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The glial antioxidant network and neuronal ascorbate: protective yet permissive for H₂O₂ signaling

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Abstract

Increasing evidence implicates reactive oxygen species, particularly hydrogen peroxide (H₂O₂), as intracellular and intercellular messengers in the brain. This raises the question of how the antioxidant network in the brain can be sufficiently permissive to allow messages to be conveyed yet, at the same time, provide adequate protection against oxidative damage. Here we present evidence that this is accomplished in part by differential antioxidant compartmentalization between glia and neurons. Based on the rationale that the glia-to-neuron ratio is higher in guinea-pig brain than in rat brain, we examined the neuroprotective role of the glial antioxidant network by comparing the consequences of elevated H₂O₂ in guinea-pig and rat brain slices. The effects of exogenously applied H₂O₂ on evoked population spikes in hippocampal slices and on edema formation in forebrain slices were assessed. In contrast to the epileptiform activity observed in rat hippocampal slices after H₂O₂ exposure, no pathophysiology was seen in guinea-pig hippocampal slices. Similarly, elevated H₂O₂ caused edema in rat brain slices, whereas this did not occur in guinea-pig brain tissue. The resistance of guinea-pig brain tissue to H₂O₂ challenge was lost, however, when glutathione (GSH) synthesis was inhibited (by buthionine sulfoximine), GSH peroxidase activity was inhibited (by mercaptosuccinate), or catalase was inhibited (by 3-amino-1,2,4,-triazole). Strikingly, exogenously applied ascorbate, a predominantly neuronal antioxidant, was able to compensate for loss of any other single component of the antioxidant network. Together, these data imply significant roles for glial antioxidants and neuronal ascorbate in the prevention of pathophysiological consequences of the endogenous neuromodulator, H₂O₂.

INTRODUCTION

Historically, reactive oxygen species (ROS) have been considered to be dangerous byproducts of cellular respiration. Indeed, increased ROS production and oxidative stress contribute in cell death following acute brain injury, including cerebral ischemia-reperfusion (Cao et al., 1988; Hyslop et al., 1995; Chan, 2004; Starkov et al., 2004), as well as in slowly progressing neurodegenerative disorders like Parkinson's disease (Ebadi et al., 1996; Olanow and Tatton, 1999; Zhang et al., 2000). This view of ROS is evolving rapidly, however. Increasing evidence indicates that ROS also act as cellular messengers that modulate processes from short-term ion-channel activation (Seutin et al., 1995; Krippeit-Drews et al., 1999; Avshalumov and Rice, 2003; Avshalumov et al., 2005) to gene transcription and expression (Suzuki et al., 1997; Haddad, 2002; Esposito et al., 2004). Of the ROS generated by mitochondrial respiration (Boveris and Chance, 1973; Peuchen et al., 1997; Liu et al., 2002), hydrogen peroxide (H₂O₂) is a particularly strong candidate as a signaling agent because it is membrane permeable

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(Ramasarma, 1983) and relatively inert, in contrast to other ROS like superoxide ($\bullet\text{O}_2^-$) or the hydroxyl radical ($\bullet\text{OH}$) (Cohen, 1994). Indeed, H_2O_2 has been shown to modulate synaptic transmission (Pellmar, 1987, 1995; Avshalumov and Rice, 2002, 2003; Chen et al., 2001, 2002; Avshalumov et al., 2000, 2003), influence the induction of long-term potentiation (Auerbach and Segal, 1997; Kamsler and Segal, 2003, 2004), and mediate nonsynaptic communication between neurons and glia (Atkins and Sweatt, 1999). Thus, the antioxidant network must be structured to allow functional levels of H_2O_2 and other ROS, while still providing protection, which adds a previously unrecognized dimension to oxidant management.

Oxidative damage by endogenous ROS is prevented by the brain antioxidant network, which includes low molecular weight antioxidants, enzymes and repair systems (Davies, 1988; Yu, 1994; Cohen, 1994; Meister, 1994; Rice, 2000; Dringen et al., 2005). However, antioxidant regulation differs between neurons and glia, with higher levels of glutathione (GSH) and GSH-related enzymes in glia than in neurons (Slivka et al., 1987; Raps et al., 1989; Makar et al., 1994; Desagher et al., 1996; Trépanier et al., 1996; Peuchen et al., 1997; Rice and Russo-Menna, 1998; Dringen and Hirrlinger, 2003) and higher levels of the low molecular weight antioxidant ascorbate in neurons than in glia (Rice and Russo-Menna, 1998). These differences might reflect the need for ROS signaling in neurons, with additional protection from oxidative damage provided by surrounding glia. Consistent with this hypothesis, evidence from cells in culture indicates that glia have a critical role in protecting neurons from oxidative stress (Desagher et al., 1996; Drukarch et al., 1997, 1998; Tanaka et al., 1999; Dringen et al., 1999). Moreover, the ability of glia to protect neurons is abolished by inhibition of GSH synthesis (Drukarch et al., 1997), GSH peroxidase or the major cellular peroxidase, catalase (Desagher et al., 1996; Dringen and Hamprecht, 1997).

OBJECTIVE

Here, we have examined the neuroprotective role of glial antioxidant network by comparing the consequences of oxidative stress caused by elevated H_2O_2 in guinea-pig and rat brain slices. This comparison is based on the rationale that the ratio of glia to neurons is higher in guinea-pig brain than in rat brain because the density of neurons is lower in the guinea-pig brain (Tower and Elliott, 1952) but glial density is relatively constant across species (Friede, 1954; Bass et al., 1971; Tower and Young, 1973; Haug, 1987; Rice and Russo-Menna, 1998). Initial studies showed that the pathophysiological consequences of H_2O_2 exposure seen in rat brain slices, including hyperexcitability (indicated by epileptiform activity in hippocampal slices) (Avshalumov and Rice, 2002), and edema (Brahma et al., 2000), are absent in guinea-pig slices. Therefore, we examined the contributions of GSH synthesis, GSH peroxidase, and catalase to the resistance of guinea-pig brain tissue to challenge with H_2O_2 and whether exogenous ascorbate compensates for the loss of any of these components of the antioxidant network.

METHODS

Brain slice preparation

All animal handling procedures were in accordance with NIH guidelines and were approved by the New York University School of Medicine Animal Care and Use Committee. Brain slices (400 μm thick) were prepared from young, male, adult rats (Long-Evans, 160–210 g; 6–8 weeks) and young, male, adult guinea pigs (Hartley, 150–250 g; 2–4 weeks); it should be noted that guinea-pig brain development at birth is comparable to that of ~3 week-old rats. Animals were anesthetized deeply with 50 mg kg^{-1} pentobarbital (i.p.), decapitated, and the brain removed into oxygenated ice-cold artificial cerebrospinal fluid (ACSF) for ~1 min. The hemispheres were bisected, blocked appropriately for either transverse slices of hippocampus (Avshalumov et al., 2000; Avshalumov and Rice, 2002) or coronal slices of forebrain (Brahma

et al., 2000; MacGregor et al., 2003) and mounted on the stage of a Vibratome (Ted Pella, St. Louis, MO). Slices were cut in ice-cold ACSF containing (in mM): NaCl (124); KCl (3.7); NaHCO₃ (26); CaCl₂ (2.4); MgSO₄ (1.3); KH₂PO₄ (1.3); and glucose (10), equilibrated with 95% O₂/5% CO₂, then transferred to a holding chamber with ACSF at room temperature for at least 1 h before experimentation.

Extracellular recording

Extracellular population spikes (PS) were evoked in hippocampal slices by stimulating the Schaffer collaterals and recorded in the stratum pyramidale of CA1 with a standard glass electrode (backfilled with 1 M NaCl) connected to an Axoprobe 1A amplifier (Molecular Devices, Foster City, CA). All measurements were made in a submersion chamber (Warner Instruments, Hamden, CT); slices were superfused with ACSF at 1.5 mL min⁻¹ at 32°C. A twisted bipolar electrode made from Teflon-insulated platinum-iridium wire was used for stimulation. Pulse duration was 100 µs, with stimulus intensity adjusted to the lowest potential (0.9–1.8 mV) required to evoke a PS of maximal amplitude (Avshalumov et al., 2000). The evoked PS was elicited at 5-s intervals using an external timing circuit (Master-8; A.M.P.I., Jerusalem, Israel) and was monitored continuously on a digital oscilloscope. Data acquisition was controlled by Clampex 7.0 software (Molecular Devices, Foster City, CA), which imported PS records to a PC via a DigiData 1200B D/A board (Molecular Devices) for averaging and analysis.

The amplitude (in mV) of PS records was measured from the mean of the positive peak preceding and the positive peak following the negative PS. Typical PS amplitude was 2 mV, as previously (Avshalumov et al., 2000; Avshalumov and Rice, 2002). Slices in which PS amplitude was <1 mV or was not stable for ≥25 min under control conditions were not tested further. Three PS records were averaged for each condition in each slice; illustrated data represent the average PS from all slices in the data set for a given condition.

Water content analysis

The water content of coronal forebrain slices was determined as described previously (Brahma et al., 2000; MacGregor et al., 2003). Briefly, slices were removed from the incubation medium and blotted between two sections of 400 µm grid nylon mesh to remove excess surface solution, without extracting water from the tissue. The slices were weighed on pre-weighed squares of aluminum foil, placed in a drying oven maintained at 95°C overnight, then re-weighed. All water content data were referenced to tissue dry weight, which is assumed to be constant under conditions of water gain (Cserr et al., 1991). Water content (ϵ) was calculated as $\epsilon = (\text{wet weight} - \text{dry weight})/(\text{dry weight})^{-1}$; data are given as g H₂O (g dw)⁻¹. As previously, water content in forebrain slices was determined after recovery and after 3 h at 35 °C.

Determination of GSH and ascorbate content

To determine tissue GSH and ascorbate contents, fresh tissue samples, hippocampal slices, and samples of cortex from coronal slices were weighed in microcentrifuge vials, frozen immediately on dry ice and stored at -80 °C until analysis (Rice et al., 1994; Brahma et al., 2000). For whole tissue analysis, three male and three female Hartley guinea pigs were anesthetized as described for brain slice preparation. The brains were rapidly removed and dissected over ice, without exposure to ACSF to avoid washout of GSH and ascorbate. The analytical method was reverse-phase HPLC with electrochemical detection using gold-amalgam electrode with a typical applied potential of +0.12 V vs. Ag/AgCl (Rice, 1999; Brahma et al., 2000). On the day of analysis, tissue samples were sonicated in ice-cold, deoxygenated eluent, centrifuged for 2 min, and 10 µL of supernatant injected directly into the HPLC system. All standards and sonicated samples were kept on ice; an argon atmosphere was maintained over the eluent throughout the analysis.

Determination of GSH peroxidase and catalase activity

The activities of GSH peroxidase (EC 1.11.1.9) and catalase (EC 1.11.1.6) were determined in forebrain slices after 30–90 min at 32 °C. Slices were sonicated in reaction buffer, centrifuged and enzyme activity determined using commercially available assays kits according to manufacturer's instructions. Initial studies indicated that similar activities were obtained in either fresh or frozen slices, so that data from both are included in means reported here. GSH peroxidase activity was determined using an OxisResearch GPx-340 kit (Oxis International, Portland, OR), in which activity is indicated by oxidation of NADPH monitored at 340 nm using a spectrophotometer (Model 340 Turner Biosystems; Sunnyvale, CA) to indicate GSH peroxidase. One unit (U) of GSH peroxidase oxidizes 1 μ mole of NADPH per min. Catalase activity was assessed using an OxisResearch Catalase-520 GPx-340 kit (Oxis), with H₂O₂ consumption indicated by formation of a quinoneimine dye detected spectrophotometrically at 520 nm. One U of catalase consumes 1 μ mole of H₂O₂ per min. The activities of GSH peroxidase and catalase are given as U per g tissue wet weight (U g⁻¹).

Experimental design and statistical analysis

In electrophysiological studies, the evoked PS was recorded in hippocampal slices for 30 min to ascertain that the response was stable; PS amplitude under control conditions was taken as 100%, with amplitude under test conditions given as % of control. Studies of H₂O₂-enhanced edema were made using coronal slices of forebrain rather than hippocampal slices because the low initial wet weight of hippocampal slices (typically 7–8 mg) precluded accurate assessment of tissue dry weight, which is ~10% of total tissue weight. For similar reasons of sensitivity, GSH peroxidase and catalase activities were also determined in forebrain slices. All data are given as means $\pm$ SEM; sample number (n) indicates the number of slices. For physiological experiments, 2–3 slices were tested per animal, so that each mean reflects data from 3–5 animals; for edema studies, 2–3 slices per condition were tested for each animal each day, so that each mean represents data from 4–10 animals. For statistical analysis, Student's *t*-test or one-way ANOVA followed by Student-Newman-Keuls *post hoc* analysis was used, as appropriate. The threshold for statistical significance was considered to be $p < 0.05$.

Reagents

ACS grade H₂O₂, sodium ascorbate, buthionine sulfoximine (BSO), mercaptosuccinic acid (mercaptosuccinate, MCS), 3-amino-1, 2,4,-triazole (ATZ) and all ACSF components were obtained from Sigma (St. Louis, MO). Solutions containing these agents were freshly prepared before each experiment. To minimize metal ion contamination, all containers were routinely washed with nitric acid (ACS Plus grade; Fisher Chemical Co., Fair Lawn, NJ), followed by thorough rinsing with deionized water (Rice, 1999).

RESULTS

Differential sensitivity of rat and guinea pig brain slices to H₂O₂ exposure

Previous studies have shown that application of exogenous H₂O₂ (1.0 – 3.0 mM) reversibly inhibits the PS evoked by Schaffer collateral stimulation in hippocampal slices from either rats or guinea pigs (Pellmar, 1995; Avshalumov et al., 2000; Avshalumov and Rice, 2002). In rat slices, recovery of the PS following H₂O₂ washout is accompanied by mild epileptiform activity, indicated by an additional PS after the primary spike (Avshalumov and Rice, 2002). This pathophysiology is a specific consequence of H₂O₂ exposure, since rat hippocampal slices that exhibit a stable, single PS during the first 10–15 min of recording do not develop additional peaks under control conditions for up to 3 h (Avshalumov and Rice, 2002). In the present studies, exposure of rat hippocampal slices to H₂O₂ (1.5 mM for 15 min) caused a significant decrease in PS amplitude to $19 \pm 2\%$ of control ($n = 9$; $p < 0.001$ vs. control); the concentration-

response for PS suppression in rat hippocampal slices is sharp because 1.2 mM H₂O₂ has no effect on PS amplitude, but 2.0 mM has no greater effect than 1.5 mM H₂O₂ (Avshalumov et al., 2001). Recovery of the PS after H₂O₂ washout (to $94 \pm 4\%$; $p > 0.05$ vs. control) was accompanied by an additional peak that was $32 \pm 3\%$ of the amplitude of the PS before H₂O₂ exposure (Fig. 1A). When guinea pig hippocampal slices were exposed to H₂O₂ under identical conditions, the evoked PS was also reversibly depressed to $19 \pm 2\%$ of control ($n = 8$; $p < 0.001$ vs. control) with recovery to $92 \pm 7\%$ of control ($p > 0.05$ vs. control) (Fig. 1A). However, unlike rat slices, only a single PS was seen in guinea-pig hippocampal slices on H₂O₂ washout, indicating the absence of lasting pathophysiology.

In parallel experiments, we assessed the effect of H₂O₂ on the water content of rat and guinea-pig forebrain slices. In rat brain slices, the water content after 1 h recovery was 5.08 ± 0.02 g H₂O (g dw)⁻¹ ($n = 48$). After 3 h incubation in ACSF at 35 °C, water content in control slices increased to 5.70 ± 0.03 g H₂O (g dw)⁻¹ ($n = 43$; $p < 0.001$ vs. recovery), which was similar to the usual water gain in rat brain slices incubated under these conditions (Brahma et al., 2000; MacGregor et al., 2003). Inclusion of H₂O₂ (1.5 mM) during incubation caused a further increase in water content ($n = 22$; $p < 0.001$ vs. control incubation in ACSF alone) (Fig. 1B). A different pattern occurred when the same experimental paradigm was repeated with guinea-pig forebrain slices. Although the water content of guinea-pig slices after recovery was similar to that in rat slices (5.02 ± 0.06 g H₂O (g dw)⁻¹; $n = 9$; $p > 0.05$ vs. rat), the final water content of guinea-pig slices after incubation was significantly lower than in incubated slices from rats (5.43 ± 0.07 g H₂O (g dw)⁻¹; $n = 12$; $p < 0.001$ vs. rat). However, in contrast to rat brain tissue, exposure of guinea-pig forebrain slices to H₂O₂ (1.5 mM) during the 3 h incubation period caused no further increase in water content ($n = 19$; $p > 0.05$ vs. incubation in ACSF alone) (Fig. 1B).

The glia:neuron ratio is reflected in the GSH:ascorbate ratio in rat and guinea pig brain

The primary difference between guinea-pig and rat brain tissue is that guinea-pig brain has a higher glia:neuron ratio (Tower and Elliot, 1952; Friede, 1954; Bass et al., 1971; Tower and Young, 1973; Haug, 1987). Because GSH is found predominantly in glia and ascorbate is predominantly in neurons (Rice and Russo-Menna, 1998), brain tissue from guinea pigs has a higher GSH content and lower ascorbate content than rat (Fig. 2). This results in a GSH:ascorbate ratio in guinea-pig cortex of 1.4 and only 0.7 in rat cortex (Rice and Russo-Menna, 1998). The pattern in rats is strain independent, with similar ratios seen in both Long-Evans (Kume Kick et al., 1996; Rice and Russo-Menna, 1998) and Sprague-Dawley (Rice et al., 1994) rats (Fig. 2A). Interestingly, cortical neuron density is higher in males than females in rats (Reid and Juraska, 1995), as well as in humans (Rabinowicz et al., 2002). In rat cortex, this is reflected in the slightly (~8%), but significantly higher ascorbate content of males vs. females (determined previously for Long-Evans rats; Kume Kick et al., 1996) (Fig. 2A), which demonstrates the ability of ascorbate content to indicate relatively subtle differences in neuron density (*see* also Rice and Russo-Menna, 1998). By contrast, rat cortical GSH content is both strain and gender invariant (Fig. 2A). In guinea-pig cortex, there was also a tendency towards higher ascorbate levels in males, but because the difference was similar to the variability in our measurements, this did not reach significance; consequently, data from male and female guinea pigs were pooled (Fig. 2A).

No data on neuron or glial density data are available for either guinea-pig or rat hippocampus. Therefore, we determined the ascorbate and GSH contents of intact guinea-pig hippocampus for comparison with previously published data from rats (Kume Kick et al., 1996; Rice and Russo-Menna, 1998) (Fig. 2B). Hippocampal ascorbate content in guinea pigs (1.78 ± 0.04 μmol g⁻¹; $n = 18$) were significantly lower than that in rats ($p < 0.001$ guinea pig vs. all rat data shown), whereas GSH content was significantly *higher* in guinea pigs (2.64 ± 0.07 μmol

g^{-1} ; $n = 18$) than in rats ($p < 0.001$ guinea pig vs. all rat data) (Fig. 2B). There is a significant gender difference in ascorbate content, but not GSH content, in rat hippocampus (Kume Kick et al., 1996). However, there were no significant differences in either ascorbate or GSH were seen in guinea-pig hippocampus so that data from males and females were again pooled (Fig. 2B). The lower ascorbate and higher GSH contents of guinea-pig vs. rat hippocampus are consistent with lower neuron density already established for guinea-pig vs. rat cortex (Tower and Young, 1973; Rice and Russo-Menna, 1998). Thus, these data provide strong evidence for a higher glia:neuron ratio in guinea-pig vs. rat hippocampus, as well as cortex, which could contribute to the higher resistance of guinea-pig brain slices to pathological consequences of H_2O_2 challenge.

***In vitro* loss of ascorbate and GSH and effects of GSH synthesis inhibition**

To examine the role of on-going GSH synthesis in preventing pathophysiological consequences of H_2O_2 in guinea-pig brain slices, slices were preincubated for 2 h at room temperature in BSO (5 mM; Pellmar et al., 1992), an irreversible inhibitor of the GSH synthesizing enzyme γ -glutamylcysteine synthetase (Griffith and Meister, 1979). During recovery in ACSF alone, both ascorbate and GSH were readily lost from guinea-pig brain slices, as described previously for rat brain slices (McIlwain et al., 1956; Rice et al., 1994). The average ascorbate content of cortical samples from forebrain slices after recovery was $0.71 \pm 0.03 \mu\text{mol g}^{-1}$ ($n = 12$), representing a ~60% decrease from intact tissue, with a further decrease to $0.63 \pm 0.03 \mu\text{mol g}^{-1}$ ($n = 12$) after 3 h incubation at 35°C ($p < 0.05$ incubation vs. recovery). In these same slices, the GSH content fell 50–55% during recovery, to $1.25 \pm 0.03 \mu\text{mol g}^{-1}$, with maintenance of these levels during incubation for 3 h at 35°C under control conditions ($1.20 \pm 0.03 \mu\text{mol g}^{-1}$; $p < 0.05$ incubation vs. recovery; $n = 12$ for each group) (Fig. 3). Preincubation of forebrain slices with BSO for 2 h at room temperature decreased cortical GSH content by a further 7% from levels in intact tissue, to $1.08 \pm 0.03 \mu\text{mol g}^{-1}$ ($p < 0.001$ vs. recovery; $n = 13$) (Fig. 3), with no change in ascorbate content ($p > 0.05$).

We then evaluated the effect of H_2O_2 exposure on cortical GSH content in control and BSO-treated guinea-pig forebrain slices during prolonged incubation under the same conditions used for edema studies. Importantly, cortical GSH content after 3 h incubation at 35°C with $1.5 \text{ mM H}_2\text{O}_2$ ($n = 13$) did not differ significantly from normal recovery levels ($p < 0.05$) (Fig. 3). GSH levels during incubation in the continued presence of BSO ($n = 13$) were also unaltered from initial levels after recovery in BSO ($p > 0.05$). However, exposure of BSO-treated slices to H_2O_2 ($n = 13$) caused a significant decrease in GSH compared to BSO-treated slices incubated without H_2O_2 ($p < 0.05$ BSO + H_2O_2 vs. BSO incubation), such that the final GSH content in BSO-treated slices after H_2O_2 incubation was 23% less than that in control slices after recovery ($p < 0.001$) (Fig. 3). Together, these data demonstrate the importance of on-going GSH synthesis in the maintenance of GSH content under conditions of oxidative challenge. Interestingly, the ascorbate content of BSO-treated slices was significantly lower after incubation in BSO than in controls, whether H_2O_2 was present or absent ($p < 0.05$): ascorbate fell by 22% from recovery levels during BSO incubation (vs. 11% in controls) and 28% in BSO + H_2O_2 (vs. 20% in controls) (data not shown). These data are consistent with the important role of GSH in maintaining tissue ascorbate levels (Meister, 1994; Rice, 2000).

Effects of GSH synthesis inhibition hippocampal slice physiology

To examine the effect of inhibiting GSH synthesis on hippocampal slice physiology, BSO-treated slices were transferred to the recording chamber where they were continually superfused with BSO in ACSF. Inhibition of GSH synthesis by BSO for 2 h at room temperature ($n = 8$) decreased hippocampal GSH content by an additional 7% from that in intact tissue ($p < 0.05$; $n = 8$), as seen in cortical slices, with no effect on ascorbate content. Tissue contents

of GSH and ascorbate in either control or BSO-treated hippocampal slices were stable for up to 1.5 h in the superfusion chamber at 32 °C, whether exposed to H₂O₂ for 15 min or not.

The amplitude of the evoked PS in GSH-depleted slices did not differ from that in ACSF alone and the suppression induced by H₂O₂ not altered by BSO ($16 \pm 7\%$; $n = 9$; $p < 0.001$ vs. control) (Fig. 4). Recovery was reversible (to $86 \pm 8\%$; $p > 0.05$ vs. control), but was accompanied by pathological epileptiform activity (Fig. 4, arrow), as seen during PS recovery in rat hippocampal slices exposed to H₂O₂ (e.g. Fig. 1A). We have reported previously that the presence of physiological levels of ascorbate (400 μ M; Rice, 2000) during H₂O₂ washout prevents the development of hyperexcitability in rat hippocampal slices (Avshalumov and Rice, 2002). We therefore tested whether ascorbate could compensate for GSH depletion in guinea-pig hippocampal slices, which it did ($n = 8$) (Fig. 4).

Effects of GSH peroxidase and catalase inhibition on hippocampal slice physiology

GSH peroxidase inhibition—The loss of H₂O₂ tolerance in guinea-pig hippocampal slices after GSH synthesis inhibition, despite a relatively small absolute decrease in GSH content, suggested that this might reflect consequences of GSH loss on the activity of GSH dependent enzymes, especially GSH peroxidase. We therefore examined whether a similar enhancement in pathophysiology occurred after GSH peroxidase inhibition by MCS (1 mM; Sokolova et al., 2001; Avshalumov et al., 2003, 2005). In these experiments, PS amplitude was recorded at 5 min intervals to provide a detailed evaluation of the time course of H₂O₂-induced PS suppression and recovery. The activity of GSH peroxidase in guinea-pig forebrain slices after 30–90 min in the recording chamber at 32 °C was 3.9 ± 0.1 U g⁻¹ ($n = 17$); after 30 min superfusion with 1 mM MCS, slice GSH peroxidase activity was not detectable ($n = 6$). Superfusion of MCS for 30 min had no effect on hippocampal PS amplitude (Fig. 5A); however, in the continued presence of MCS, exposure to H₂O₂ caused a larger suppression of the evoked PS (to $7 \pm 3\%$; $n = 7$; $p < 0.001$ vs. control) than in ACSF alone ($p < 0.01$ vs. ACSF) (Fig. 5A). The recovery of PS amplitude during H₂O₂ washout in MCS was accompanied by secondary H₂O₂-induced pathology (Fig. 5A, arrow). Moreover, recovery was delayed compared to recovery in ACSF alone ($p < 0.01$ washout in MCS vs. ACSF; ANOVA) and was incomplete (to $74 \pm 8\%$; $p < 0.05$ vs. control) (Fig. 5B). Inclusion of ascorbate (400 μ M) in the washout solution prevented these pathophysiological consequences of H₂O₂ exposure when GSH peroxidase was inhibited by MCS ($n = 5$) (Fig. 5A,B).

Catalase inhibition—We examined the role of catalase in preventing pathophysiological consequences of H₂O₂ in guinea pig brain tissue using the catalase inhibitor, ATZ (10 mM; Dringen and Hamprecht, 1997; Avshalumov et al., 2005). Basal catalase activity in guinea pig forebrain slices incubated for 30–90 min in the recording chamber at 32 °C was 321 ± 20 U g⁻¹ ($n = 20$); after 30 min of superfusion with ATZ, catalase activity was undetectable ($n = 5$). In slices of guinea-pig hippocampus, catalase inhibition alone did not alter PS amplitude (Fig. 6A); however, in ATZ, H₂O₂-induced PS suppression (to $5 \pm 2\%$ of ATZ control; $n = 7$; $p < 0.001$) was enhanced compared to that seen in ACSF alone ($p < 0.001$ vs. ACSF) (Fig. 6B). As with MCS, PS recovery after H₂O₂ washout in ATZ was accompanied by epileptiform activity (Fig. 6A) and was delayed ($p < 0.01$ ATZ vs. ACSF; ANOVA) and incomplete ($67 \pm 9\%$; $p < 0.05$ vs. control) (Fig. 6B). Ascorbate in the washout solution again prevented these pathological consequences of H₂O₂ exposure ($n = 8$) (Fig. 6A,B).

Effects of GSH depletion and peroxidase inhibition on brain-slice edema

Having found that each of several components of the brain antioxidant network contribute to prevention of H₂O₂-induced pathophysiology in hippocampal slices, we then examined the role of GSH synthesis, GSH peroxidase and catalase in the prevention of brain tissue edema during prolonged exposure to H₂O₂ (Fig. 1B). For these studies, slices were preincubated at

room temperature with a given inhibitor (2 h for 5 mM BSO or 30 min for 1 mM MCS or 10 mM ATZ), before further incubation for 3 h at 35 °C in the continued presence of the inhibitor. Consistent with the lack of effect of these agents on the evoked PS in guinea-pig hippocampal slices, slice water content after incubation with BSO (n = 17), MCS (n = 12), or ATZ (n = 16) did not differ from that in ACSF alone ($p > 0.05$ vs. control for each) (Fig. 7A). However, inclusion of H₂O₂ caused significant water gain in the presence of each of these agents ($p < 0.001$ vs. control for each) (Fig. 7A). Water content after incubation with H₂O₂ was similar in slices incubated with BSO (n = 21) or MCS (n = 11) ($p > 0.05$ BSO vs. MCS), but significantly higher than in guinea-pig slices incubated with H₂O₂ alone ($p < 0.001$ BSO or MCS vs. H₂O₂ alone). However, the final water content in the absence of ongoing GSH synthesis or GSH peroxidase activity was lower than that in rat forebrain slices incubated with H₂O₂ (Fig. 7A) ($p < 0.001$ BSO or MCS vs. rat H₂O₂). When catalase was inhibited in guinea-pig slices, the water content after H₂O₂ incubation (n = 20) was not only greater than in guinea-pig slices with an intact antioxidant network ($p < 0.001$), but also indistinguishable from that seen in rat slices exposed to H₂O₂ ($p > 0.05$) (Fig. 7A). The final water content after H₂O₂ incubation with ATZ exceeded that seen with either BSO or MCS ($p < 0.001$ vs. BSO or MCS) (Fig. 7A). However, when ascorbate (400 mM initial concentration) was included with H₂O₂ in ATZ-treated slices, the H₂O₂-dependent water gain was prevented, such that final water content (5.53 ± 0.05 g H₂O (g dw)⁻¹ (n = 9)), did not differ from that of guinea-pig controls slices incubated either with or without ATZ alone ($p > 0.05$) (data not shown).

We then compared absolute H₂O₂-enhanced gain in water content under each condition. As noted above, incubation of guinea-pig forebrain slices with H₂O₂ alone resulted in a non-significant water gain of 0.12 ± 0.05 g H₂O (g dw)⁻¹ (n = 19), which contrasted markedly with the H₂O₂-enhanced increase in rat slices of 0.57 ± 0.04 g H₂O (g dw)⁻¹; n = 22) ($p < 0.001$ guinea pig vs. rat) (Fig. 7B). The H₂O₂-enhanced water gain when guinea-pig slices were exposed to H₂O₂ after inhibition of GSH synthesis or GSH peroxidase were comparable to that seen in rat brain slices after H₂O₂ exposure alone ($p > 0.05$ BSO or MCS vs. rat H₂O₂) (Fig. 7B). Although the absolute water contents in BSO- or MCS- treated slices were lower than that in rat, the greater H₂O₂-dependent increase reflected the lower control values in guinea-pig slices (see Fig. 1B). The H₂O₂-enhanced water gain after inhibition of catalase by ATZ (n = 20) was greater than that with either BSO or MCS ($p < 0.01$) and exceeded that in rat brain slices ($p < 0.05$) (Fig. 7B), indicating a key role for catalase in preventing pathological consequences of elevated H₂O₂.

CONCLUSIONS

We show that guinea-pig brain tissue is resistant to pathophysiological consequences of H₂O₂, which contrasts markedly to the consequences of comparable H₂O₂ exposure in rat brain. Loss of any single component of the brain antioxidant network examined left guinea-pig brain slices as vulnerable as rat brain tissue to H₂O₂-induced pathology.

- The primary difference between guinea-pig and rat brain tissue is the higher glia:neuron ratio of guinea-pig brain (Tower and Elliot, 1952; Friede, 1954; Bass et al., 1971; Tower and Young, 1973; Haug, 1987; Rice and Russo-Menna, 1998). Thus, these data support previous findings from neuron-glia co-cultures that glia protect neurons from H₂O₂ toxicity (Desahger et al., 1996; Drukarch et al., 1997, 1998; Tanaka et al., 1999; Dringen et al., 1999; Dringen and Hamprecht, 1997).
- Previous studies have shown that ascorbate, a predominantly neuronal antioxidant (Rice and Russo-Menna, 1998; Rice, 2000), compensates for loss of GSH after synthesis inhibition by BSO (Mårtensson and Meister, 1991; Meister, 1994). Here, we show that ascorbate also compensates for loss of other components of the antioxidant network, including GSH peroxidase and catalase.

- These findings indicate the importance of both glial and neuronal antioxidants in providing a permissive, yet protective environment for signaling by H_2O_2 and other ROS.

DISCUSSION

Brain cells, like all cells in the body, have an intricate and inter-dependent network of low molecular weight antioxidants and antioxidant enzymes to provide protection against oxidative damage. ROS are generated by several cellular processes, the most important of which is mitochondrial respiration, which produces superoxide anion, $\bullet O_2^-$, from a single-electron reduction of molecular oxygen (Boveris and Chance, 1973; Peuchen et al., 1997; Liu et al., 2002). Additional sources of ROS include enzymes like NADPH oxidase (Sauer et al., 2001; Serrano et al., 2003), which produce $\bullet O_2^-$ that can participate in signaling cascades. Cellular $\bullet O_2^-$ is managed by mitochondrial and cytosolic forms of superoxide dismutase, which produce H_2O_2 . In turn, H_2O_2 is regulated by GSH peroxidase, which is free in the cytosol, and catalase, which is confined to subcellular peroxisomes (Cohen, 1994; Peuchen et al., 1997; Dringen et al., 2005). Interaction of either $\bullet O_2^-$ or H_2O_2 with trace metal ions, including iron and copper, can produce the aggressive hydroxyl radical, $\bullet OH$, which is neutralized primarily by GSH and ascorbate (Cohen, 1994). Thus, both enzymes and low-molecular weight antioxidants work together to regulate ROS levels and prevent oxidative damage.

The present studies demonstrate the importance of the antioxidant network of glia in providing protection against pathophysiological consequences of H_2O_2 elevation. Such consequences include epileptiform activity and edema, which can only be addressed in the complex microenvironment of brain slices. Although the primary effect of H_2O_2 in hippocampal slices (suppression of the evoked PS) readily occurred in guinea-pig slices, the secondary pathology seen in rat slices when electrical activity returns upon H_2O_2 washout was absent (Fig. 1A). Selective pharmacological deletion of the major components of the brain antioxidant network revealed that GSH, GSH peroxidase and catalase contribute to preventing these secondary effects of H_2O_2 exposure guinea-pig brain tissue, and that ascorbate can compensate for the loss of each of these components.

ROS as signaling agents in the brain

Increasing evidence that H_2O_2 and other ROS modulate key aspects of brain function indicates that oxidant regulation must be more subtle than previously thought. For example, our laboratory discovered that endogenously generated H_2O_2 regulates the activity of dopaminergic neurons in the substantia nigra and modulates dopamine release throughout the nigrostriatal pathway (Avshalumov et al., 2003; Avshalumov and Rice, 2003; Avshalumov et al., 2005). Regulation of the nigrostriatal dopamine system is important because of the central role this pathway plays in the control of movement by the basal ganglia. In dopaminergic neurons, intracellular H_2O_2 modulates neuronal excitability via ATP-sensitive K^+ (K_{ATP}) channels (Avshalumov et al., 2005). By contrast, modulatory H_2O_2 in the striatum is generated downstream from glutamatergic AMPA receptors, which are not present on dopaminergic axons, indicating that H_2O_2 must act as a diffusible messenger generated postsynaptically to modulate presynaptic dopamine release (Avshalumov et al., 2003).

Neuromodulation by H_2O_2 is not limited to the nigrostriatal system. Other studies show that H_2O_2 influences characteristics of LTP in the hippocampus (Auerbach and Segal, 1997; Kamsler and Segal, 2003; 2004), which has implications for memory formation. Diffusible H_2O_2 also plays a role in neuron-glia signaling in the hippocampus, in which neuronal activation leads to H_2O_2 -dependent phosphorylation of myelin basic protein in oligodendrocytes (Atkins and Sweatt, 1999). Thus, H_2O_2 can act as an intracellular signaling agent and as a diffusible messenger in the brain. For H_2O_2 to act at both intracellular and

potentially distant targets requires that the brain antioxidant network is both permissive and protective. We propose that key features of a permissive environment are the predominance of cytosolic GSH peroxidase activity in glia, with sub-compartmentalization of catalase in peroxisomes in both glia and neurons, and the predominance of ascorbate in neurons (Cohen, 1994; Rice and Russo-Menna, 1998; Dringen et al., 2005).

The glial antioxidant network

Considerable evidence, including data presented here, indicates that GSH synthesizing enzymes and GSH peroxidase are predominantly expressed in glia (Slivka et al., 1987; Raps et al., 1989; Maker et al., 1994; Desagher et al., 1996; Trépanier et al., 1996; Peuchen et al., 1997). The higher tolerance of guinea-pig brain than rat brain tissue to H₂O₂ exposure implies that the higher glia:neuron ratio in this species provides additional antioxidant protection from glia. Although GSH synthesis occurs in all cells from its substituent amino acids, glutamate, cysteine, and glycine (Meister, 1994), in the brain, synthetic enzymes for GSH are more abundant in glia than in neurons (Maker et al., 1994). Consistent with this localization, cellular GSH levels in intact brain tissue are ~50% higher in glia (~4 mM) than in neurons (2.5 mM) (Rice and Russo-Menna, 1998). Neurons also synthesize GSH (Chen and Swanson, 2003; Himi et al., 2003), however, and have additional mechanisms, including shuttling of GSH and its precursors from glia to neurons, for maintenance of neuronal GSH content (Sagara et al., 1993; Wang and Cynader, 2000; Dringen and Hirrlinger, 2003).

In the present studies, both GSH and ascorbate were lost readily from guinea-pig brain slices *in vitro*, with tissue contents that were typically 60% lower than those in intact tissue, as described previously for rat brain slices (McIlwain et al., 1956; Rice et al., 1994). Nonetheless, the additional small, but significant, decreases in GSH (and ascorbate) content in both hippocampal and cortical tissue seen after inhibition of GSH synthesis by BSO led to epileptiform activity and enhanced edema formation after H₂O₂ exposure. Even after BSO incubation, however, tissue GSH and ascorbate contents in cortical samples from guinea-pig brain slices remained higher than previously reported values for cortical levels in rat brain slices of $0.65 \pm 0.04 \mu\text{mol g}^{-1}$ for GSH (Rice et al., 1994) and $0.36 \pm 0.06 \mu\text{mol g}^{-1}$ for ascorbate after 3 h at 34 °C (Brahma et al., 2000). This indicates that the enhanced pathophysiology after GSH synthesis inhibition does not depend primarily on the absolute tissue contents of these low molecular weight antioxidants. Rather, the small difference in GSH levels, coupled with the similarity of the H₂O₂-enhanced water gain seen after inhibition of either GSH synthesis or GSH peroxidase (Fig. 7), suggests that the protective effect of endogenous GSH is mediated through its role as a co-factor for GSH peroxidase, as well as by maintenance of ascorbate levels by recycling oxidized ascorbate (Meister, 1994; Rice, 2000).

The relative distribution of catalase in neurons and glia is less defined than of GSH and related enzymes. Although catalase is expressed highly in glial cells (Sokolova et al., 2001), catalase activities in glia and neurons in culture are similar (*see* Dringen et al., 2005 for review). Importantly, however, the confinement of catalase to peroxisomes in both neurons and glia adds a diffusional component to the efficacy of this peroxidase (Dringen et al., 2005), which might facilitate the 'escape' of H₂O₂ signals from a cell of origin. In neuron-glia cocultures, loss of the ability of glia to protect neurons from H₂O₂ toxicity after catalase inhibition (Dringen et al., 1999) reflects loss of neuronal and glial catalase (Dringen et al., 2005). The present findings indicate that catalase also provides key protection from elevated H₂O₂ in the intact neuropil of brain slices. The pathophysiological consequences of H₂O₂ challenge in guinea-pig brain slices after catalase inhibition by ATZ equaled or exceeded that seen in rat brain slices in both models examined. This enhanced pathology presumably reflects contributions from several factors, including inhibition of catalase activity in both neurons and glia, the overall higher activity of catalase than GSH peroxidase in brain tissue in general, and

the lower affinity of catalase than GSH peroxidase for H_2O_2 , which would amplify the role of catalase in removal of the high exogenous concentrations tested here.

Neuronal ascorbate

One other difference between guinea pigs and rats is that guinea pigs, like humans and non-human primates, but unlike rats, cannot synthesize ascorbate. However, synthesis in mammals occurs only in the liver, so that in all species, ascorbate is transported throughout the body via plasma and is taken up at cell-specific levels by the sodium-dependent ascorbate (vitamin C) transporters, SVCT1 and SVCT2 (Rice, 2000); the SVCT2 isoform is expressed in brain (Tsukaguchi et al., 1999). Consequently, cellular regulation of ascorbate is species independent, so that whether an animal can synthesize ascorbate should not influence other components of the antioxidant network. In the brain, ascorbate is compartmentalized between neurons and glia, with much higher concentrations in neurons (~10 mM) than in glia (1 mM) (Rice and Russo-Menna, 1998). This distribution is consistent with the selective expression of SVCT2 by neurons, but not glia (Tsukaguchi et al., 1999; Berger and Hediger, 2000).

Previous studies *in vitro* show that application of exogenous H_2O_2 does not affect the evoked PS in hippocampal slices when ascorbate is present at its normal extracellular concentration; similar protection is provided by deferoxamine, indicating that the ROS required for PS suppression is $\bullet OH$ (Avshalumov et al., 2000). H_2O_2 -dependent PS suppression is also prevented by isoascorbate (D-ascorbate) (Avshalumov et al., 2000), the non-biologically active stereoisomer of L-ascorbate that is not transported by stereoselective SVCT2 (Tsukaguchi et al., 1999). This indicates an extracellular site of antioxidant action. Ascorbate also prevents long-lasting consequences of H_2O_2 exposure. This is indicated by the absence of epileptiform activity in rat hippocampal slices when H_2O_2 is washed out with ascorbate-containing media (Avshalumov and Rice, 2002) or in guinea-pig slices when the antioxidant network is compromised, as shown here. This effect of ascorbate must occur intracellularly in neurons, however, since non-transported isoascorbate is not protective under these conditions (Avshalumov and Rice, 2002).

In contrast to the efficacy of ascorbate in preventing pathological consequences of H_2O_2 exposure, this antioxidant has no effect on the modulation of striatal dopamine release by *endogenous* H_2O_2 (Avshalumov et al., 2003). This indicates that ascorbate permits H_2O_2 -dependent signaling in striatum, which is mediated by K_{ATP} -channel activation (Avshalumov and Rice, 2003) as it in dopaminergic neurons (Avshalumov et al., 2005), and that inhibition of dopamine release is a direct action of H_2O_2 , rather than $\bullet OH$. Thus, the present findings, coupled with these previous results suggest that ascorbate is ideally suited as a key antioxidant in neurons because of its ability to permit H_2O_2 signaling, yet prevent pathological consequences that could occur from unregulated H_2O_2 generation and $\bullet OH$ production.

Implications

Here we present evidence that glia provide protection against H_2O_2 toxicity. Previous studies in neuron-glia cocultures shown that that the glial antioxidant network can protect neurons from H_2O_2 toxicity; the present findings suggest that this is also true in acute brain slices, in which neuronal and glial integrity are maintained. Importantly, we have reported previously that regulation of dopamine release by endogenously generated H_2O_2 is similar in guinea-pig and rat striatum (Avshalumov et al., 2003), which demonstrates that H_2O_2 signaling is not impaired in the presence of the stronger antioxidant network afforded by the higher glia:neuron ratio of guinea-pig brain. Together, these findings support our hypothesis that neuron-glia compartmentalization of antioxidants is critical for neuronal signaling as well as neuronal protection. Indeed, the higher glia:neuron ratio in guinea-pig than rat brain might better manage discrete signaling by diffusible messengers like H_2O_2 because ensheathing glia limit the

effective radius of a locally broadcast ROS signal. Overall, the present findings provide encouraging news for humans, since the neuron density is lower (Tower and Elliott, 1952), and, thus, the glia:neuron ratio higher (Friede, 1954; Bass et al., 1971; Tower and Young 1973; Haug, 1987), than in either rodent species examined here.

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Fig. 1. Greater tolerance of guinea-pig than rat brain tissue to H_2O_2 elevation

A) Electrophysiological recordings of the extracellular PS evoked by stimulation of the Schaffer collaterals in CA1 before H_2O_2 exposure (Control), after 15 min superfusion with H_2O_2 (1.5 mM), and after 30 min washout of H_2O_2 (Wash) in rat and guinea hippocampal slices. Recovery of the primary PS was accompanied by mild epileptiform activity, indicated by an additional PS after washout (arrow; $n = 9$) in rat, but not guinea pig ($n = 8$). Shown are the averaged PS responses for each species under each condition. **B)** Tissue water content in rat and guinea-pig forebrain slices incubated for 3 h at 35 °C in ACSF alone (Control) and with H_2O_2 (1.5 mM). The water content of rat brain slices was significantly higher after H_2O_2 challenge than in control slices incubated in ACSF alone (*** $p < 0.001$; $n = 22-44$). Although guinea-pig brain slices also gained water during incubation ($n = 12$), final water content was

significantly lower than that seen in rat brain slices incubated under identical conditions ($p < 0.001$ guinea pig *vs.* rat incubation in ACSF). Moreover, no further water gain occurred in guinea-pig slices incubated with H_2O_2 ($n = 19$) ($p > 0.05$), so the final water content remained below that in rat control slices (indicated by dashed line; $p < 0.01$ *vs.* rat control).

Figure 2. Ascorbate (Asc) and GSH contents in intact rat and guinea-pig cortex and hippocampus
A) The GSH:Asc ratio in rat cortex is strain independent (Long-Evans, L-E, from Kume Kick et al., 1996; Sprague-Dawley, S-D, from Rice et al., 1994); Asc content is also gender dependent (+++ $p < 0.001$ female vs. male L-E; Kume Kick et al., 1996) ($n = 42-43$ for each). GSH levels are higher and ascorbate levels lower in guinea-pig cortex ($n = 15$) than in the cortex of any rat group examined ($***p < 0.001$ vs. any rat group; data from male and female guinea pigs are pooled (from Rice and Russo-Menna, 1998)). **B)** GSH and ascorbate contents in rat and guinea-pig hippocampus. In rat hippocampus, GSH:Asc ratio is strain independent (Kume Kick et al., 1996; Rice et al., 1994), with lower Asc content in female than male L-E rats (+++ $p < 0.001$ female vs. male; Kume Kick et al., 1996) (rat data $n = 35-41$). In guinea-pig hippocampus ($n = 18$), GSH content was higher and ascorbate content lower than in the hippocampus of the rats examined ($***p < 0.001$; data from male and female guinea pigs are pooled).

Fig. 3. Effect of GSH synthesis inhibition on cortical GSH content in guinea-pig brain slices
 Normalized data are given as percent of GSH content after recovery in ACSF alone (% rec; left y-axis), with absolute tissue content indicated (right y-axis). To achieve and maintain GSH synthesis inhibition, BSO (5 mM) was present throughout recovery (2 hours at room temperature) and incubation (3 h at 35 °C). GSH content was unaltered by prolonged incubation whether in ACSF alone or in the presence of H₂O₂ (+H₂O₂) ($p > 0.05$ versus recovery) ($n = 13$ per group). However, GSH levels were significantly lower in BSO-treated than control slices for each condition tested (**, $P < 0.01$; ***, $P < 0.001$; $n = 12-13$). Exposure to H₂O₂ caused a significant decrease in GSH content in BSO-treated slices, but not in control slices (+ $p < 0.05$, BSO incubated vs. BSO + H₂O₂; $p > 0.05$ control incubated vs. control H₂O₂). Ascorbate content in cortical samples from guinea-pig brain slices after recovery was not altered by BSO; however, ascorbate loss during incubation with and without H₂O₂ was enhanced in BSO-treated slice (see text).

Figure 4. H_2O_2 -induced pathophysiology in guinea-pig hippocampal slices after GSH-synthesis inhibition by BSO

Evoked PS in guinea-pig hippocampal slices under control conditions, after 15 min exposure to H_2O_2 (1.5 mM), and after 30 min H_2O_2 washout. GSH synthesis was inhibited by BSO (5 mM), which was present throughout the experiment. Neither the initial evoked PS nor H_2O_2 -induced PS suppression were altered by BSO, however, epileptiform activity was seen after H_2O_2 washout ($n = 9$) (**arrow**). Inclusion of ascorbate (400 μ M; +Asc) during H_2O_2 washout prevented this secondary pathophysiology (**lower panel**) ($n = 8$). Shown are the averaged PS responses for each condition; $n = 8-9$.

Figure 5. H_2O_2 -induced pathophysiology in guinea-pig hippocampal slices after GSH-peroxidase inhibition

A) Evoked PS in guinea-pig hippocampal slices under control conditions, after 15 min exposure to H_2O_2 (1.5 mM), and after 30 min H_2O_2 washout in ACSF alone (ACSF), in the presence of MCS (1 mM) to inhibit GSH peroxidase, and in MCS when H_2O_2 was washed out with ACSF plus ascorbate (400 μ M; +Asc). Superfusion of MCS for 30 min had no effect on hippocampal PS amplitude. However, in the continued presence of MCS, exposure to H_2O_2 caused a larger suppression of the evoked PS than in slices with an intact antioxidant network exposed to H_2O_2 in ACSF alone ($p < 0.01$ MCS vs. ACSF; $n = 7$). Recovery of the PS was accompanied by epileptiform activity (arrow); the presence of ascorbate during H_2O_2 washout prevented this secondary pathophysiology (lower panel) ($n = 5$). Shown are the averaged responses for each condition ($n = 5-8$). **B)** Time course of PS amplitude changes during H_2O_2 exposure and washout in guinea-pig hippocampal slices with and without MCS, and with ascorbate present during H_2O_2 washout. PS suppression during H_2O_2 application was more pronounced after

GSH peroxidase inhibition by MCS, and recovery was also delayed compared with companion slices exposed to H_2O_2 in ACSF alone ($p < 0.01$ washout in MCS *vs.* ACSF; ANOVA). When ascorbate was present during H_2O_2 washout in GSH-peroxidase inhibited slices, the time course of recovery was indistinguishable from that in ACSF alone ($n = 5$) ($p > 0.05$ washout in MCS + Asc *vs.* ACSF; ANOVA).

Figure 6. H₂O₂-induced pathophysiology in guinea-pig hippocampal slices after catalase inhibition
A) Evoked PS in guinea-pig hippocampal slices under control conditions, after 15 min exposure to H₂O₂ (1.5 mM), and after 30 min H₂O₂ washout in ACSF alone (ACSF), in the presence of ATZ (10 mM), in the presence of ATZ to inhibit catalase, and in ATZ when H₂O₂ was washed out with ACSF plus ascorbate (400 μM; +Asc). Superfusion of ATZ (10 mM) for 30 min had no effect on hippocampal PS amplitude; however, in the continued presence of ATZ, exposure to H₂O₂ caused a larger suppression of the evoked PS than during H₂O₂ exposure in slices with an intact antioxidant network exposed to H₂O₂ in ACSF alone ($p < 0.001$ ATZ vs. ACSF; $n = 7$). Recovery of the PS was accompanied by epileptiform activity (arrow); the presence of ascorbate during H₂O₂ washout prevented this secondary pathophysiology (lower panel) ($n = 8$). Shown are the averaged PS responses for each condition ($n = 7-8$). **B)** Time course of PS-amplitude changes during H₂O₂ exposure and washout in guinea-pig hippocampal slices with and without ATZ, and with ascorbate present during H₂O₂ washout. PS suppression during H₂O₂ application was more pronounced after catalase inhibition by ATZ. Moreover, recovery was less complete ($p < 0.05$ ATZ vs. ACSF) and delayed compared to that seen during H₂O₂

washout in companion slices exposed to H_2O_2 in ACSF alone ($p < 0.01$ washout in ATZ *vs.* ACSF; ANOVA). The presence of ascorbate during H_2O_2 washout in catalase-inhibited slices returned the time course of recovery to that seen in control slices ($n = 8$) ($p > 0.05$ washout in ATZ + Asc *vs.* ACSF; ANOVA).

Figure 7. Roles of GSH, GSH peroxidase, and catalase in prevention of H₂O₂-enhanced edema in guinea-pig slices

A) Water content of guinea-pig forebrain slices after incubation for 3 h at 35 °C with or without H₂O₂ (1.5 mM) in ACSF alone (Control) and after inhibition of GSH synthesis (BSO, 5 mM), GSH peroxidase (MCS, 1 mM), or catalase (ATZ, 10 mM). Water content after incubation with BSO, MCS, or ATZ did not differ from those in ACSF alone ($p > 0.05$). Incubation with H₂O₂ did not increase water content in ACSF alone; however, edema was enhanced significantly by H₂O₂ in slices treated with either BSO, MCS, or ATZ ($***p < 0.001$ for H₂O₂ + inhibitor vs. inhibitor alone); the final water contents of antioxidant-compromised slices after H₂O₂ challenge were also significantly greater than in H₂O₂-exposed control slices

(+++ $p < 0.001$) ($n = 12-21$). Dotted line indicates the H_2O_2 -enhanced water content of rat brain slices (*see* Fig. 1B). **B**) Absolute water gain after H_2O_2 incubation in rat brain slices compared to that in guinea-pig slices with and without antioxidant inhibition. The H_2O_2 -enhanced water gain in control guinea-pig was significantly less than in rat or in guinea-pig after inhibition of GSH synthesis (+BSO), GSH peroxidase (+MCS), or catalase (+ATZ) (*** $p < 0.001$ vs. guinea-pig H_2O_2). In guinea-pig brain slices, the H_2O_2 -enhanced water gain in the presence of BSO or MCS did not differ from the H_2O_2 -enhanced water gain in rat slices. However, after catalase inhibition in guinea-pig slices, the H_2O_2 -enhanced water gain was greater than that in rat brain slices (++) $p < 0.01$, (+++ $p < 0.001$ vs. rat H_2O_2 alone).