Controlled enzymatic production of astrocytic hydrogen peroxide protects neurons from oxidative stress via an Nrf2-independent pathway

Renée E. Haskew-Layton[a,1], Jimmy B. Payappilly[a], Natalya A. Smirnova[a], Thong C. Ma[a], Kelvin K. Chan[a], Timothy H. Murphy[b], Hengchang Guo[a], Brett Langley[a], Rukhsana Sultana[c], D. Allan Butterfield[c], Sandro Santagata[d,e], Melissa J. Alldred[f], Irina G. Gazaryan[a], George W. Bell[d], Stephen D. Ginsberg[f], and Rajiv R. Ratan[a,1]

[a]The Burke Medical Research Institute, Department of Neurology and Neuroscience, Weill Medical College of Cornell University, White Plains, NY 10605; [b]Kinsmen Laboratory of Neurological Research, Department of Psychiatry, University of British Columbia, Vancouver, BC, Canada V6T 1Z3; [c]Department of Chemistry, Sanders-Brown Center on Aging, University of Kentucky, Lexington, KY 40536; [d]The Whitehead Institute for Biomedical Research, Cambridge, MA 02142; [e]Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115; and [f]Center for Dementia Research, Nathan Kline Institute and the New York University Langone Medical Center, Orangeburg, NY 10962

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Neurons rely on their metabolic coupling with astrocytes to combat oxidative stress. The transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) appears important for astrocyte-dependent neuroprotection from oxidative insults. Indeed, Nrf2 activators are effective in stroke, Parkinson disease, and Huntington disease models. However, key endogenous signals that initiate adaptive neuroprotective cascades in astrocytes, including activation of Nrf2-mediated gene expression, remain unclear. Hydrogen peroxide (H_2O_2) plays an important role in cell signaling and is an attractive candidate mediator of adaptive responses in astrocytes. Here we determine (*i*) the significance of H_2O_2 in promoting astrocyte-dependent neuroprotection from oxidative stress, and (*ii*) the relevance of H_2O_2 in inducing astrocytic Nrf2 activation. To control the duration and level of cytoplasmic H_2O_2 production in astrocytes cocultured with neurons, we heterologously expressed the H_2O_2-producing enzyme *Rhodotorula gracilis* D-amino acid oxidase (rgDAAO) selectively in astrocytes. Exposure of rgDAAO-astrocytes to D-alanine lead to the concentration-dependent generation of H_2O_2. Seven hours of low-level H_2O_2 production (~3.7 nmol·min·mg protein) in astrocytes protected neurons from oxidative stress, but higher levels (~130 nmol·min·mg protein) were neurotoxic. Neuroprotection occurred without direct neuronal exposure to astrocyte-derived H_2O_2, suggesting a mechanism specific to astrocytic intracellular signaling. Nrf2 activation mimicked the effect of astrocytic H_2O_2 yet H_2O_2-induced protection was independent of Nrf2. Astrocytic protein tyrosine phosphatase inhibition also protected neurons from oxidative death, representing a plausible mechanism for H_2O_2-induced neuroprotection. These findings demonstrate the utility of rgDAAO for spatially and temporally controlling intracellular H_2O_2 concentrations to uncover unique astrocyte-dependent neuroprotective mechanisms.

Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), are products of cellular respiration and enzymatic activity (1). H_2O_2 acts as a second messenger molecule by inhibiting protein tyrosine phosphatases (PTPs), activating kinases (i.e., MAP kinases), and by inducing transcription factor activation (i.e., NFκB, FOXO, and p53) (2–6). Not surprisingly, H_2O_2 plays an integral role in diverse biological processes, such as chemotaxis, apoptosis, axon repulsion, and neurotransmitter regulation (3, 6–9). However, ROS accumulation beyond a homeostatic set point can lead to the net oxidation of cellular constituents leading to cell dysfunction or death, termed oxidative stress. The CNS is particularly susceptible to oxidative stress and thus oxidative damage is a characteristic of almost all acute and neurodegenerative disorders, including Alzheimer's disease, Parkinson disease, stroke, and traumatic brain and spinal cord injury (10). However, because neurons have limited antioxidant capacity, they rely heavily on their metabolic coupling with astrocytes to combat oxidative stress (11).

In the CNS the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) plays an integral role in astrocyte-mediated protection of neurons from oxidative stress. Nrf2 facilitates the transcription of antioxidant genes containing the antioxidant response element (ARE) in their promoters (12). Examples of ARE-containing genes include heme oxygenase 1 (*HO-1*), NADPH-quinone oxidoreductase (*NOQ1*), and those involved in glutathione (GSH) biosynthesis, use, and export (i.e., GSH synthase, glutathione *S*-transferase, and multidrug resistance protein 1) (11). Indeed, Nrf2-mediated GSH biosynthesis and release from astrocytes protects neurons from oxidative stress (11), and Nrf2 overexpression specifically in astrocytes leads to neuroprotection in in vivo models, such as Parkinson disease and amyotrophic lateral sclerosis (ALS) (13, 14).

Thus, the established role that astrocytic-Nrf2 plays in ameliorating CNS disease models has prompted intense interest in identifying endogenous signals that trigger astrocytic Nrf2 activation. Electrophilic agents have been extensively characterized as Nrf2 activators via their ability to disrupt Keap1 (Kelch-like ECH-associated protein 1)-facilitated Nrf2 proteasomal degradation by targeting critical Keap1 cysteine residues (15). However, whether ROS, such as H_2O_2, also represent key agents capable of stabilizing Nrf2 remains a matter of controversy. Several lines of evidence show that exogenous H_2O_2 or enzymatic models of extracellular H_2O_2 production promote Nrf2 activation; yet other studies have found either no effect or a limited effect of H_2O_2 on Nrf2 activation, requiring high concentrations to promote Nrf2 activation (16–19).

In the present study we have developed a system that allows for the precise spatial, temporal, and quantitative control of astrocytic H_2O_2 in an astrocyte-neuron coculture model. Our results show that low-level astrocyte-derived H_2O_2 confers neuroprotection from GSH depletion-induced oxidative stress independent of Nrf2 activation.

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[1]To whom correspondence may be addressed. E-mail: rrr2001@med.cornell.edu or rhaskew@burke.org.

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Results

Temporal and Quantitative Regulation of Astrocytic H_2O_2 Production via *Rhodotorula gracilis* D-Amino Acid Oxidase. D-amino acid oxidase (DAAO) is a peroxisomal flavoenzyme that oxidatively deaminates D-amino acids into their corresponding imino acids, producing H_2O_2 as a byproduct (20, 21) (Fig. 1*A*). In contrast to other enzymatic models of H_2O_2 production, such as glucose oxidase, monoamine oxidase, or superoxide dismutase, DAAO permits precise H_2O_2 production via the manipulation of substrate (D-amino acids) availability, which are generally scarce in mammalian cells. We constructed adenoviruses containing flag-tagged cDNA for *Rhodotorula gracilis* (red yeast) DAAO (rgDAAO) lacking its peroxisomal targeting sequence, which directs rgDAAO expression to the cytoplasm and thus circumvents the avid scavenging of H_2O_2 in peroxisomes by catalase (21). We selected rgDAAO as a H_2O_2 source because it has higher catalytic activity and is less prone to auto-oxidation–induced inactivation than mammalian DAAO (20).

Cytoplasmic rgDAAO staining (via its flag epitope) was observed in ~80% of primary rat astrocytes transduced with rgDAAO adenoviruses (adDAAO), but was not detectable in empty vector transduced (adEV) astrocytes (Fig. 1*B*). As expected, the addition of the rgDAAO substrate D-alanine (2 mM, D-Ala) to the bathing media stimulated H_2O_2 production in adDAAO astrocytes (Fig. 1*C*). Because D-Ala alone was not sufficient to maintain continuous H_2O_2 production beyond 10 min, the bathing media was further supplemented with the DAAO cofactor flavin adenine dinucleotide (FAD) at a range from 0.08 μM to 5 μM (Fig. 1*C*). It was found that 2.5 μM FAD was required for sustained H_2O_2 production in the presence of D-Ala (Fig. 1*C*), and thus for subsequent experiments D-Ala treatments were supplemented with 2.5 μM FAD. In the absence of D-Ala, rgDAAO expression did not evoke H_2O_2 production but adding 0.016 to 4 mM D-Ala to the media led to a concentration-dependent increase in the rate of H_2O_2 production (Fig. 1*D*). We did not detect any increases in H_2O_2 production from control (nontransduced) or adEV-astrocytes with the addition of FAD or any of the D-Ala concentrations tested (Fig. 1*D*). Thus, H_2O_2 production in cells expressing rgDAAO can be manipulated in a concentration dependent manner by the addition of varying concentrations of D-Ala.

GSH Depletion-Induced Oxidative Stress in Astrocyte-Neuron Cocultures. Glutamate or its analogs (i.e., homocysteic acid, HCA) induce the neurotoxicity of immature neurons independent of NMDA receptor-mediated excitotoxicity (Fig. S1 *A–C*) (22). Rather, glutamate inhibits cystine transport and thus leads to the subsequent loss of intracellular cysteine and depletion of the antioxidant GSH, culminating in oxidative stress (22). We sought to define experimental conditions in which the oxidative glutamate toxicity of neurons could be studied using a physiologically relevant ratio of astrocytes to neurons. Thus, primary rat neurons (1 day in vitro, DIV) were plated on top of a confluent monolayer of astrocytes (14–21 DIV) with 5 mM HCA in the bathing medium. Astrocyte-neuron cocultures exposed to 1.25 through 20 mM HCA for 24 h (time when no cell death is observed) showed a concentration-dependent decrease in intracellular GSH levels (Fig. 2*A*). As expected, exposure to ≥5 mM HCA for 48 h resulted in significant neuronal death (Fig. 2*B* and Fig. S1*D*) but no astrocytic death (Fig. 2*B* and Fig. S1*E*). To verify a specific effect of GSH depletion in mediating the neuronal death, the bathing media was supplemented with cystine (to overcome competitive inhibition of cystine transport by HCA) and *N*-acetylcysteine (NAC, an antioxidant that supports GSH synthesis by delivering cysteine independent of the X_c^- transporter), both of which partially blocked HCA-induced GSH depletion (Fig. S1*F*). To verify that the neurons ultimately die because of oxidative stress, vitamin E [a lipophilic antioxidant incapable of preventing GSH-depletion (Fig. S1*F*)] was added to the cocultures. All of these conditions abrogated HCA-induced neuronal death (Fig. 2*C*). These studies establish astrocyte-neuron coculture conditions under which neurons can be selectively killed by HCA in the presence of confluent astrocytes.

Astrocytic H_2O_2 Production Evokes Resistant of Neurons to Oxidative Stress. We next sought to identify kinetic parameters of H_2O_2 production in astrocytes capable of protecting neurons from oxidative stress. To determine the amplitude and duration of astrocytic H_2O_2 production necessary to modulate oxidative

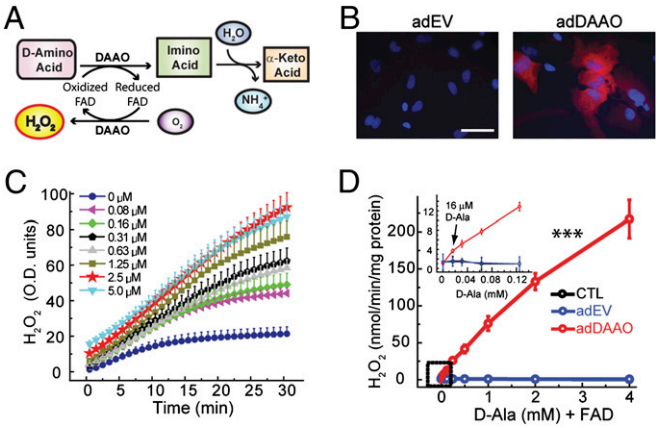

Fig. 1. Heterologous expression of rgDAAO as a tool for regulating intracellular H_2O_2 production in astrocytes. (*A*) Schematic representation of the oxidative deamination of D-amino acids into imino acids by DAAO. As O_2 is used to recycle reduced FAD back to oxidized FAD, H_2O_2 is produced as a byproduct. Ammonia (NH_4^+) is also produced as a byproduct following the conversion of imino acids to α-keto acids. (*B*) Astrocytes were transduced with adEV or adDAAO for 4 d. Nuclear DAPI staining is blue; anti-flag (rgDAAO) is red. (Scale bar, 50 μm.) Images are representative of four independent experiments. (*C*) AdDAAO transduced astrocytes were treated with 2 mM D-Ala ± FAD and, ~30 s later, extracellular H_2O_2 levels were measured. Values represent optical density units averaged from five experiments ± SEM. (*D*) Control (nontransduced), adEV, or adDAAO astrocytes were treated with D-Ala + 2.5 μM FAD and ~30 s later continuous H_2O_2 production was measured for 1 h. The control values (black symbols) are obscured by the adEV values (blue symbols). Data are the means of the rate of H_2O_2 production for five experiments ± SEM. ***, $P \leq 0.001$, adDAAO vs. CT and adEV. (*Inset*) A reduced scale of data within the hatched box.

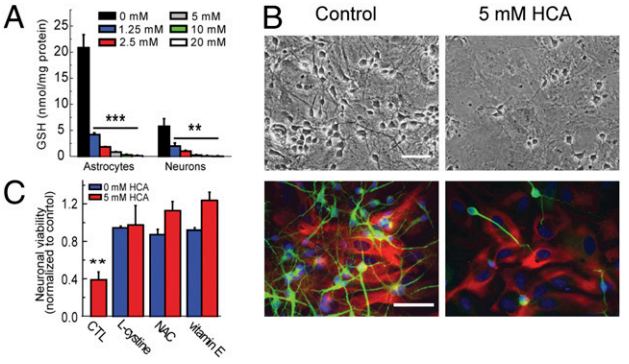

Fig. 2. Glutathione depletion in an astrocyte-neuron coculture model induces neuronal death via oxidative stress. (*A*) Astrocyte-neuron cocultures were treated ± HCA for 24 h. ***$P \leq 0.001$, HCA concentrations vs. control (astrocytes). **$P \leq 0.01$ HCA concentrations vs. control (neurons). (*B*) Astrocyte-neuron cocultures were treated ± HCA for 48 h. Representative phase-contrast images of live cells from four independent experiments show neurons plated ± HCA on astrocytes (*Upper*). Representative images from four experiments show the astrocyte specific marker GFAP (red), the neuron specific marker MAP2 (green), and the nuclear marker DAPI (blue). (Scale bars, 50 μm.) (*C*) Neurons were plated ± HCA coapplied with 300 μM L-cystine, 100 μM NAC, or 100 μM vitamin E (α-tocopherol) on astrocytes for 48 h. **, $P \leq 0.01$, HCA alone vs. all other groups. Values are the averages of four to five experiments ± SEM for all data within the figure.

neuronal death, we cocultured neurons with adDAAO astrocytes and bathed the cocultures with D-Ala (Fig. 3*A*). Low-level H_2O_2 production induced by 0.016 mM D-Ala (~3.7 nmol·min·mg protein) (Fig. 1*D*) over 7 h protected the neurons from HCA (Fig. 3*B*). Although low-level H_2O_2 production in adDAAO astrocytes did not affect neuronal viability in the absence of HCA, higher levels of H_2O_2 produced in astrocytes (~133 nmol·min·mg protein) killed neighboring neurons, even without HCA present (Fig. 3*B*). D-Ala treatment in control or adEV astrocytes had no effect on neuronal viability (Fig. 3*C*), and all of the conditions tested did not alter neuronal adherence to the astrocytes (Fig. S2*A*).

We next used 10 U/mL of the H_2O_2 scavenging-enzyme catalase (CAT), added to the bathing media, to abrogate extracellular adDAAO-generated H_2O_2 released from the astrocytes (Fig. 3*D*). Indeed, the extracellular catalase prevented neuronal death caused by 2 mM D-Ala–induced H_2O_2 production in the absence of HCA (Fig. 3*E*). However, the protective effect of low-level astrocytic H_2O_2 production (0.016 mM D-Ala) was not altered by scavenging extracellular H_2O_2 with catalase in HCA-treated cultures (Fig. 3*E*). These data suggest that high-level H_2O_2 production from astrocytes diffuses out of the cells to exert a neurotoxic effect. In contrast, the neuroprotective effect of low-level astrocytic H_2O_2 production is likely astrocyte-specific. Of note, forced expression of rgDAAO in astrocytes was sufficient to protect mature neurons (14 DIV) from NMDA-induced toxicity independent of D-Ala exposure (Fig. S2 *B* and *C*). Such an effect may be the result of long-term endogenous D-serine metabolism by rgDAAO, yielding increased H_2O_2 generation in astrocytes or diminished D-serine (an NMDA coagonist) levels in synaptically active neurons.

To evaluate if DAAO/D-Ala-generated H_2O_2 is working via an intracellular astrocyte-specific mechanism to protect immature neurons, we generated H_2O_2 for 4, 7, or 24 h in adDAAO astrocytes in the absence of neurons (Fig. 4*A*). We removed the D-Ala from the medium, washed to remove residual H_2O_2 production (Fig. S3*A*), and added neurons (plus HCA to induce oxidative stress) to the H_2O_2 "primed" astrocytes. Despite never having "seen" H_2O_2, the neurons plated with adDAAO astrocytes (primed for 7 h with 0.016 mM D-Ala to induce low-level H_2O_2 production) were resistant to GSH depletion-induced death (Fig. 4 *B* and *C*). The neuroprotective effect was diminished at 24 h and completely absent at 4 h preconditioning (Fig. 4*C*). There was also a lack of neuroprotection at all time points by high H_2O_2 levels (Fig. 4*C*) (0.25 and 2 mM D-Ala) that was not a result of astrocyte toxicity (Fig. S3*B*). Indeed, D-Ala treatment in control or adEV astrocytes had no effect on neuronal viability (Fig. 4*D*) and all of the conditions tested did not alter neuronal adherence to the astrocytes (Fig. S3*C*). These studies establish the amplitude and duration-dependence required for astrocytic H_2O_2's effect on neuronal protection, suggesting that a low level of sustained H_2O_2

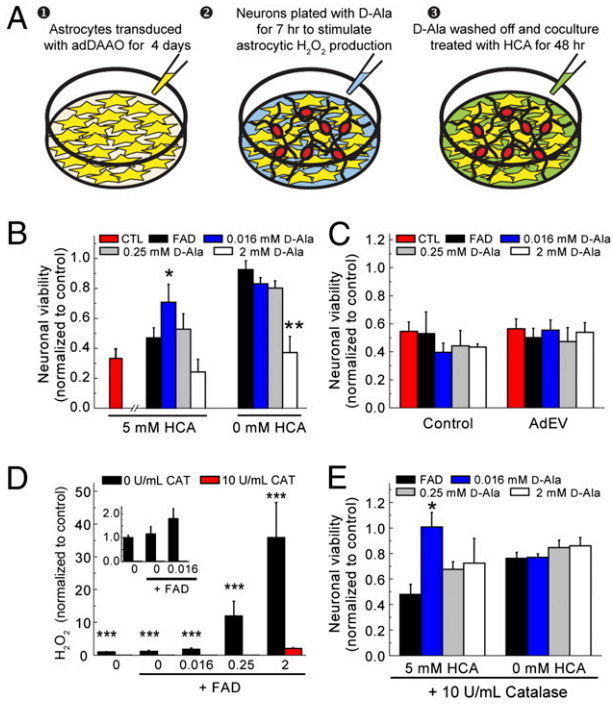

Fig. 3. DAAO-induced astrocytic H_2O_2 production confers resistance of adjacent neurons from oxidative stress. (*A*) Schematic representation of experimental design. (*B*) Neurons were plated ± D-Ala + 2.5 μM FAD on adDAAO astrocytes for 7 h followed by wash-off and 48 h HCA treatment. *, $P \leq 0.05$, 0.016 mM D-Ala vs. HCA control; **, $P \leq 0.01$, 2 mM D-Ala vs. all other groups within 0 mM HCA group. (*C*) Neurons were plated ± D-Ala + 2.5 μM FAD on control or adEV astrocytes for 7 h followed by 48-h HCA treatment. (*D*) AdDAAO astrocytes were treated with ± D-Ala + 2.5 μM FAD in the presence or absence of extracellular catalase (CAT) for 30 min. The media was removed after 30 min and extracellular H_2O_2 measured. (*Inset*) Reduced scale, for clarity, of low level H_2O_2 production. ***, $P < 0.001$, 0 U/mL CAT vs. 10 U/mL CAT. (*E*) Neurons were plated with D-Ala + 2.5 μM FAD + extracellular CAT on adDAAO astrocytes for 7 h followed by wash-off and 48 h ± HCA treatment. *, $P \leq 0.05$, 0.016 mM D-Ala vs. FAD + HCA control. Values are the averages of four to five experiments ± SEM for all data within figure.

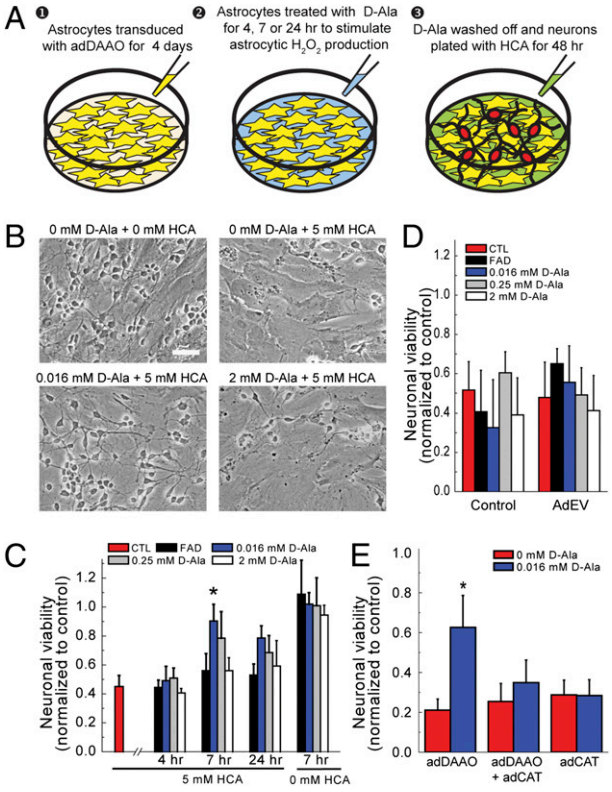

Fig. 4. Low-level H_2O_2 confers neuroprotection via an astrocyte-specific mechanism. (*A*) Schematic representation of experimental design. (*B*) AdDAAO astrocytes were pretreated with ± D-Ala + 2.5 μM FAD for 7 h. Following D-Ala wash-off, neurons were plated ± HCA and phase-contrast images of live cells taken 48 h later, representative of three independent experiments. (Scale bar, 50 μm.) (*C*) AdDAAO astrocytes were pretreated ± D-Ala + 2.5 μM FAD for 4, 7, or 24 h. Following D-Ala wash-off, neurons were plated ± HCA for 48 h. *, $P \leq 0.05$, 0.016 mM D-Ala vs. HCA control. (*D*) Control or adEV astrocytes were pretreated ± D-Ala + 2.5 μM FAD for 7 h. Following D-Ala wash-off, neurons were plated ± 5 mM HCA on the astrocytes for 48 h. (*E*) AdDAAO, adDAAO + adCAT, or adCAT astrocytes were preteated ± 0.016 mM D-Ala + 2.5 μM FAD for 7 h. Following D-Ala wash-off, neurons were plated with 5 mM HCA for 48 h. *, $P \leq 0.05$, adDAAO + 0.016 mM D-Ala vs. adDAAO control. Values are the averages of four to five experiments ± SEM for all data within the figure.

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production for 7 h (not 4 or 24 h) induces an optimal "state change" in astrocytes that renders them capable of protecting neurons from oxidative death.

To verify that the metabolism of D-Ala by DAAO imparts its effects on astrocytes via H_2O_2, we coexpressed CAT, using an adenoviral vector (adCAT), with adDAAO specifically in astrocytes to scavenge intracellular H_2O_2 production only in astrocytes. Immunoblotting verified increased CAT expression in adCAT-astrocytes and showed that this increased expression persisted in the presence of rgDAAO (Fig. S3*D*), and the cotransduction of adCAT did not interfere with rgDAAO expression (Fig. S3*E*). AdDAAO-astrocytes cotransduced with adCAT attenuated H_2O_2 production (Fig. S3*F*), and this reduction in H_2O_2 by adCAT abrogated the neuroprotective effect of D-Ala in adDAAO astrocytes (Fig. 4*E*). These results, along with our inability to measure a change in the DAAO-byproduct ammonia at the neuroprotective concentration of D-Ala (0.016 mM D-Ala) (Fig. S3*G*), support a critical role of H_2O_2 in mediating the astrocyte-dependent neuroprotection.

Nrf2 Activation in Astrocytes Induces Neuroprotection. Prior studies have suggested that H_2O_2 can activate Nrf2 in astrocytes (18). We therefore examined whether Nrf2, which stimulates the transcription of antioxidant genes and has been implicated in astrocyte-dependent neuroprotection (11, 14), is necessary for the neuroprotective effect of astrocytic H_2O_2. As a positive control for these studies we pretreated astrocytes, before neuronal plating, with the small molecule electrophilic Nrf2 activators sulforaphane (SF), tert-butylhydroquinone (tBHQ) and 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2), or overexpressed Nrf2 using an adenoviral vector. As expected, the electrophiles and Nrf2 overexpression mimicked the neuroprotective effect seen with astrocytic H_2O_2 (Fig. S4 *A* and *B*). To verify that the Nrf2 activators induce transcription via the ARE, the binding site for Nrf2, we constructed adenoviruses that express the ARE promoter sequence upstream of a luciferase reporter (ARE-Luc). Indeed, SF activated the ARE in astrocytes (Fig. S4*C*). To verify that astrocytic Nrf2 is required for the neuroprotection observed with SF, we treated astrocytes with siRNAs targeted against Nrf2 (which decreased Nrf2 protein levels and mRNA levels by 50 to 60%, and abrogated both SF-induced ARE-Luc activity and SF-induced protein levels of HO-1 (a downstream target of Nrf2) (Fig. S4 *D*–*G*). Thus, as expected, Nrf2-siRNA blocked the SF-induced astrocytic neuroprotection (Fig. S4*H*) (which was not a result of siRNA-induced toxicity) (Fig. S4 *I* and *J*). In addition, overexpression of the Nrf2 repressor Keap1 abrogated the astrocyte-dependent SF-mediated neuroprotection (Fig S4*K*).

Astrocytic H_2O_2 Induces Neuroprotection Independent of Nrf2. With confirmation in hand that Nrf2 is necessary for astrocyte-mediated electrophilic neuroprotection, we next asked whether Nrf2 is also necessary for H_2O_2-induced neuroprotection. Surprisingly, the Nrf2-siRNAs failed to block the neuroprotective effect of adDAAO-mediated astrocytic H_2O_2 production (Fig. 5*A*). Despite higher levels of neuronal death in cultures exposed to both transfection reagent and HCA, the fold-protection induced by DAAO plus D-Ala treatment in the presence of transfection reagent and Nrf2-siRNAs remained consistent with the previously observed protection (compare Fig. 4*C* with Fig. 5*A*). We believe it unlikely that the persistent neuroprotection reflects the incomplete knockdown of Nrf2, because adDAAO-induced H_2O_2 production failed to augment markers of Nrf2 activation, such as HO-1 protein levels (Fig. 5*B*) and ARE-Luc activity (Fig. 5*C*). We also confirmed that the lack of effect of adDAAO-mediated H_2O_2 production on ARE-Luc activity was not a result of diminished rgDAAO expression (Fig. S5*A*), diminished adARE-Luc transduction (Fig. S5*B*), or a lack of H_2O_2 production (Fig. S5*C*) in astrocytes cotransduced with adDAAO and adARE-Luc. We also confirmed that exogenous treatment of astrocytes with H_2O_2 (up to 30 μM H_2O_2) failed to initiate significant ARE-Luc activity (Fig. 5*D*). We were unable

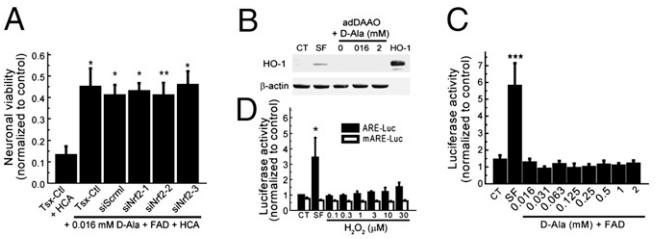

Fig. 5. Low-level, intracellular H_2O_2 generated via heterologous DAAO expression in astrocytes confers neuroprotection independent of Nrf2. (*A*) AdDAAO-transduced astrocytes were transfected with vehicle (Tsx-Ct) or siRNAs (siScrml or siNrf2-1, -2, or -3) and treated for 7 h with 0.016 mM D-Ala. Following wash-off, neurons were plated + 5 mM HCA for 48 h. *, $P \leq 0.05$, Tsx-CT, siScrml, siNrf2-1 and -3 vs. HCA; **, $P \leq 0.01$ siNrf2-2 vs. HCA. (*B*) Immunoblot of nontransduced (CT) astrocytes ± 5 μM SF (24 h) or adDAAO-transduced astrocytes ± D-Ala + 2.5 μM FAD (7 h). The last lane is recombinant HO-1 protein. The blot is representative of three independent experiments. (*C*) AdDAAO + adARE-Luc astrocytes were treated with 5 μM SF for 24 h or D-Ala for 7 h. ***, $P \leq 0.001$, SF vs. control. (*D*) adARE-Luc or mARE-Luc astrocytes were treated with 5 μM SF or H_2O_2 for 7 h. *, $P \leq 0.05$, SF vs. control ARE-Luc. Values are the averages of three to five experiments ± SEM for all data within the figure.

to test higher levels of exogenous H_2O_2 on ARE-Luc activity because of astrocyte toxicity (Fig. S5*D*). Finally, the Nrf2 electrophilic activators SF or tBHQ maintain their astrocytic protective effect even with overexpression of the H_2O_2 scavenging enzyme CAT, further supporting disparate mechanisms of the electrophile and H_2O_2 protective pathways (Fig S5*E*).

Because Nrf2-dependent neuroprotection correlates strongly with enhanced astrocytic GSH release (11), we examined if the astrocyte-dependent neuroprotective effect could be sustained with astrocyte conditioned media, and if astrocytic GSH levels increase in response to adDAAO-generated H_2O_2. Consistent with a lack of Nrf2 involvement in the H_2O_2 effect, conditioned media from SF-treated astrocytes protected the neurons from oxidative stress, but the media from H_2O_2-primed astrocytes failed to induce neuroprotection (Fig. S6*A*). In addition, SF, but not adDAAO-derived H_2O_2, enhanced astrocytic intracellular GSH levels (Fig. S6*B*). Finally 7-h D-Ala pretreatment in adDAAO astrocytes before neuronal plating with HCA failed to preserve GSH levels in the cocultures (Fig. S6*C*).

Microarray Analysis of Astrocytic Low-Level DAAO-induced H_2O_2 Generation. As other prosurvival transcription factors, such as NF-κB, are also redox-regulated, we performed an unbiased analysis of the transcriptional changes induced by low-level H_2O_2 (induced by 0.016 mM D-Ala) in adDAAO astrocytes using two microarray approaches: (*i*) high density genome-wide and (*ii*) custom-designed microarray platform. The genome-wide approach showed no significant transcriptional changes, including no changes in a battery of Nrf2-regulated genes, in response to low-level H_2O_2 generation (Figs. S6*D* and S7). However, the more sensitive custom-designed microarray approach uncovered several significant changes induced by low-level H_2O_2, most notably the up-regulation of interleukin 1-β (*IL-1β*) and caveolin 1 (*CAV1*), and the down-regulation of transglutaminase 2, an enzyme whose selective inhibition has been associated with neuroprotection (23) (Table S1). The H_2O_2-induced regulated genes were almost entirely independent from genes controlled by the Nrf2-activator SF, such as *GFAP* (Tables S2 and S3). Together, these microarray data further support the lack of involvement of Nrf2 in mediating the H_2O_2 effect and provide insight into potential low level H_2O_2-dependent mechanisms.

PTP Inhibition Mimicks the Neuroprotective Effect of Astrocytic H_2O_2. PTPs are uniquely and highly sensitive to oxidation-induced inactivation by physiological H_2O_2 levels because of the low pK_a of cysteine residues found within their catalytic domain (2). Indeed,

PTP inhibition can enhance interleukin levels (24), and IL-1β was up-regulated in our gene-array analysis (Table S1). Thus, to establish if low-level H_2O_2-induced PTP inactivation is a plausible mechanism responsible for the astrocyte-dependent neuroprotective effect, astrocytes were pretreated with three structurally diverse PTP inhibitors before neuronal plating. Indeed, sodium orthovanadate, phenylarsine oxide, and BVT-948 mimicked the astrocytic H_2O_2-induced neuroprotection (Fig. 6 *A–C* and Fig S8 *A–C*). Consistent with the H_2O_2-induced neuroprotection, BVT-948 (which inhibits PTPs via H_2O_2 generation) was most effective at the lowest concentration tested.

Discussion

Herein we provide evidence that regulatable, continuous, low-level astrocytic H_2O_2 production for 7 h mediates the protection of adjacent neurons from oxidative stress (Figs. 3*B* and 4 *B* and *C*). Although previous studies employing bolus H_2O_2 have provided important insights (4, 6, 7), bolus application does not emulate localized and continuous cellular H_2O_2 production. The rgDAAO method overcomes many of the potential confounds introduced by bolus addition of H_2O_2 and allows easy control of the level and duration of intracellular H_2O_2 production not offered by other enzymatic models (Fig. 1*D*). Our ability to manipulate astrocytic H_2O_2 production demonstrates how H_2O_2 can act as a protective messenger or a lethal toxin for adjacent neurons, depending on the concentration generated (Figs. 3*B* and 4 *B* and *C*). Of note, H_2O_2's neurotoxic effects are attributable to diffusion into the extracellular space (Fig. 3*E*), but H_2O_2's protective functions reflect its local role in astrocytes (Fig. 4*E*). We used several independent strategies to show that H_2O_2 does not activate Nrf2 to mediate its salutary, astrocyte-dependent effects (Fig. 5 and Fig. S6).

Central to Nrf2 regulation is the repressor protein Keap1 (12). Debate surrounds the exact mechanism by which Nrf2 evades Keap1-dependent proteasomal degradation and the precise cellular signals that prompt Nrf2 activation. Keap1 is a cysteine-rich protein containing 25 to 27 cysteine residues (15). The cysteine thiol group is nucleophilic, providing an ideal target for electrophiles that readily accept electron pairs. Electrophiles, such as SF, menadione, 15d-PGJ2, isoliquiritigenin, and tBHQ readily react with Keap1 cysteines and have thus been extensively characterized as potent Nrf2 activators (15, 25). Although H_2O_2 possesses the capacity to oxidize cysteine residues, information on H_2O_2's role in modulating the Keap1/Nrf2 system is not consistent. An attractive hypothesis is that H_2O_2 accumulation beyond a homeostatic threshold is the intracellular signal initiating Nrf2-dependent gene transcription and providing feedback regulation to limit oxidative damage. Indeed, the treatment of numerous cell types (including astrocytes) with H_2O_2 leads to Nrf2 activation (17, 18, 26, 27). However, the levels of exogenous H_2O_2 required to induce Nrf2 activation (mid micromolar to low millimolar) are unlikely to be produced by most cells. In fact, H_2O_2 only transiently oxidizes Keap1 (28) and is unable to overcome the inhibitory effect of Keap1 overexpression on Nrf2 activation (25). Not surprisingly, others have found no effect of H_2O_2 on Nrf2 in astrocytes and keratinocytes (16, 19). Thus, our data showing that enzymatic low level H_2O_2 production in astrocytes protects neurons independent of Nrf2 activation is in-line with numerous reports showing minimal to no effect of H_2O_2 on the Nrf2/Keap1 system.

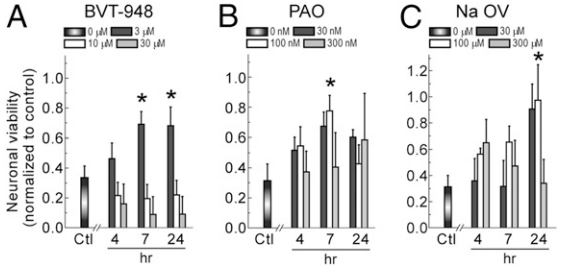

Fig. 6. PTP inhibition in astrocytes induces the neuroprotection of adjacent neurons. Astrocytes were treated at the indicated time points (data are grouped by hour of astrocyte pretreatment) with (*A*) BVT-948, (*B*) phenylarsine oxide (PAO), or (*C*) sodium orthovanadate (Na OV). Following washoff, neurons were plated + 5 mM HCA for 48 h and neuronal viability quantified. Values are the averages of four to five experiments ± SEM. *, $P \leq 0.05$, PTP inhibitor vs. HCA Ctl. Data in the absence of HCA are shown in Fig. S8.

Accumulating evidence points to a protective role of ROS, such as H_2O_2, in the CNS. ROS/H_2O_2 are necessary for neuroprotective preconditioning in in vitro models of ischemia and oxidative stress (29–31). In brain slices subjected to hypoxia, H_2O_2 spares neurons from irreversible firing inhibition and induces neuroprotection (32, 33). In retinal cells, H_2O_2-initiated AKT signaling is necessary to prevent apoptosis (34). Our current results expand the established protective effect of H_2O_2 to show that enzymatic H_2O_2 production in one cell-type (astrocytes) affords protection to a neighboring cell population (neurons).

In the CNS and other tissues, cells respond to hypoxic or oxidative stress by initiating endogenous protective cascades often regulated at the transcriptional level. Thus, we used two microarray approaches (high-density genome-wide and custom-designed) to explore potential transcriptional changes initiated by low-level H_2O_2. Both microarray analyses support the lack of Nrf2 involvement (Fig. S6*D* and Tables S1, S2, and S3). In addition, the more sensitive custom-designed microarray uncovered transcriptional changes in response to low-level H_2O_2, providing potential mechanistic insight into the astrocyte-dependent neuroprotective pathway. Two of the most up-regulated genes were *IL-1β* and *CAV1* (Table S1). Although interleukins are normally associated with inflammation-induced neurotoxicity, IL-6 was recently found to mediate astrocyte-dependent neuroprotection (35). Caveolins are components of lipid rafts and play a large role in facilitating cell signaling by compartmentalizing proteins involved in signal transduction (36), thus representing an attractive candidate protein in mediating the astrocyte-dependent neuroprotective effect.

Other plausible mechanisms include the H_2O_2-induced post-translational modification of proteins, such as PTPs, which are avidly and reversibly oxidized by low-level H_2O_2 (2). Indeed, the inhibition of PTPs specifically in astrocytes induced potent and durable neuroprotection (Fig. 6 *A–C*), mimicking the H_2O_2 effect. In fact, PTP inhibition has been shown to be neuroprotective in spinal cord injury (37), suggesting that PTPs are potential candidates in mediating H_2O_2's astrocytic neuroprotective effect. PTP inhibition-mediated astrocytic neuroprotection was not restricted to a 7-h time-window, as seen with low-level H_2O_2. This finding suggests that low-level H_2O_2 generation for 7 h induces the favorable reversible oxidative modification of target proteins (i.e., sulfenic acidification of PTPs), but diminished protection by 24 h indicates a possible switch to the irreversible sulfinic and sulfonic acidification of proteins by H_2O_2, which may hamper the neuroprotective effect. Future studies will address this question. Regardless of a transcriptional or posttranslation mechanism, our findings that 7-h low-level H_2O_2 production in astrocytes is neuroprotective challenges the current dogma that pre- or postconditioning is a biphasic response that involves a short-lived (2–4 h) posttranscriptional change in proteins and a longer-lived (24–72 h) transcriptional change involving the expression of hundreds of adaptive genes (38).

In closing, our data demonstrate that astrocytic H_2O_2 contributes to neurosurvival or death, depending on the levels generated, adding to a growing body of literature showing that astrocyte-specific mechanisms influence neuronal fate (13, 14). Prior studies have revealed a salutary, Nrf2-mediated role for astrocytes in overcoming neuronal death in Parkinson disease and ALS (13, 14). Our studies confirm the importance of Nrf2 in the CNS but point to unexpected, unique astrocytic mechanisms that work independently of Nrf2. The important role of low-level astrocytic H_2O_2 in protecting neurons from oxidative stress underscores the complexity of redox biology and provides insight into the beneficial role of astrocyte-derived oxidants in the CNS.

Materials and Methods

More detailed descriptions of the methods and materials used can be found in *SI Materials and Methods*.

Cell Culture. Primary astrocyte cultures were prepared from the cerebral cortices of rat pups (P1–3). Primary neuronal cultures were prepared from the forebrains of rat embryos (E17). Dissociation was performed as described by Ratan et al. (22).

Adenoviral Transduction. Cultured astrocytes were treated with the adenoviral vectors at a multiplicity of infection of 15 for 4 h in serum-free medium.

Extracellular H_2O_2 Measurement. A HRP/Amplex Red substrate system was used to monitor extracellular H_2O_2 levels in live astrocytes.

Immunocytochemistry. Cells were paraformaldehyde-fixed and incubated with primary/secondary antibodies and mounted with a DAPI-containing solution.

GSH Measurements. Intracellular total GSH was determined using the GSH-Glo(TM) Glutathione Assay kit (Promega).

Neuronal Viability. Fixed cocultures were incubated with antibodies for the neuronal specific marker MAP2 followed by HRP-conjugated secondary antibodies. HRP activity was then monitored using an Amplex Red reaction buffer.

Astrocyte Viability. Astrocyte viability was quantified using the CellTiter 96 Assay (Promega).

Live-Cell Imaging. Phase contrast images were taken with a Nikon Eclipse TS 100.

Western Blots. Following SDS/PAGE, transfer to nitrocellulose membrane and primary/secondary antibody incubation, astrocyte protein bands were detected with an infrared imaging system (LI-COR Biosciences).

ARE-Luciferase Reporter Assay. Astrocytes were transduced with adenoviral vectors containing the ARE sequence upstream of a luciferase reporter. Luciferase activity was measured using a luciferase assay kit (Promega).

Small Interfering RNA Transfections. Nrf2 knock down was achieved using siRNA targeted against Nrf2. Cultured astrocytes were transfected with the siRNAs plus the transfection reagent *Trans*IT-siQUEST (Mirus) overnight.

Quantitative PCR. Total RNA was isolated from primary rat astrocytes using the NucleoSpin RNA II kit. A TaqMan RNA-to-CR_TR *1-Step* Kit was used for real-time PCR.

Statistical Analysis. Unless otherwise stated, statistical significance was determined by a one-way ANOVA followed by posthoc Newman Keuls or Dunnett's test using Statistica software (StatSoft). To determine the significance of the rate of H_2O_2 production, a main-effects ANOVA was performed.

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