25	18.66 $\pm$ 3.22	19.04 $\pm$ 3.08	6
	normalized	1.00 $\pm$ 0.00	0.95 $\pm$ 0.06	0.85 $\pm$ 0.08	0.88 $\pm$ 0.12	0.91 $\pm$ 0.13	
R _i	raw [m Ω]	628.01 $\pm$ 104.29	632.15 $\pm$ 110.96	649.18 $\pm$ 134.14	655.46 $\pm$ 126.27	707.62 $\pm$ 132.96	6
	normalized	1.00 $\pm$ 0.00	1.02 $\pm$ 0.05	1.02 $\pm$ 0.10	1.04 $\pm$ 0.11	1.24 $\pm$ 0.24	
Rheobase	raw [pA]	21.08 $\pm$ 6.49	18.01 $\pm$ 4.49	14.04 $\pm$ 4.64	20.65 $\pm$ 9.50	14.98 $\pm$ 3.54	6
	normalized	1.00 $\pm$ 0.00	0.93 $\pm$ 0.17	0.85 $\pm$ 0.22	1.12 $\pm$ 0.25	1.02 $\pm$ 0.30	
H ₂ O ₂							
THR	raw [mV]	-26.48 $\pm$ 1.07	(-24.62 $\pm$ 1.83)	-28.57 $\pm$ 2.44	-31.63 $\pm$ 4.50 *	-30.65 $\pm$ 3.20 *	(4),7
	normalized	1.00 $\pm$ 0.00	(0.95 $\pm$ 0.03)	1.07 $\pm$ 0.05	1.17 $\pm$ 0.12 *	1.14 $\pm$ 0.08 *	
RMP	raw [mV]	-63.34 $\pm$ 0.89	-68.34 $\pm$ 4.54	-59.46 $\pm$ 2.06	-60.30 $\pm$ 4.35	-54.36 $\pm$ 2.80 *	7
	normalized	1.00 $\pm$ 0.00	1.08 $\pm$ 0.06	0.94 $\pm$ 0.03	0.95 $\pm$ 0.06	0.86 $\pm$ 0.04 *	
[THR-RMP]	raw [mV]	36.86 $\pm$ 0.97	(38.05 $\pm$ 3.10)	30.89 $\pm$ 2.64 *	28.66 $\pm$ 2.51 *	23.70 $\pm$ 2.70 *	(4),7

	baseline/T _{5min}	treatment/T _{10min}	wash/T _{20min}	treatment/T _{30min}	wash/T _{40min}	n
	normalized	1.00 ±0.00	(1.03 ±0.06)	0.84 ±0.07 *	0.77 ±0.06 *	0.64 ±0.07 *
R_i	raw [mΩ]	609.47 ±91.9	467.36 ±105.44	587.34 ±88.17	550.19 ±96.29	561.21 ±128.60
	normalized	1.00 ±0.00	0.76 ±0.13	0.98 ±0.06	0.89 ±0.07	0.88 ±0.10
Rheobase	raw [pA]	73.52 ±13.73	(78.87 ±29.79)	55.53 ±15.57 *	53.49 ±14.69 *	40.66 ±8.63 *
	normalized	1.00 ±0.00	(1.32 ±0.20)	0.75 ±0.12	0.68 ±0.10	0.57 ±0.09

Two-way RM ANOVA; comparisons shown within groups;

* $p < 0.05$; compared to baseline. “n” represents the number of observations at each time point. Note three cells did not discharge during the initial bout of H₂O₂ so that certain parameters could not be measured; values in parentheses indicate the number of cells that continued to discharge during the 1st H₂O₂.