Cellular/Molecular

Hydrogen Peroxide Increases $GABA_A$ Receptor-Mediated Tonic Current in Hippocampal Neurons

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Hydrogen peroxide (H_2O_2), a key reactive oxygen species, is produced at low levels during normal cellular metabolism and at higher concentrations under pathological conditions such as ischemia-reperfusion injury. The mechanisms by which H_2O_2 contributes to physiological and pathological processes in the brain remain poorly understood. Inhibitory GABA type A ($GABA_A$) receptors critically regulate brain function by generating tonic and synaptic currents; however, it remains unknown whether H_2O_2 directly modulates $GABA_A$ receptor function. Here, we performed patch-clamp recordings, together with pharmacological and genetic approaches, to investigate the effects of H_2O_2 on $GABA_A$ receptor-mediated tonic and synaptic currents recorded in cultured mouse hippocampal neurons and CA1 pyramidal neurons in hippocampal slices. We found that H_2O_2 caused a dramatic increase in tonic current, whereas synaptic currents were unaffected. This increase in tonic current resulted from an extracellular oxidative reaction, which increased the potency of GABA, but only when $GABA_A$ receptors were activated by low concentrations of GABA. Oxygen-glucose deprivation, which produces high endogenous levels of H_2O_2, similarly increased the tonic current. These results suggest that $GABA_A$ receptor-mediated tonic current, which is potentiated by H_2O_2, might contribute to H_2O_2-induced brain dysfunction.

Key words: **$GABA_A$ receptor; hippocampus; hydrogen peroxide; ischemia-reperfusion; mouse; tonic current**

Introduction

Reactive oxygen species (ROS) are by-products generated during normal cellular oxidative metabolism in the brain and other organs (Giorgio et al., 2007; Rice, 2011). Excessive production of ROS occurs in a variety of neurological disorders including ischemic-reperfusion injury, stroke, and neurodegenerative disease (Andersen, 2004; Allen and Bayraktutan, 2009; Uttara et al., 2009). High levels of ROS can lead to oxidative stress, which in turn causes cellular dysfunction and neuronal death (Giorgio et al., 2007; Rice, 2011).

One of the most important ROS is hydrogen peroxide (H_2O_2), a relatively stable molecule that diffuses through the cell membrane to interact with distant molecular targets (Giorgio et al., 2007; Rice, 2011). Low concentrations of H_2O_2 physiologically regulate synaptic plasticity (Kamsler and Segal, 2003; Rice, 2011), whereas higher concentrations are associated with neuronal dysfunction (Abramov et al., 2007; Giorgio et al., 2007). Concentrations of H_2O_2 as high as 200 μM occur during the early period of postischemic reperfusion (Hyslop et al., 1995). Despite being involved in a wide variety of physiological and pathological functions, the molecular substrates underlying H_2O_2 effects on neuronal function remain poorly understood.

GABA type A ($GABA_A$) receptors mediate the majority of inhibition in the brain and perturbations in GABAergic inhibition contribute to a variety of neurological disorders (Brickley and Mody, 2012). $GABA_A$ receptors generate two forms of inhibition: synaptic and tonic, which play distinct roles in regulating network synchrony and neuronal excitability (Farrant and Nusser, 2005; Brickley and Mody, 2012). Synaptic inhibition is generated by postsynaptic $GABA_A$ receptors that are activated by transient, near-saturating concentrations of GABA. In contrast, tonic inhibition is generated by extrasynaptic $GABA_A$ receptors that are activated by persistent, low ambient concentrations of GABA (Farrant and Nusser, 2005; Brickley and Mody, 2012).

H_2O_2 modulation of $GABA_A$ receptor function is of considerable interest as alterations in inhibitory neurotransmission dramatically change network activity (Farrant and Nusser, 2005;

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Brickley and Mody, 2012). Previous studies that investigated the effects of H_2O_2 on GABAergic inhibition have produced conflicting results. Application of H_2O_2 (300 μM) to hippocampal slices increases the intracellular concentrations of chloride (Sah and Schwartz-Bloom, 1999). In contrast, very high concentrations of H_2O_2 (1.5–3.5 mM), reduce the amplitude of IPSPs recorded in the hippocampus, cerebral cortex, and thalamus (Pellmar, 1995; Frantseva et al., 1998). This reduction of IPSPs could result from a decrease in the release of GABA from presynaptic terminals or from direct inhibition of $GABA_A$ receptors. No previous studies have examined the direct effects of H_2O_2 on $GABA_A$ receptors that generate tonic and synaptic currents.

Here we show that H_2O_2 dramatically increases the amplitude of the tonic current in hippocampal neurons, whereas synaptic currents are unaffected. This increase in tonic current is due to an increase in potency of GABA, which results from an extracellular oxidative reaction. Oxygen-glucose deprivation, a pathological condition that generates high levels of endogenous H_2O_2 also increases the tonic current. Collectively, the results suggest that H_2O_2 could cause neuronal dysfunction, at least in part, by increasing $GABA_A$ receptor-mediated tonic current.

Materials and Methods

Experimental animals. All experimental procedures were approved by the Animal Care Committee of the University of Toronto (Toronto, ON, Canada). Three strains of mice of either sex were used to produce cultures of hippocampal neurons. Swiss White mice were obtained from Charles River Laboratories. $GABA_A$ receptor α5 subunit null-mutant (*Gabra5*$^{-/-}$) mice and wild-type (WT) mice (C57BL/6 × SvEv129) were obtained from Merck Sharp and Dohme Research Laboratories. $GABA_A$ receptor δ subunit null-mutant (*Gabrd*$^{-/-}$) mice and WT mice (C57BL/6 × SvJ129) were obtained from Dr Gregg E. Homanics at the University of Pittsburgh (Pittsburgh, PA). The generation, genotyping, and characterization of *Gabra5*$^{-/-}$ and *Gabrd*$^{-/-}$ mice were previously described (Mihalek et al., 1999; Collinson et al., 2002). Mice were housed in the animal care facility of the University of Toronto.

Cell culture. Cultures of embryonic hippocampal neurons were prepared as previously described (Wang et al., 2012). Briefly, fetal pups (embryonic day 18) were removed from mice killed by cervical dislocation. The hippocampi were dissected from each fetus and placed in an ice-cooled culture dish. Neurons were then dissociated by mechanical titration using two Pasteur pipettes (tip diameter, 150–200 μm) and plated on 35 mm culture dishes at a density of $\sim 1 \times 10^6$ cells/ml. The culture dishes were coated with collagen or poly-D-lysine (Sigma-Aldrich). For the first 5 d *in vitro*, cells were maintained in minimal essential media (MEM) supplemented with 10% fetal bovine serum and 10% horse serum (Life Technologies). The neurons were cultured at 37°C in a 5% CO_2-95% air environment. After the cells had grown to confluence, 0.1 ml of a mixture of 4 mg 5-fluorodeoxyuridine and 10 mg uridine in 20 ml MEM was added to the extracellular solution to reduce the number of dividing cells. Subsequently, the media was supplemented with 10% horse serum and changed every 3 or 4 d. Cells were maintained in culture for 14–20 d before use.

Whole-cell voltage-clamp recordings in cultured neurons. Before electrophysiological recordings, cultured neurons were rinsed with extracellular recording solution containing the following (in mM): 140 NaCl, 1.3 $CaCl_2$, 1 $MgCl_2$, 2 KCl, 25 *N*-2-hydroxy-ethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES), 20 glucose, pH 7.4, 320–330 mOsm). 6-Cyano-7-nitroquinoxaline-2,3-dione (10 μM), and (2R)-amino-5-phosphonovaleric acid (20 μM) were added to the extracellular solution to block ionotropic glutamate receptors and tetrodotoxin (0.3 μM) was used to block voltage-dependent sodium channels. Patch pipettes (3–4 MΩ) were fabricated from borosilicate glass capillary tubing and filled with an intracellular solution containing the following (in mM): 140 CsCl, 10 HEPES, 11 EGTA, 4 MgATP, 2 $MgCl_2$, 1 $CaCl_2$, and 10 TEA (pH 7.3 with CsOH, 285–295 mOsm). All experiments were conducted at room temperature and whole-cell currents were recorded using the Axopatch 200B amplifier (Molecular Devices) controlled with pClamp 8.0 software via a Digidata 1322 interface (Molecular Devices). Membrane capacitance was measured using the membrane test protocol in pClamp 8.0. Currents were sampled at 10 kHz and filtered at 2 kHz by using an 8-pole low-pass Bessel filter. The extracellular solution was applied to neurons by a computer-controlled multibarreled perfusion system (SF-77B; Warner Instruments).

To measure the amplitude of the tonic current, the $GABA_A$ receptor competitive antagonist, bicuculline (100 μM), was applied as described previously (Caraiscos et al., 2004; Wang et al., 2012). All cells were recorded at a holding potential of −60 mV except for those used to study the current–voltage (*I–V*) relationship. *I–V* plots were constructed using a voltage step protocol, as previously described (Pavlov et al., 2009). Voltage steps of 20 mV were applied from −80 to +20 mV for 4 s, in the absence and presence of bicuculline (100 μM). The difference between current measured at the different membrane potentials, in the absence and presence of bicuculline, was used to generate the *I–V* plot. For each neuron, the voltage step protocol was repeated before and 5 min after the application of H_2O_2.

Whole-cell voltage-clamp recordings in hippocampal slices. Three-week old WT mice (C57BL/6 × SvEv129) of either sex were used for the recordings. After live decapitation, brains were removed and placed in ice-cold, oxygenated (95% O_2, 5% CO_2) artificial CSF (ACSF) that contained the following (in mM): 124 NaCl, 3 KCl, 1.3 $MgCl_2$, 2.6 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 10 D-glucose with the solution osmolarity adjusted to 300–310 mOsm. Coronal brain slices (350 μm) were prepared with a VT1200S vibratome (Leica). The slices were allowed to recover for at least 1 h at room temperature (23–25°C) before being transferred to a submersion recording chamber where they were perfused with ACSF at 3–4 ml/min. All recordings were performed at room temperature using a MultiClamp 700B amplifier (Molecular Devices) that was controlled by pClamp 9.0 software via a Digidata 1322A interface (Molecular Devices). For whole-cell voltage-clamp recordings, the pipettes (2–3 MΩ) were filled with the same intracellular solution as described above for cell culture. Currents were sampled at 10 kHz. All cells were recorded at a holding potential of −60 mV. To measure the tonic current, the $GABA_A$ receptor competitive antagonist, bicuculline (20 μM) was applied. The tonic current was quantified by measuring the change in the holding current calculated from a Gaussian fitting of 30 s segments. Exogenous GABA (5 μM) was added to ACSF. The recorded neurons were perfused with ACSF containing H_2O_2 (1 mM) through a square-shaped barrel (ID 0.7 mm; Warner Instruments) whose tip was located in close proximity to the recorded neurons ($\approx$ 100 μM).

Oxygen-glucose deprivation. Ischemia-reperfusion injury was induced using the *in vitro* model of oxygen-glucose deprivation as previously described (Ohsawa et al., 2007). After establishing a stable baseline response, neurons were perfused for 10 min with a glucose- and O_2-deficient extracellular solution, in which the glucose was replaced with 2-deoxy-D-glucose (20 mM) and N_2 was continuously bubbled to remove O_2. The recorded neuron was then re-perfused with regular oxygenated extracellular solution for 5 min. The magnitude of the tonic current during the reperfusion period was compared with the baseline current. Only one neuron was used from each culture dish.

Statistical analyses. Data are represented as mean ± SEM. A Student's *t* test (paired or unpaired) was used to compare groups, where appropriate. For comparing three or more groups, one-way ANOVA followed by Newman–Keuls *post hoc* test was used. Two-way ANOVA (treatment × concentration) followed by Bonferroni *post hoc* test was also used, as indicated. Cumulative distributions of the amplitude and frequency of miniature IPSCs (mIPSCs) were compared using the Kolmogorov–Smirnov test. Statistical significance was set at $p < 0.05$.

Results

H_2O_2 markedly increases the tonic but not synaptic currents in hippocampal neurons

The tonic current was quantified by measuring the change in the holding current that occurred following an application of the competitive $GABA_A$ receptor antagonist bicuculline (BIC; 100

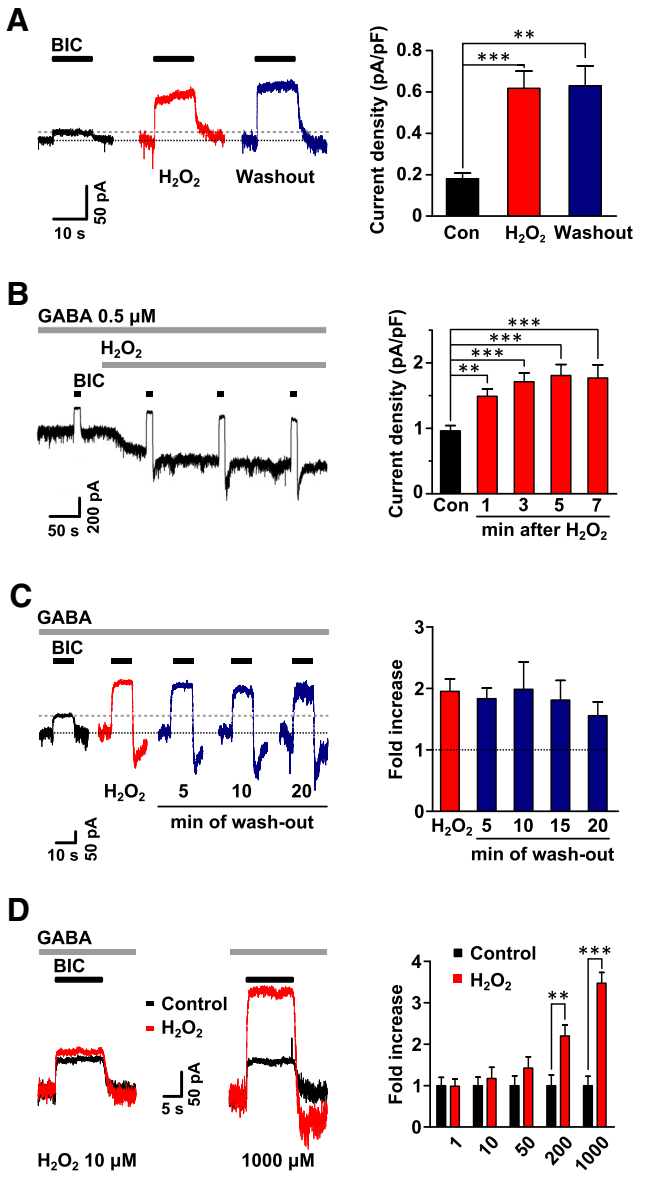

Figure 1. H_2O_2 increased tonic GABA currents in hippocampal neurons. ***A***, Representative traces and summarized data showing that the tonic current revealed by BIC (100 μM) was increased after 5 min of H_2O_2 (200 μM, $n = 21$) and this increase persisted for at least 5 min after washout ($n = 6$). ***B***, Representative trace and summarized data showing the time-dependent effects of H_2O_2 (200 μM) on tonic current in the presence of a low concentration of GABA (0.5 μM, $n = 17–23$). ***C***, Representative traces and summarized data demonstrate that tonic current was persistently increased even after 20 min of washout in the presence of GABA (0.5 μM, $n = 5–7$). One-way ANOVA: $F_{(2,47)} = 16.1$, for ***A***; $F_{(4,102)} = 6.7$ for ***B***, $F_{(4,24)} = 0.4$; and $p = 0.4$ for ***C***; Newman–Keuls *post hoc* test, $^{**}p < 0.01$ and $^{***}p < 0.001$. ***D***, Left, Representative traces showing the increase in tonic current induced by a low-concentration (10 μM) and a high-concentration (1000 μM) of H_2O_2 in the presence of GABA (0.5 μM). Right: Quantified data demonstrating the dose-dependent enhancing effects of H_2O_2 on tonic current ($n = 4$ for each concentration). Two-way ANOVA, effect of H_2O_2: $F_{(1,30)} = 32.0$, $p < 0.0001$; effect of concentration: $F_{(4,30)} = 9.2$, $p < 0.0001$; effect of interaction: $F_{(4,30)} = 9.2$, $p < 0.0001$; Bonferroni *post hoc* test, $^{**}p < 0.01$, $^{***}p < 0.001$. Con, Control (for here and in subsequent figures).

μM; Fig. 1*A*). For these initial experiments, no exogenous GABA was added to the extracellular solution. Pretreating the neurons with H_2O_2 (200 μM) for 5 min increased the amplitude of the tonic current 3.4 ± 0.5-fold (control: 0.18 ± 0.03 pA/pF; H_2O_2: 0.62 ± 0.08 pA/pF, $n = 21–23$, $p < 0.001$, one-way ANOVA followed by Newman–Keuls *post hoc* test; Fig. 1*A*). This increase persisted for at least 5 min after H_2O_2 was washed out.

The increase in tonic current by H_2O_2 could result from an increase in either the extracellular concentration of GABA or the $GABA_A$ receptor function. To exclude the possibility that changes in the extracellular concentration accounted for the increase, a low concentration of exogenous GABA (0.5 μM) was added to the extracellular perfusion solution. Under these conditions, neurons were "concentration clamped" at a stable, known concentration of agonist. GABA (0.5 μM) was selected as this concentration is similar to physiological levels of extracellular GABA that occur *in vivo* (Farrant and Nusser, 2005; Bright and Smart, 2013).

In the presence of exogenous GABA (0.5 μM), H_2O_2 (200 μM) gradually increased the holding current (control: −81.6 ± 8.7 pA; H_2O_2: −189.8 ± 21.8 pA, $n = 21–23$, $p < 0.001$, paired *t* test). Therefore, the amplitude of the tonic current revealed by BIC was enhanced in a time-dependent manner and peaked at 5–7 min (Fig. 1*B*). The enhancing effect of H_2O_2 was not reversible and was sustained for at least 20 min after washout (Fig. 1*C*). Notably, a transient, inward "rebound" current was observed after termination of the BIC application in H_2O_2-treated neurons. This rebound current has been attributed to BIC protecting $GABA_A$ receptors from entering an agonist-bound desensitized state (Bianchi and Macdonald, 2001; Chang et al., 2002). H_2O_2 dose-dependently increased the current with a threshold concentration of 10 μM and maximum effective concentration at 1 mM (3.5 ± 0.3-fold increase; Fig. 1*D*). Concentrations of H_2O_2 >1 mM were not studied because they caused instability of the recordings and cell death as reported previously (Schraufstatter et al., 1986; Kaneko et al., 2006).

To determine whether H_2O_2 similarly potentiated postsynaptic $GABA_A$ receptor currents, spontaneous mIPSCs were recorded from the cultured neurons. Surprisingly, pretreatment with H_2O_2 (200 μM) failed to change the amplitude or frequency of mIPSCs (Fig. 2; Table 1). The time course and charge transfer of the mIPSCs were also unaffected (Table 1).

We next investigated whether H_2O_2 also increased the tonic GABA current in CA1 pyramidal neurons of acutely isolated hippocampal slices. H_2O_2 (1 mM, 10 min) increased the amplitude of the tonic current recorded in the presence of 5 μM GABA (control: 0.05 ± 0.02 pA/pF; H_2O_2: 0.28 ± 0.08 pA/pF; $n = 6$ for each group, $p = 0.02$, unpaired *t* test; Fig. 3) but did not change the amplitude or time course of spontaneous synaptic GABA currents (Table 1). Collectively, these results show that H_2O_2 selectively increases the tonic but not synaptic currents in hippocampal neurons.

H_2O_2-induced increase in tonic current does not require α5$GABA_A$ receptors or $\delta$$GABA_A$ receptors

Several factors could contribute to the differential sensitivity of tonic and synaptic currents to H_2O_2 including the subunit composition of the underlying $GABA_A$ receptors or the conditions under which the receptors are activated. To further investigate H_2O_2 enhancement of the tonic current, additional experiments were performed with cultured hippocampal neurons, as this preparation is a relatively high-throughput assay. In addition, cells can be effectively concentration-clamped and modulators can be applied and washed out rapidly.

A variety of $GABA_A$ receptor subtypes generate the tonic current although, most tonic current is generated by α5 subunit-containing $GABA_A$ (α5$GABA_A$) receptors and δ subunit-containing $GABA_A$ ($\delta$$GABA_A$) receptors (Caraiscos et al., 2004; Farrant and Nusser, 2005; Mortensen and Smart, 2006; Brickley and Mody, 2012; Lee and Maguire, 2014). To study the subtypes

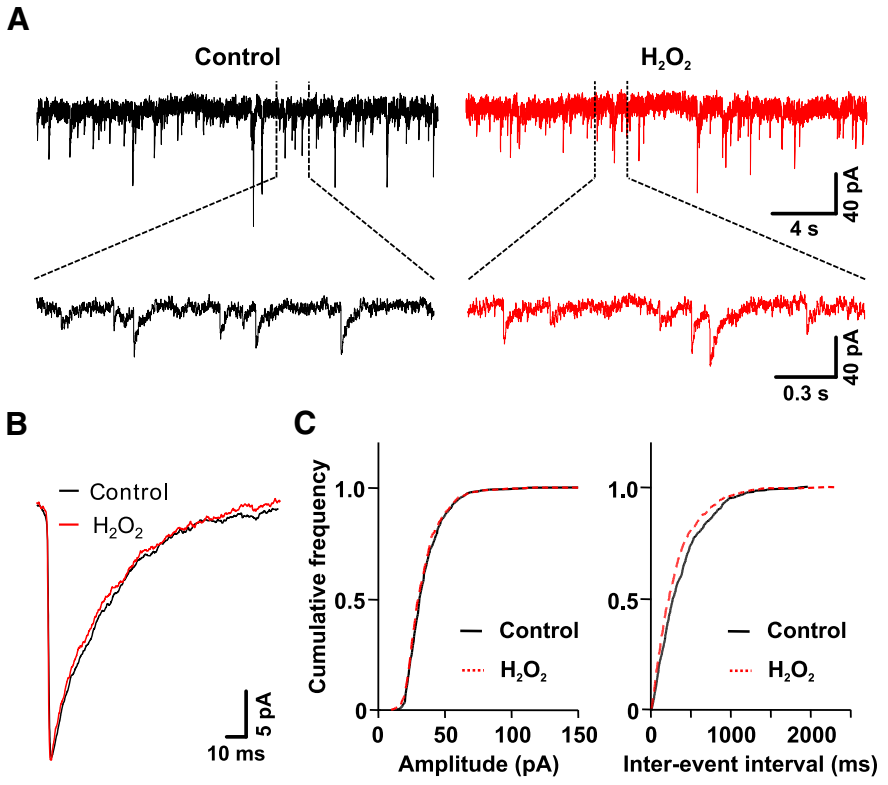

Figure 2. H_2O_2 had no effect on synaptic GABA currents. ***A***, Representative recordings of $GABA_A$ receptor-mediated mIPSCs recorded at −60 mV before (left) and after (right) 5 min of H_2O_2 (200 μM) treatment from the same neuron. ***B***, Traces were averaged from 475 (control) and 527 (H_2O_2) individual mIPSCs. ***C***, Cumulative amplitude (left) and frequency (right) distributions of mIPSCs showing that both the amplitude and the frequency were not affected by H_2O_2; $p = 0.24$ for the amplitude, $p = 0.06$ for the frequency, Kolmogorov–Smirnov test.

of $GABA_A$ receptors that mediate the H_2O_2 enhancement, a combination of pharmacological and genetic approaches was used. First, to investigate the role of α5$GABA_A$ receptors, the inhibitory effects of L-655,708 (L6, 20 nM), a selective inverse agonist for α5$GABA_A$ receptors (Quirk et al., 1996) were studied. The change in holding current produced by an application of L6 was measured before and after application of H_2O_2. BIC (100 μM) was applied after L6 to reveal the total tonic current generated by all subtypes of $GABA_A$ receptors. Despite the expected large increase in the BIC-sensitive current after H_2O_2, the amplitude of the L6-sensitive current was not significantly increased (control: 0.08 ± 0.02 pA/pF; H_2O_2: 0.10 ± 0.03 pA/pF; $n = 6$, $p = 0.36$, paired t test; Fig. 4*A*) and the ratio of the L6-sensitive to BIC-sensitive current was reduced from 47.1 ± 13.7% for control to 15.4 ± 3.9% for H_2O_2 ($n = 6$, $p = 0.03$, paired t test). These results show that α5$GABA_A$ receptors are not a major contributor to the H_2O_2-enhanced tonic current. To confirm that α5$GABA_A$ receptors are not necessary for the H_2O_2-sensitive current, hippocampal neurons were harvested from *Gabra5*$^{-/-}$ mice and WT mice (Fig. 4*B*). The relative magnitude of the tonic current recorded in these neurons was similarly increased by H_2O_2 between genotypes (*Gabra5*$^{-/-}$: 3.7 ± 0.7-fold increase, WT: 4.1 ± 0.6-fold increase; $n = 9$ and 10, respectively; $p = 0.70$, unpaired t test; Fig. 4*B*) confirming that α5$GABA_A$ receptors are not required for enhancement of tonic current. Note, the low amplitude tonic current observed in both genotypes under control conditions, may be attributed to a low ambient concentration of GABA (Caraiscos et al., 2004; Glykys and Mody, 2007; Wang et al., 2012).

To determine whether δ$GABA_A$ receptors generated the H_2O_2-enhanced tonic current, we applied the δ$GABA_A$ receptor-preferring agonist THIP (Brown et al., 2002). THIP-evoked inward current was measured before and after H_2O_2. The THIP (0.5 μM)-evoked current increased 3.3 ± 0.7-fold after H_2O_2 (control: 0.09 ± 0.04 pA/pF; H_2O_2: 0.30 ± 0.06 pA/pF; $n = 6$, $p = 0.007$, paired t test; Fig. 4*C*). The tonic current revealed by BIC was also greatly increased; but H_2O_2 did not change the THIP to BIC current ratio (Control: 59.6 ± 15.7%, H_2O_2: 34.6 ± 3.4%, $n = 5$; $p = 0.21$, paired t test). These results show that δ$GABA_A$ receptors contribute to, but do not exclusively generate the H_2O_2-enhanced tonic current. Next, to determine whether δ$GABA_A$ receptors were necessary for the H_2O_2 effects, the amplitude of the tonic current was measured in neurons harvested from *Gabrd*$^{-/-}$ and WT mice. H_2O_2 caused a similar increase in current in the two genotypes (WT: 9.6 ± 1.5-fold increase, *Gabrd*$^{-/-}$: 9.2 ± 1.4-fold increase; $n = 9$ for each genotype, $p = 0.85$, unpaired t test; Fig. 4*D*), indicating that δ$GABA_A$ receptors are not necessary for the H_2O_2 effects. Thus, the subtypes of $GABA_A$ receptors that typically generate the tonic current in hippocampal neurons are not required for H_2O_2 enhancement of the tonic current.

H_2O_2 increases the potency of GABA at the $GABA_A$ receptors

Given that the unique population of $GABA_A$ receptors that typically generates tonic current does not confer sensitivity to H_2O_2, we next studied whether the conditions of receptor activation contributed to the increase in tonic but not synaptic currents. The tonic current is generated by low concentrations of GABA whereas synaptic currents are activated by near-saturating concentrations of GABA (Farrant and Nusser, 2005; Brickley and Mody, 2012). Thus, we postulated that H_2O_2 increased the potency of GABA at the $GABA_A$ receptors and thereby enhanced the tonic current. If this was true, then the potentiating effects of H_2O_2 should increase with decreasing concentrations of GABA, and decrease with increasing GABA concentrations. Accordingly, we studied the effects of H_2O_2 on current evoked by a brief application of several concentrations of GABA. H_2O_2 (200 μM) increased the amplitude of GABA (0.5 μM)-evoked current 3.4 ± 0.5-fold (control: 1.6 ± 0.3 pA/pF; H_2O_2: 5.4 ± 0.7 pA/pF; $n = 13$ for each group, $p < 0.001$, one-way ANOVA followed by Newman–Keuls *post hoc* test). The increase in current developed slowly over 5 min and was irreversible (Fig. 5*A*). Decreasing GABA from 0.5 μM to 0.3 μM increased the potentiating effect of H_2O_2 (5.3 ± 1.1-fold increase; $n = 5$, $p < 0.001$, two-way ANOVA followed by Bonferroni *post hoc* test; Fig. 5*B*). Conversely, increasing GABA from 0.5 to 10 μM reduced the magnitude of the H_2O_2 effect (1.3 ± 0.2-fold increase; $n = 6$, $p > 0.05$, two-way ANOVA followed by Bonferroni *post hoc* test). Importantly, H_2O_2 caused no change in the current when a near-saturating concentration of GABA (100 μM) was applied. Thus,

Table 1. H_2O_2 did not modify mIPSCs recorded in cultured hippocampal neurons (200 μM, 5 min) or in CA1 pyramidal neurons from hippocampal slices (1 mM, 10 min)

	Treatment	Amplitude (pA)	Frequency (Hz)	Rise time (ms)	Decay time (ms)	Area (pA · ms)
Culture ($n = 9$)	Control	36.4 ± 1.5	2.3 ± 0.6	4.8 ± 0.2	12.4 ± 1.0	487.8 ± 48.5
	H_2O_2	36.1 ± 1.6	1.8 ± 0.4	4.9 ± 0.2	11.9 ± 0.8	454.5 ± 36.3
Slice ($n = 6$)	Control	34.5 ± 2.9	2.9 ± 0.4	3.1 ± 0.2	10.5 ± 0.7	360.5 ± 34.1
	H_2O_2	46.9 ± 4.8	2.8 ± 0.4	3.2 ± 0.2	10.9 ± 0.7	490.3 ± 52.8

Data are presented as mean ± SEM for all the parameters; $p > 0.05$, Student's paired t test.

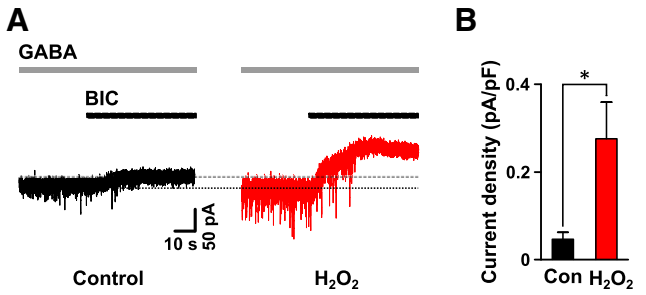

Figure 3. H_2O_2 increased tonic GABA currents in hippocampal slices. Representative traces (***A***) and summarized data (***B***) showing the increase in tonic current after 10 min of H_2O_2 (1 mM) treatment ($n = 6$); *$p = 0.02$, Student's unpaired t test.

H_2O_2 modifies the $GABA_A$ receptors at low but not high concentrations of GABA.

Next, GABA concentration-response plots were constructed for currents evoked by GABA (0.3–1000 μM) in the absence and presence of H_2O_2 (200 μM). H_2O_2 shifted the concentration-response plot to the left, but only for the lower components of the curve (Fig. 5*C*). The EC_{20} value was reduced from 5.5 ± 0.4 μM for control to 3.5 ± 0.4 μM for H_2O_2 ($n = 8$ and 11, respectively; $p = 0.003$, unpaired t test), whereas the EC_{50} values (control: 15.6 ± 1.3 μM; H_2O_2: 12.6 ± 1.1 μM; $n = 8$ and 11, respectively; $p = 0.11$, unpaired t test) and EC_{80} values (control: 44.5 ± 4.8 μM; H_2O_2: 47.3 ± 3.9 μM; $n = 8$ and 11, respectively; $p = 0.65$, unpaired t test) were unchanged. H_2O_2 also reduced the Hill coefficient (control: 1.35 ± 0.04; H_2O_2: 1.07 ± 0.05; $n = 8$ and 11, respectively; $p = 0.0005$, unpaired t test). Collectively, these results show that H_2O_2 increases the potency of GABA at the $GABA_A$ receptors, but only at low GABA concentrations.

H_2O_2 does not change the desensitization or voltage sensitivity of $GABA_A$ receptors

We next asked whether H_2O_2 modified the desensitization and voltage sensitivity of $GABA_A$ receptors, two factors that can modify the magnitude of tonic current (Chang et al., 2002; Pavlov et al., 2009). If H_2O_2 simply increased the potency of GABA without changing desensitization, then the extent of desensitization of the increased current should be similar to that observed with a higher concentration of GABA. Also, no change in current desensitization should be observed at a saturating concentration of GABA (Colquhoun, 1998; Chang et al., 2002). We quantified the extent of desensitization by recording whole-cell currents evoked by longer applications of GABA (20 s). The ratio of current at 20 s to peak current was measured before and after application of H_2O_2 (200 μM). In these experiments, H_2O_2 increased the extent of desensitization for current evoked by low concentrations of GABA (Fig. 5*D*), but did not influence desensitization of currents evoked by higher concentrations of GABA (e.g., 10 μM). We next plotted the relationship between the peak current and current at 20 s application of GABA (over a concentration range of 0.3–10 μM; Fig. 5*E*). A linear regression fit to data for the two conditions

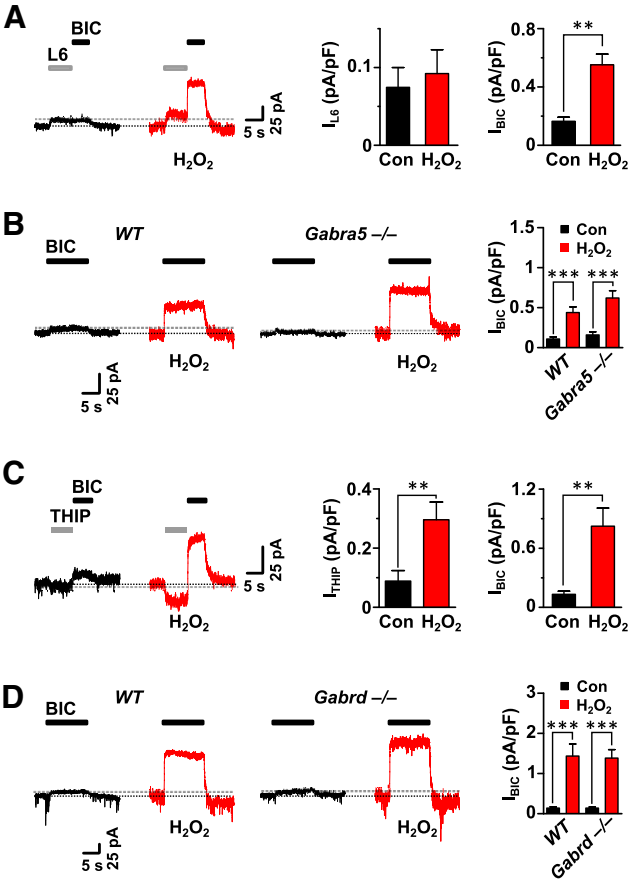

Figure 4. $\alpha 5GABA_A$ and $\delta GABA_A$ receptors were not required for the H_2O_2-dependent increase in current. ***A***, Left, Representative traces showing the tonic current revealed by a selective inverse agonist of $\alpha 5GABA_A$ receptors (L6, 20 nM) and BIC (100 μM). The L6-sensitive tonic current was unaffected, whereas BIC-sensitive current was greatly increased by H_2O_2. Right, Quantification of L6- and BIC-sensitive tonic current, before and after H_2O_2 ($n = 6$); **$p = 0.002$, Student's paired t test. ***B***, Left, Representative traces showing the tonic current revealed by BIC (100 μM) in neurons from WT and *Gabra5*$^{-/-}$ mice. Right, Quantification of the tonic current before and after H_2O_2 ($n = 10$ for WT, $n = 9$ for *Gabra5*$^{-/-}$). ***C***, Left, Representative traces showing the current activated by $\delta GABA_A$ receptor-preferring agonist THIP (0.5 μM) and the tonic current revealed by BIC (100 μM). Right, Quantification of THIP-activated inward current ($n = 6$) and the tonic current revealed by BIC ($n = 5$) before and after H_2O_2; **$p = 0.007$ for THIP, **$p = 0.008$ for BIC, Student's paired t test. ***D***, Left, Representative traces showing the tonic current revealed by BIC in neurons from WT and *Gabrd*$^{-/-}$ mice. Right, Quantification of the tonic current before and after H_2O_2 ($n = 9$ for each genotype).

showed that the slopes of the lines were similar (control: 1.2 ± 0.05; H_2O_2: 1.2 ± 0.06; $n = 23$ for each group, $p = 0.87$). Thus, H_2O_2 does not alter the intrinsic desensitization properties of $GABA_A$ receptors.

If H_2O_2 simply increases the potency of GABA, then no change in the voltage sensitivity of $GABA_A$ receptors should be observed (Colquhoun, 1998; Legendre et al., 2000; Pavlov et al., 2009). To test this prediction, *I–V* plots were constructed for

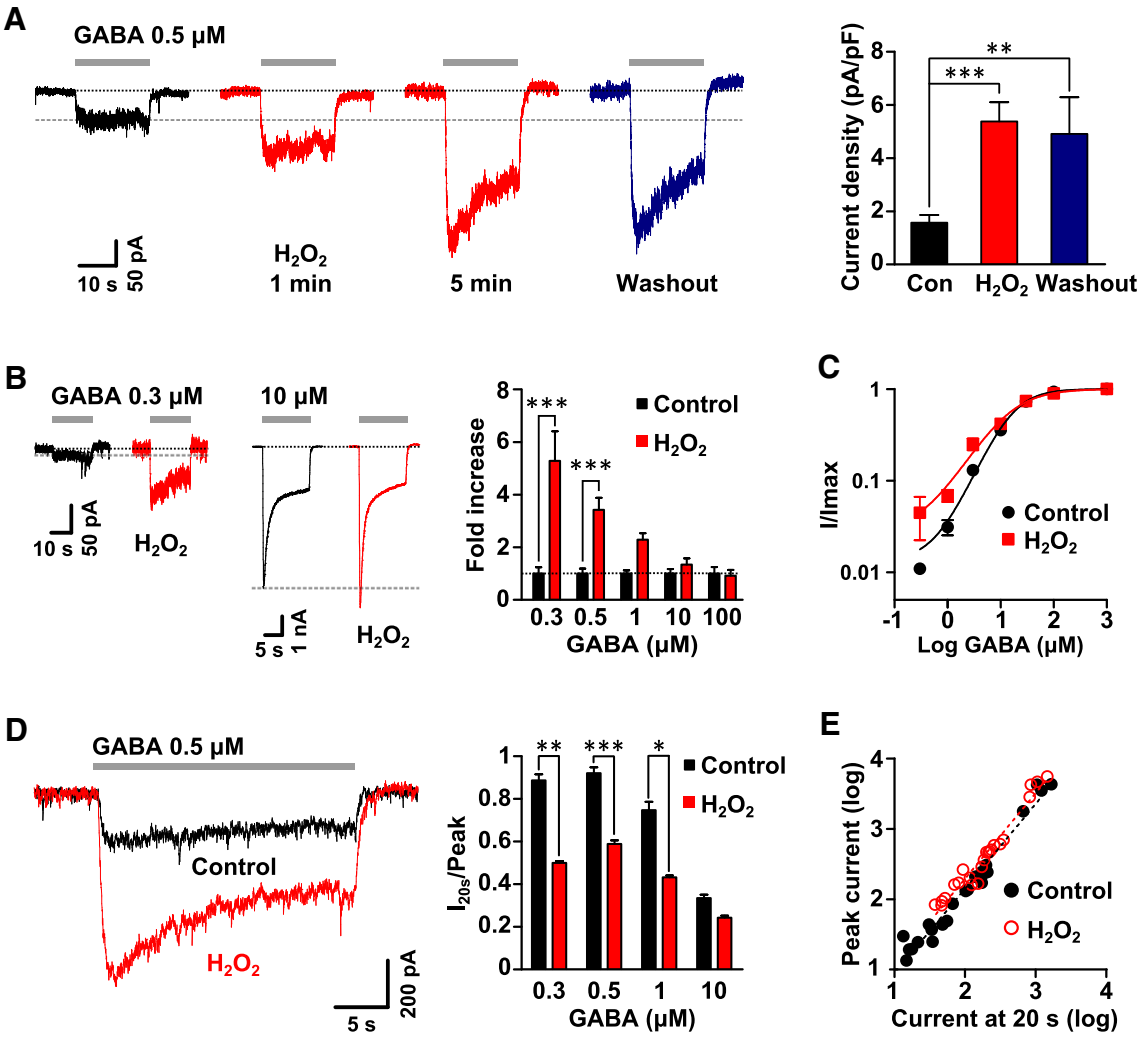

Figure 5. H_2O_2 increased the potency of low concentrations of GABA without modifying the desensitization properties. ***A***, Left, Representative traces showing the time-dependent effects of H_2O_2 (200 μM) on currents evoked by a low concentration of GABA (0.5 μM). Right, Summarized data ($n = 4–13$). One-way ANOVA: $F_{(2,27)} = 11.8$; Newman–Keuls *post hoc* test; $^{**}p < 0.01$, $^{***}p < 0.001$. ***B***, Left, Representative traces showing the effects of H_2O_2 (200 μM) on the currents evoked by 0.3 and 10 μM GABA. Right, Summarized data showing the fold increase in currents evoked by different concentrations of GABA by H_2O_2 (200 μM; $n = 5–13$). Two-way ANOVA, effect of H_2O_2: $F_{(1,58)} = 33.2$, $p < 0.0001$; effect of concentration: $F_{(4,58)} = 7.6$, $p < 0.0001$; effect of interaction: $F_{(4,58)} = 7.6$, $p < 0.0001$; Bonferroni *post hoc* test, $^{***}p < 0.001$. ***C***, Concentration-response plots in the absence and presence of H_2O_2 fitted with Hill equation ($n = 8–11$). ***D***, Left, Representative traces showing currents evoked by GABA (0.5 μM) before and after H_2O_2 (200 μM). Right, Summarized data showing the effects of H_2O_2 on desensitization of currents evoked by increasing concentrations of GABA. H_2O_2 decreased the ratio of current at 20 s to peak current only at low GABA concentrations but not at 10 μM ($n = 4–9$). Two-way ANOVA, effect of H_2O_2: $F_{(1,38)} = 33.5$, $p < 0.0001$; effect of concentration: $F_{(3,38)} = 18.0$, $p < 0.0001$; effect of interaction: $F_{(3,38)} = 1.8$, $p = 0.16$; Bonferroni *post hoc* test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. ***E***, Correlation between the current at 20 s and peak current, before and after H_2O_2 (200 μM). The linear regression fit generated similar slopes between control and H_2O_2 treatment ($n = 23$ for each condition).

tonic current recorded before and after application of H_2O_2 (200 μM). Specifically, GABA (0.5 μM) was added to the extracellular solution and currents were recorded using a voltage step protocol (−80 to +20 mV; Fig. 6*A*). BIC (100 μM) was then applied, and currents were recorded using the same voltage step protocol. The magnitude of the tonic current at the various potentials was calculated as the difference in current measured in the absence and presence of BIC. The control *I–V* plot exhibited a slight outward rectification (Fig. 6*A*), as previously described (Pavlov et al., 2009). H_2O_2 increased the slope of the *I–V* plot but did not change the outward rectifying properties or reversal potential (control: −1.9 ± 3.2 mV; H_2O_2: −6.3 ± 1.7 mV, $n = 6$, $p = 0.44$, paired *t* test) of the tonic current. The H_2O_2-induced increase in current was equivalent at all potentials between −80 mV and +20 mV (2.3 ± 0.1-fold; Fig. 6*B*), suggesting that H_2O_2 does not alter the voltage sensitivity of $GABA_A$ receptors.

H_2O_2 effects are independent of Zn^{2+}, Ca^{2+}, and $GABA_B$ receptors

The divalent cation Zn^{2+} inhibits subpopulations of high-affinity $GABA_A$ receptors that generate a tonic current in hippocampal neurons (Saxena and Macdonald, 1994; Mortensen and Smart, 2006), and trace levels of Zn^{2+} are present in the extracellular solution (Smart et al., 1994). Thus, it is plausible that H_2O_2 increased the current by relieving Zn^{2+}-induced inhibition of $GABA_A$ receptors. We tested this hypothesis in two ways. First, if Zn^{2+} inhibited the tonic current, then reducing the extracellular concentration of Zn^{2+} should mimic the effect of H_2O_2. The Zn^{2+} chelator TPEN (10 μM) was added to the extracellular solution to markedly reduce the concentration of Zn^{2+}. TPEN produced no change in the amplitude of the tonic current (control: 1.3 ± 0.3 pA/pF; TPEN: 1.3 ± 0.2 pA/pF; $n = 4$, $p = 0.55$, paired *t* test; Fig. 7*A*). Next, we studied whether increasing the extracel-

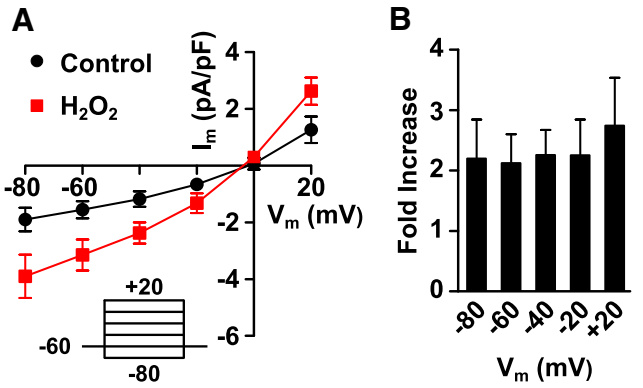

Figure 6. H_2O_2 did not modify the voltage sensitivity of the tonic GABA current. ***A***, The *I–V* relationship of tonic current before and after H_2O_2 treatment was studied using a voltage step protocol for holding potentials between −80 and +20 mV that had a duration of 4 s (n = 5–6). ***B***, Summarized data showing similar increase in tonic current by H_2O_2 at all membrane potentials (n = 5). One-way ANOVA: $F_{(4,20)} = 0.17$, $p = 0.95$.

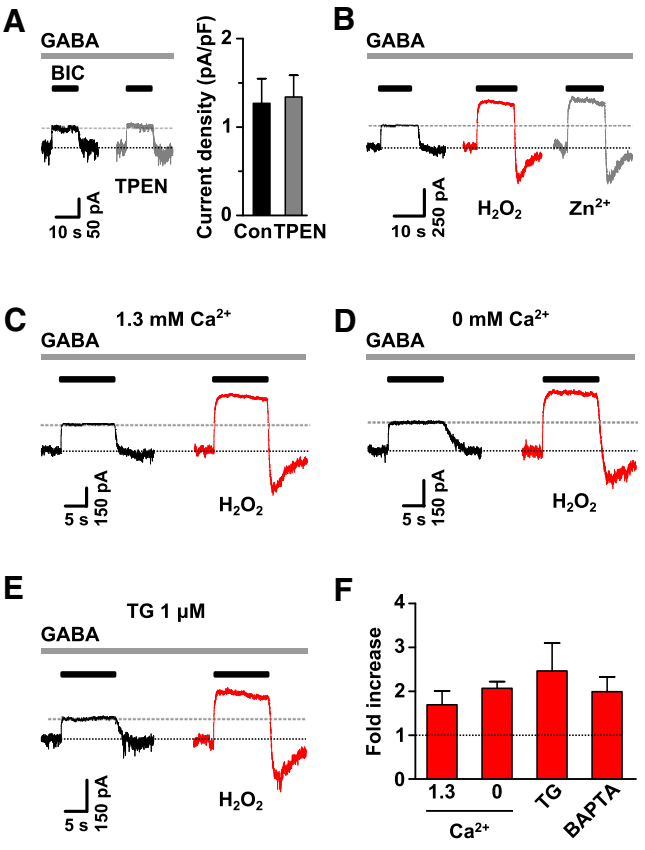

Figure 7. Changes in the concentration of Zn^{2+} and Ca^{2+} did not modify the effects of H_2O_2. ***A***, Left, Representative traces showing the effects of TPEN (10 μM) on the tonic current. Right, Quantification of the increase in tonic current before and after TPEN (n = 4). ***B***, Representative traces showing the effects of Zn^{2+} (10 μM) on tonic current after being potentiated by H_2O_2 (200 μM). Similar results were obtained from another three cells. ***C–E***, Representative traces showing the increase in tonic current by H_2O_2 (200 μM) in the presence of normal extracellular Ca^{2+} (***C***), in the absence of extracellular Ca^{2+} (***D***), or when intracellular Ca^{2+} was depleted with thapsigargin (TG, 1 μM; ***E***). ***F***, Summarized data for experiments in ***C–E*** (n = 5–9 for each condition). One-way ANOVA: $F_{(3,20)} = 0.7$, $p = 0.55$.

lular concentration of Zn^{2+} decreased the H_2O_2 effects on current. The addition of Zn^{2+} (10 μM) to the extracellular solution had no effect on H_2O_2 (200 μM) enhancement of the current (H_2O_2: 2.6 ± 0.5 pA/pF; Zn^{2+}: 2.4 ± 0.6 pA/pF; n = 4, p = 0.3, paired t test; Fig. 7*B*). Thus, H_2O_2 relief of Zn^{2+} blockade does not account for the increase in tonic current.

H_2O_2 is known to alter the intracellular concentration of Ca^{2+} (Smith et al., 2003) and the cytosolic concentration of Ca^{2+} alters the potency of GABA at $GABA_A$ receptors (Inoue et al., 1986; Martina et al., 1994). To determine whether H_2O_2-dependent changes in Ca^{2+} contributed to the increased current, extracellular and intracellular Ca^{2+} concentrations were modified. First, we reduced the extracellular concentration of Ca^{2+} and observed no change in the ability of H_2O_2 to increase the current (Fig. 7*C*,*D*,*F*). Depleting intracellular Ca^{2+} stores by treating the neurons with thapsigargin (TG; 1 μM; Fig. 7*E*,*F*) also had no effect. Similarly, chelating Ca^{2+} by perfusing the neurons with BAPTA-AM (100 μM; Fig. 7*F*) failed to change the H_2O_2-enhanced current. Thus, the H_2O_2 effects are independent of the extracellular and intracellular concentrations of Ca^{2+}.

It is plausible that the effects of H_2O_2 are secondary to its modulatory effects on other receptors or proteins. Upregulation of $GABA_B$ receptors increases tonic GABA current (Connelly et al., 2013; Tao et al., 2013) and H_2O_2 could increase the tonic current indirectly by increasing $GABA_B$ receptor function. However, when a $GABA_B$ receptor blocker CGP 55845 (1 μM) was added to the bath, H_2O_2 increased tonic GABA current 2.0 ± 0.2-fold (CGP: 1.2 ± 0.2 pA/pF; H_2O_2 and CPG: 2.4 ± 0.3 pA/pF; n = 5; p = 0.002, paired t test) suggesting that H_2O_2 does not act on $GABA_B$ receptors. Collectively, these results suggest that the increase in GABA potency by H_2O_2 does not result from relief of Zn^{2+}-induced inhibition and is independent of Ca^{2+} concentrations, as well as $GABA_B$ receptors.

H_2O_2 enhancement is mediated by an extracellular oxidative reaction

H_2O_2 regulates the function of several ion channels that span the cell membrane including TRPM4, TRPC6, and purinergic (P2X2 and P2X4) receptors via an oxidative reaction (Coddou et al., 2009; Graham et al., 2010; Simon et al., 2010). To determine whether H_2O_2 similarly regulated $GABA_A$ receptors via an oxidative reaction, the antioxidant glutathione (GSH, 1 mM) was added to the pipette solution and current was recorded before and after treatment with H_2O_2 (200 μM). Under these conditions, H_2O_2 increased the tonic current as reported above (control: 1.4 ± 0.2 pA/pF; H_2O_2: 3.6 ± 0.7 pA/pF; n = 6 for each group, p = 0.008, paired t test; Fig. 8*A*). In contrast, the effects of H_2O_2 were completely abolished when GSH was added to the extracellular solution (Fig. 8*B*). Similarly, when the antioxidant dithiothreitol (DTT, 1 mM) was added to the extracellular solution, H_2O_2 had no effect on the current (Fig. 8*C*). Collectively, the results show that the potentiating effects of H_2O_2 result from an extracellular oxidative reaction.

The oxidative reaction could be mediated either directly by H_2O_2 or indirectly through the production of hydroxyl radical ($\cdot$OH). H_2O_2 generates $\cdot$OH by interacting with ferrous irons (Fe^{2+}) via the Fenton reaction (Haber and Weiss, 1934). We tested whether inhibiting the Fenton reaction by adding the iron chelator deferoxamine (DFO; 100 μM) to the extracellular solution modified the effect of H_2O_2. DFO completely abolished the effect of H_2O_2 (Fig. 8*D*) confirming that the increase in current was dependent on the Fenton reaction.

Endogenous ROS increase tonic current

Finally, we investigated whether endogenous H_2O_2 increased the tonic current. Ischemia-reperfusion, which produces high levels of H_2O_2 and other ROS (Hyslop et al., 1995) can be mimicked *in*

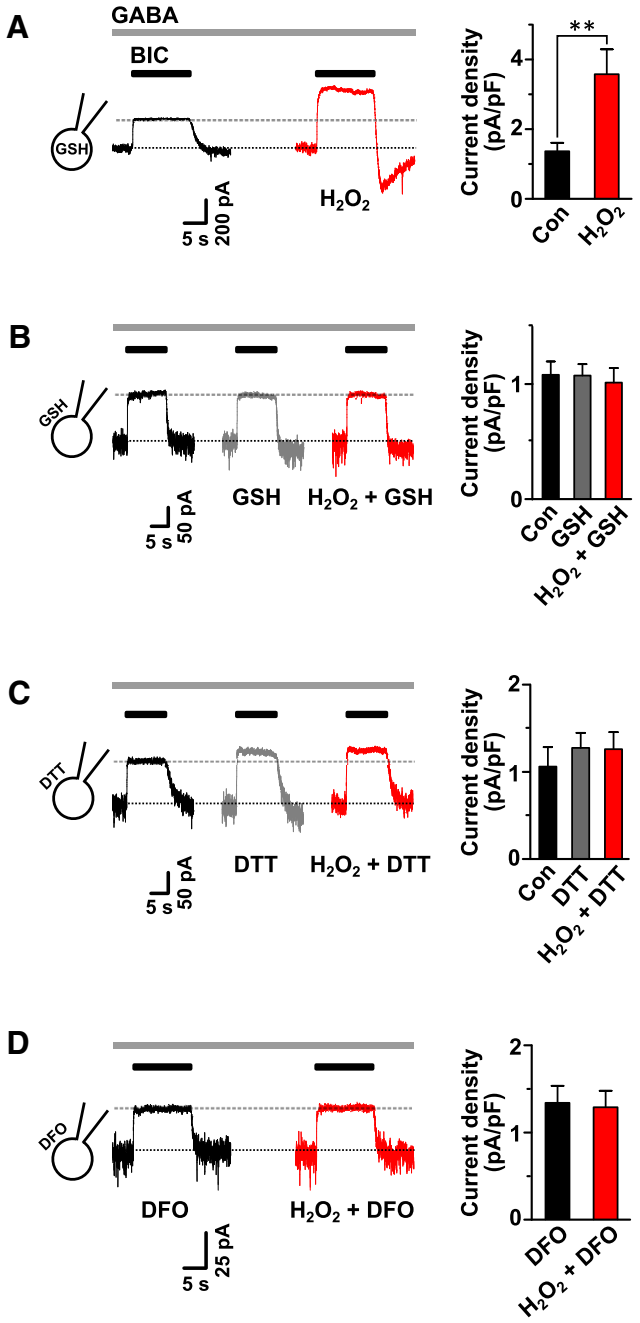

Figure 8. Extracellular oxidative reaction mediated the effects of H_2O_2. ***A***, Addition of the antioxidant GSH (1 mM) to the pipette solution did not modify the increase in tonic current by H_2O_2 (200 μM). $n = 6$ for each group, $^{**}p = 0.008$, Student's unpaired t test. ***B***, ***C***, Addition of the antioxidants GSH (1 mM) and DTT (1 mM) to the extracellular solution abolished the increase in tonic current by H_2O_2 (200 μM); $n = 4$. ***D***, DFO (100 μM) prevented the increase in tonic current by H_2O_2 (200 μM); $n = 6$.

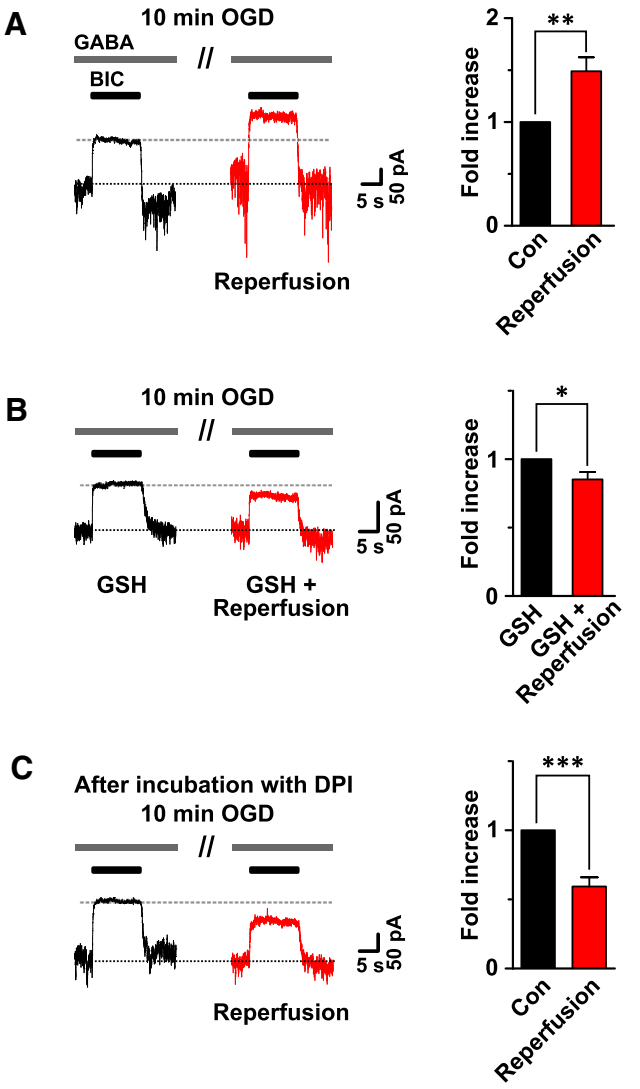

Figure 9. OGD treatment increased tonic current through an oxidative reaction. ***A***, Reperfusion after OGD increased tonic current; $n = 9$, $^{**}p = 0.006$, Student's paired t test. ***B***, ***C***, The increase in tonic current by reperfusion was abolished and a decrease after reperfusion was observed for neurons treated with the antioxidant GSH (1 mM; ***B***) or pretreated for 30 min with NADPH oxidase inhibitor DPI (10 μM; ***C***). Student's paired t test; $n = 7$, $^{*}p = 0.03$ for ***B***; $n = 7$, $^{***}p < 0.001$ for ***C***.

vitro using an oxygen-glucose deprivation (OGD) protocol (Abramov et al., 2007). Following 10 min of OGD, cultures were re-perfused with the normal, oxygenated extracellular solution. OGD increased the amplitude of the current by 49 ± 13% ($n = 9$, $p = 0.006$, paired t test; Fig. 9*A*). Next, to confirm that this increase in current after OGD resulted from an oxidative reaction, the antioxidant GSH (1 mM) was added to the extracellular solution during period of ischemia. GSH completely abolished the increase in current following OGD (Fig. 9*B*). The current was also slightly reduced by GSH (15 ± 5%, $n = 7$, $p = 0.04$, paired t test).

We next sought to identify the source of the endogenous H_2O_2 produced during OGD and reperfusion. NADPH oxidase is the principal source of ROS during reperfusion and inhibition of this enzyme prevents the production of ROS in hippocampal neurons (Abramov et al., 2004; Riquelme et al., 2011). To examine whether NADPH oxidase generated H_2O_2, which increased the tonic current after OGD, cultured neurons were incubated with the NADPH oxidase inhibitor diphenyleneiodonium (DPI; 10 μM) for 30 min before being treated with OGD. DPI completely abolished the increase in tonic current after OGD and indeed, reduced tonic GABA current by 41 ± 1% ($n = 7$, $p < 0.0001$, paired t test; Fig. 9*C*). These results suggest that endogenous H_2O_2 produced by NADPH oxidase during ischemia-reperfusion might increase the tonic current via an oxidative reaction. The decrease in tonic current after GSH and DPI suggests that OGD may also have an inhibitory effect on tonic current that is masked by the potentiating effects of H_2O_2.

Discussion

The results show that H_2O_2 causes a robust increase in $GABA_A$ receptor-mediated tonic current whereas synaptic currents are unaffected. This selective increase in tonic current does not require the expression of typical extrasynaptic $GABA_A$ receptors and is due to an extracellular oxidative reaction that increases the potency of GABA. In addition, ischemia-reperfusion injury, which produces high endogenous levels of H_2O_2, similarly increases the tonic current.

H_2O_2 increases the potency of GABA, but only when $GABA_A$ receptors are activated by low concentrations of GABA. The GABA concentration-response plot is shifted to the left; however, only the EC_{20} value but not the EC_{50} or EC_{80} value is reduced. Several additional lines of evidence further support the conclusion that H_2O_2 increases the potency of GABA. First, a transient, inward "rebound" current is observed in H_2O_2-treated neurons (Fig. 1*B*). A similar rebound current can occur when currents are activated by high concentrations of GABA (Bianchi and Macdonald, 2001). Second, H_2O_2 did not modify the desensitization or voltage sensitivity, two intrinsic properties of the $GABA_A$ receptors that alter the current amplitude. Thirdly, the peak amplitude for current evoked by saturating concentration of GABA and mIPSCs was unaffected, which is consistent with the increased potency of GABA by H_2O_2 at only low concentrations of GABA. Two possible mechanisms might underlie the increased potency at low GABA concentrations. H_2O_2 can increase the open probability of $GABA_A$ receptors that reside in a monoliganded state, as occurs at low GABA concentrations (Petrini et al., 2011). H_2O_2 may stabilize such an open state and thereby increase the tonic current. Also, H_2O_2 may enhance the spontaneous opening probability of $GABA_A$ receptors that only activates the tonic current (McCartney et al., 2007; Wlodarczyk et al., 2013). Future studies, such as single channel recording, may provide insights into the underlying mechanisms responsible for the selective increase in potency of GABA at low concentrations.

The increase in GABA potency is attributed to an extracellular oxidative reaction as the addition of extracellular antioxidants (GSH and DTT) to the perfusion solution completely abolishes H_2O_2 effects, whereas intracellular application of GSH has no effect. Such oxidation by H_2O_2 may result from the production of · OH by the Fenton reaction (Haber and Weiss, 1934) as inhibiting the Fenton reaction abolishes the effect. This result is consistent with previous reports that show H_2O_2, via the Fenton reaction, modulates the intracellular concentration of Cl^- (Sah and Schwartz-Bloom, 1999) and synaptic potentials (Pellmar, 1995) in hippocampal slices.

The onset of H_2O_2 enhancement of tonic current is slow and irreversible and occurs within the time frame of minutes. Most positive allosteric modulators of $GABA_A$ receptors, including benzodiazepines, barbiturates, and general anesthetics, increase current within the time frame of milliseconds to seconds and are fully reversible (Twyman et al., 1989; Orser et al., 1994; Walters et al., 2000). The slow time course of H_2O_2 increase in $GABA_A$ receptor activity is similar to H_2O_2 effects on P2X2 and TRPM4 channels, which results from oxidation of cysteine residues in the ion channels themselves (Coddou et al., 2009; Simon et al., 2010). For example, H_2O_2 oxidation of Cys^{430} in P2X2 channels increases receptor activity by increasing agonist potency (Coddou et al., 2009), whereas oxidation of Cys^{1093} in TRPM4 channels abolishes receptor desensitization (Simon et al., 2010).

It is plausible that the slow onset and irreversibility of H_2O_2 effects in our study are due to oxidation of cysteine residues of $GABA_A$ receptors. H_2O_2 predominantly oxidizes cysteine residues, but can also modify methionine, tyrosine, phenylalanine, histidine, and lysine residues (Chu et al., 2006; Calero and Calvo, 2008). H_2O_2 may oxidize amino acids that are located either in the binding pocket for GABA, or in a region close to the binding domain (Boileau et al., 1999). Alternatively, H_2O_2 may oxidize $GABA_A$ receptor-associated proteins, such as the transmembrane cell adhesion molecules dystroglycan (Pribiag et al., 2014) and neuroligin 2 (Hines et al., 2012), which regulate $GABA_A$ receptor function. It is of future interest to determine whether H_2O_2 via oxidation, acts directly on $GABA_A$ receptors or indirectly on receptor-associated proteins.

Extrasynaptic $\alpha5GABA_A$ receptors and $\delta GABA_A$ receptors are not necessary for the H_2O_2 effects. However, tonic current in neurons is not generated by a single population of $GABA_A$ receptors but rather by multiple subtypes including those containing $\alpha\beta$, $\alpha1\beta\delta$, $\alpha3\beta\gamma2$, $\alpha4\delta$, $\alpha6\delta$, and $\alpha\beta\varepsilon$ subunits (Mortensen and Smart, 2006; Lee and Maguire, 2014). These different receptor subtypes could contribute to the increased current produced by H_2O_2. Therefore, the major factor that confers sensitivity to H_2O_2 is unlikely the subunit composition of the receptor *per se*, but rather receptor occupancy by the agonist. When these multiple $GABA_A$ receptors are activated by low concentrations of GABA, oxidation by H_2O_2 increases the potency of GABA, which enhances the tonic current. H_2O_2 may also selectively potentiate yet-to-be-identified subpopulations of high affinity $GABA_A$ receptors. In support of this hypothesis, the increase in tonic current by H_2O_2 varied from 3.4-fold to 9.6-fold in cultured neurons from Swiss White mice and C57BL/6 × SvJ129 mice, respectively, studied in the absence of exogenous GABA (Figs. 1*B*, Fig. 4*D*). Different strains of mice may express different combinations of $GABA_A$ receptor subtypes (Caldji et al., 2004; Schlussman et al., 2013) that exhibit different sensitivities to H_2O_2. Future studies of combination of recombinant $GABA_A$ receptors might offer insights into possible mechanisms that account for the variable effects of H_2O_2.

Interestingly, several studies have suggested that H_2O_2 decreases $GABA_A$ receptor function. High concentrations of H_2O_2 (1.5–3.5 mM) reduce the amplitude of IPSPs in hippocampal, cerebral, cortical and thalamic neurons possibly due to a reduction in transmitter increase (Pellmar, 1995; Frantseva et al., 1998). In contrast, H_2O_2 (300 μM) induces an increase in intracellular Cl^- (Sah and Schwartz-Bloom, 1999), possibly by increasing the tonic current. We found that H_2O_2 has no effect on mIPSCs recorded in hippocampal neurons. This result is consistent with a study showing that H_2O_2 (100 μM) does not modify mIPSCs in spinal cord neurons, although, higher concentration of H_2O_2 (1 mM) increases both the amplitude and the frequency of mIPSCs possibly by increasing release of GABA (Takahashi et al., 2007).

H_2O_2 upregulation of tonic current may be particularly important under conditions where the concentration of H_2O_2 and other ROS increase dramatically (Andersen, 2004; Allen and Bayraktutan, 2009; Uttara et al., 2009). During ischemia-reperfusion injury, H_2O_2 levels in the brain can reach as high as 200 μM (Hyslop et al., 1995). OGD, which produces high endogenous levels of H_2O_2 as well as ROS, also increases the tonic current. Notably, the relative increase in current after OGD is similar to that produced by concentrations of H_2O_2 of 50–200 μM (Fig. 1*C*).

In summary, to the best of our knowledge, this study provides the first evidence that H_2O_2 dramatically increases the amplitude of tonic current generated by native $GABA_A$ receptors in neurons. This novel regulatory effect may have important clinical

implications, given that perturbations in tonic inhibition has been implicated in multiple neurological disorders and age-associated cognitive deficits (Brickley and Mody, 2012; Wang et al., 2012). Suppressing the H_2O_2-enhanced tonic GABA current might represent a novel strategy for preventing and/or treating such neurological disorders.

References

Abramov AY, Canevari L, Duchen MR (2004) β-Amyloid peptides induce mitochondrial dysfunction and oxidative stress in astrocytes and death of neurons through activation of NADPH oxidase. J Neurosci 24:565–575. CrossRef Medline

Abramov AY, Scorziello A, Duchen MR (2007) Three distinct mechanisms generate oxygen free radicals in neurons and contribute to cell death during anoxia and reoxygenation. J Neurosci 27:1129–1138. CrossRef Medline

Allen CL, Bayraktutan U (2009) Oxidative stress and its role in the pathogenesis of ischaemic stroke. Int J Stroke 4:461–470. CrossRef Medline

Andersen JK (2004) Oxidative stress in neurodegeneration: cause or consequence? Nat Med 10:S18–S25. CrossRef Medline

Bianchi MT, Macdonald RL (2001) Agonist trapping by $GABA_A$ receptor channels. J Neurosci 21:9083–9091. Medline

Boileau AJ, Evers AR, Davis AF, Czajkowski C (1999) Mapping the agonist binding site of the $GABA_A$ receptor: evidence for a β-strand. J Neurosci 19:4847–4854. Medline

Brickley SG, Mody I (2012) Extrasynaptic $GABA_A$ receptors: their function in the CNS and implications for disease. Neuron 73:23–34. CrossRef Medline

Bright DP, Smart TG (2013) Methods for recording and measuring tonic $GABA_A$ receptor-mediated inhibition. Front Neural Circuits 7:193. CrossRef Medline

Brown N, Kerby J, Bonnert TP, Whiting PJ, Wafford KA (2002) Pharmacological characterization of a novel cell line expressing human $\alpha4\beta3\delta$ $GABA_A$ receptors. Br J Pharmacol 136:965–974. CrossRef Medline

Caldji C, Diorio J, Anisman H, Meaney MJ (2004) Maternal behavior regulates benzodiazepine/$GABA_A$ receptor subunit expression in brain regions associated with fear in BALB/c and C57BL/6 mice. Neuropsychopharmacology 29:1344–1352. CrossRef Medline

Calero CI, Calvo DJ (2008) Redox modulation of homomeric ρ1 GABA receptors. J Neurochem 105:2367–2374. CrossRef Medline

Caraiscos VB, Elliott EM, You-Ten KE, Cheng VY, Belelli D, Newell JG, Jackson MF, Lambert JJ, Rosahl TW, Wafford KA, MacDonald JF, Orser BA (2004) Tonic inhibition in mouse hippocampal CA1 pyramidal neurons is mediated by α5 subunit-containing γ-aminobutyric acid type A receptors. Proc Natl Acad Sci U S A 101:3662–3667. CrossRef Medline

Chang Y, Ghansah E, Chen Y, Ye J, Weiss DS (2002) Desensitization mechanism of GABA receptors revealed by single oocyte binding and receptor function. J Neurosci 22:7982–7990. Medline

Chu XP, Close N, Saugstad JA, Xiong ZG (2006) ASIC1a-specific modulation of acid-sensing ion channels in mouse cortical neurons by redox reagents. J Neurosci 26:5329–5339. CrossRef Medline

Coddou C, Codocedo JF, Li S, Lillo JG, Acuña-Castillo C, Bull P, Stojilkovic SS, Huidobro-Toro JP (2009) Reactive oxygen species potentiate the P2X2 receptor activity through intracellular Cys^{430}. J Neurosci 29:12284–12291. CrossRef Medline

Collinson N, Kuenzi FM, Jarolimek W, Maubach KA, Cothliff R, Sur C, Smith A, Otu FM, Howell O, Atack JR, McKernan RM, Seabrook GR, Dawson GR, Whiting PJ, Rosahl TW (2002) Enhanced learning and memory and altered GABAergic synaptic transmission in mice lacking the α5 subunit of the $GABA_A$ receptor. J Neurosci 22:5572–5580. Medline

Colquhoun D (1998) Binding, gating, affinity and efficacy: the interpretation of structure-activity relationships for agonists and of the effects of mutating receptors. Br J Pharmacol 125:924–947. CrossRef Medline

Connelly WM, Fyson SJ, Errington AC, McCafferty CP, Cope DW, Di Giovanni G, Crunelli V (2013) $GABA_B$ receptors regulate extrasynaptic $GABA_A$ receptors. J Neurosci 33:3780–3785. CrossRef Medline

Farrant M, Nusser Z (2005) Variations on an inhibitory theme: phasic and tonic activation of $GABA_A$ receptors. Nat Rev Neurosci 6:215–229. CrossRef Medline

Frantseva MV, Perez Velazquez JL, Carlen PL (1998) Changes in membrane and synaptic properties of thalamocortical circuitry caused by hydrogen peroxide. J Neurophysiol 80:1317–1326. Medline

Giorgio M, Trinei M, Migliaccio E, Pelicci PG (2007) Hydrogen peroxide: a metabolic by-product or a common mediator of ageing signals? Nat Rev Mol Cell Biol 8:722–728. CrossRef Medline

Glykys J, Mody I (2007) Activation of $GABA_A$ receptors: views from outside the synaptic cleft. Neuron 56:763–770. CrossRef Medline

Graham S, Ding M, Ding Y, Sours-Brothers S, Luchowski R, Gryczynski Z, Yorio T, Ma H, Ma R (2010) Canonical transient receptor potential 6 (TRPC6), a redox-regulated cation channel. J Biol Chem 285:23466–23476. CrossRef Medline

Haber F, Weiss J (1934) The catalytic decomposition of hydrogen peroxide by iron salts. Proc R Soc Lond A 147:332–351. CrossRef

Hines RM, Davies PA, Moss SJ, Maguire J (2012) Functional regulation of $GABA_A$ receptors in nervous system pathologies. Curr Opin Neurobiol 22:552–558. CrossRef Medline

Hyslop PA, Zhang Z, Pearson DV, Phebus LA (1995) Measurement of striatal H_2O_2 by microdialysis following global forebrain ischemia and reperfusion in the rat: correlation with the cytotoxic potential of H_2O_2 *in vitro*. Brain Res 671:181–186. CrossRef Medline

Inoue M, Oomura Y, Yakushiji T, Akaike N (1986) Intracellular calcium ions decrease the affinity of the GABA receptor. Nature 324:156–158. CrossRef Medline

Kamsler A, Segal M (2003) Hydrogen peroxide modulation of synaptic plasticity. J Neurosci 23:269–276. Medline

Kaneko S, Kawakami S, Hara Y, Wakamori M, Itoh E, Minami T, Takada Y, Kume T, Katsuki H, Mori Y, Akaike A (2006) A critical role of TRPM2 in neuronal cell death by hydrogen peroxide. J Pharmacol Sci 101:66–76. CrossRef Medline

Lee V, Maguire J (2014) The impact of tonic $GABA_A$ receptor-mediated inhibition on neuronal excitability varies across brain region and cell type. Front Neural Circuits 8:3. CrossRef Medline

Legendre P, Ali DW, Drapeau P (2000) Recovery from open channel block by acetylcholine during neuromuscular transmission in zebrafish. J Neurosci 20:140–148. Medline

Martina M, Kilić G, Cherubini E (1994) The effect of intracellular Ca^{2+} on GABA-activated currents in cerebellar granule cells in culture. J Membr Biol 142:209–216. CrossRef Medline

McCartney MR, Deeb TZ, Henderson TN, Hales TG (2007) Tonically active $GABA_A$ receptors in hippocampal pyramidal neurons exhibit constitutive GABA-independent gating. Mol Pharmacol 71:539–548. CrossRef Medline

Mihalek RM, Banerjee PK, Korpi ER, Quinlan JJ, Firestone LL, Mi ZP, Lagenaur C, Tretter V, Sieghart W, Anagnostaras SG, Sage JR, Fanselow MS, Guidotti A, Spigelman I, Li Z, DeLorey TM, Olsen RW, Homanics GE (1999) Attenuated sensitivity to neuroactive steroids in γ-aminobutyrate type A receptor δ subunit knockout mice. Proc Natl Acad Sci U S A 96:12905–12910. CrossRef Medline

Mortensen M, Smart TG (2006) Extrasynaptic αβ subunit $GABA_A$ receptors on rat hippocampal pyramidal neurons. J Physiol 577:841–856. CrossRef Medline

Ohsawa I, Ishikawa M, Takahashi K, Watanabe M, Nishimaki K, Yamagata K, Katsura K, Katayama Y, Asoh S, Ohta S (2007) Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals. Nat Med 13:688–694. CrossRef Medline

Orser BA, Wang LY, Pennefather PS, MacDonald JF (1994) Propofol modulates activation and desensitization of $GABA_A$ receptors in cultured murine hippocampal neurons. J Neurosci 14:7747–7760. Medline

Pavlov I, Savtchenko LP, Kullmann DM, Semyanov A, Walker MC (2009) Outwardly rectifying tonically active $GABA_A$ receptors in pyramidal cells modulate neuronal offset, not gain. J Neurosci 29:15341–15350. CrossRef Medline

Pellmar TC (1995) Use of brain slices in the study of free-radical actions. J Neurosci Methods 59:93–98. CrossRef Medline

Petrini EM, Nieus T, Ravasenga T, Succol F, Guazzi S, Benfenati F, Barberis A (2011) Influence of $GABA_AR$ monoliganded states on GABAergic responses. J Neurosci 31:1752–1761. CrossRef Medline

Pribiag H, Peng H, Shah WA, Stellwagen D, Carbonetto S (2014) Dystroglycan mediates homeostatic synaptic plasticity at GABAergic synapses. Proc Natl Acad Sci U S A 111:6810–6815. CrossRef Medline

Quirk K, Blurton P, Fletcher S, Leeson P, Tang F, Mellilo D, Ragan CI, McKernan RM (1996) [^{3}H]L-655,708, a novel ligand selective for the benzodiazepine site of $GABA_A$ receptors which contain the α5 subunit. Neuropharmacology 35:1331–1335. CrossRef Medline

Rice ME (2011) H_2O_2: a dynamic neuromodulator. Neuroscientist 17:389–406. CrossRef Medline

Riquelme D, Alvarez A, Leal N, Adasme T, Espinoza I, Valdés JA, Troncoso N, Hartel S, Hidalgo J, Hidalgo C, Carrasco MA (2011) High-frequency field stimulation of primary neurons enhances ryanodine receptor-mediated Ca^{2+} release and generates hydrogen peroxide, which jointly stimulate NF-κB activity. Antioxid Redox Signal 14:1245–1259. CrossRef Medline

Sah R, Schwartz-Bloom RD (1999) Optical imaging reveals elevated intracellular chloride in hippocampal pyramidal neurons after oxidative stress. J Neurosci 19:9209–9217. Medline

Saxena NC, Macdonald RL (1994) Assembly of $GABA_A$ receptor subunits: role of the δ subunit. J Neurosci 14:7077–7086. Medline

Schlussman SD, Buonora M, Brownstein AJ, Zhang Y, Ho A, Kreek MJ (2013) Regional mRNA expression of GABAergic receptor subunits in brains of C57BL/6J and 129P3/J mice: strain and heroin effects. Brain Res 1523:49–58. CrossRef Medline

Schraufstatter IU, Hyslop PA, Hinshaw DB, Spragg RG, Sklar LA, Cochrane CG (1986) Hydrogen peroxide-induced injury of cells and its prevention by inhibitors of poly(ADP-ribose) polymerase. Proc Natl Acad Sci U S A 83:4908–4912. CrossRef Medline

Simon F, Leiva-Salcedo E, Armisén R, Riveros A, Cerda O, Varela D, Eguiguren AL, Olivero P, Stutzin A (2010) Hydrogen peroxide removes TRPM4 current desensitization conferring increased vulnerability to necrotic cell death. J Biol Chem 285:37150–37158. CrossRef Medline

Smart TG, Xie X, Krishek BJ (1994) Modulation of inhibitory and excitatory amino acid receptor ion channels by zinc. Prog Neurobiol 42:393–441. CrossRef Medline

Smith MA, Herson PS, Lee K, Pinnock RD, Ashford ML (2003) Hydrogen-peroxide-induced toxicity of rat striatal neurones involves activation of a non-selective cation channel. J Physiol 547:417–425. CrossRef Medline

Takahashi A, Mikami M, Yang J (2007) Hydrogen peroxide increases GABAergic mIPSC through presynaptic release of calcium from IP3 receptor-sensitive stores in spinal cord substantia gelatinosa neurons. Eur J Neurosci 25:705–716. CrossRef Medline

Tao W, Higgs MH, Spain WJ, Ransom CB (2013) Postsynaptic $GABA_B$ receptors enhance extrasynaptic $GABA_A$ receptor function in dentate gyrus granule cells. J Neurosci 33:3738–3743. CrossRef Medline

Twyman RE, Rogers CJ, Macdonald RL (1989) Differential regulation of γ-aminobutyric acid receptor channels by diazepam and phenobarbital. Ann Neurol 25:213–220. CrossRef Medline

Uttara B, Singh AV, Zamboni P, Mahajan RT (2009) Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. Curr Neuropharmacol 7:65–74. CrossRef Medline

Walters RJ, Hadley SH, Morris KD, Amin J (2000) Benzodiazepines act on $GABA_A$ receptors via two distinct and separable mechanisms. Nat Neurosci 3:1274–1281. CrossRef Medline

Wang DS, Zurek AA, Lecker I, Yu J, Abramian AM, Avramescu S, Davies PA, Moss SJ, Lu WY, Orser BA (2012) Memory deficits induced by inflammation are regulated by α5-subunit-containing $GABA_A$ receptors. Cell Rep 2:488–496. CrossRef Medline

Wlodarczyk AI, Sylantyev S, Herd MB, Kersanté F, Lambert JJ, Rusakov DA, Linthorst AC, Semyanov A, Belelli D, Pavlov I, Walker MC (2013) GABA-independent $GABA_A$ receptor openings maintain tonic currents. J Neurosci 33:3905–3914. CrossRef Medline