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Forum Review

H_2O_2 Signaling in the Nigrostriatal Dopamine Pathway via ATP-Sensitive Potassium Channels: Issues and Answers

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ABSTRACT

The role of reactive oxygen species (ROS) as signaling agents is increasingly appreciated. Studies of ROS functions in the central nervous system, however, are only in their infancy. Using fast-scan cyclic voltammetry and fluorescence imaging in brain slices, the authors discovered that hydrogen peroxide (H_2O_2) is an endogenous regulator of dopamine release in the dorsal striatum. Given the key role of dopamine in motor, reward, and cognitive pathways, regulation by H_2O_2 has implications for normal dopamine function, as well as for dysfunction of dopamine transmission. In this review, data are summarized to show that H_2O_2 is a diffusible messenger in the striatum, generated downstream from glutamate receptor activation, and an intracellular signal in dopamine neurons of the substantia nigra, generated during normal pacemaker activity. The mechanism by which H_2O_2 inhibits dopamine release and dopamine cell activity is activation of ATP-sensitive K^+ (K_{ATP}) channels. Characteristics of the neuronal and glial antioxidant networks required to permit H_2O_2 signaling, yet prevent oxidative damage, are also considered. Lastly, estimates of physiological H_2O_2 levels are discussed, and strengths and limitations of currently available methods for H_2O_2 detection, including fluorescence imaging using dichlorofluorescein (DCF) and the next generation of fluorescent probes, are considered.
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INTRODUCTION

Reactive oxygen species (ROS) are often considered to be toxic 'byproducts' of cell metabolism. Indeed, increased ROS production and oxidative stress contribute in cell death following acute brain injury, including cerebral ischemia–reperfusion (29, 34, 74, 127), as well as in slowly progressing neurodegenerative disorders such as Parkinson's disease (57, 97, 144). This view of ROS is evolving rapidly, however, in light of increasing evidence that ROS can also act as cellular messengers that target physiological processes from short-term ion channel activation (11, 14, 75, 132) and regulation of cell phosphorylation pathways (78, 110, 128) to gene transcription (58, 67). Hydrogen peroxide (H_2O_2) is a particularly intriguing candidate as a signaling molecule because it is neutral and membrane permeable (107) and can therefore diffuse freely from a site of generation, as does the established diffusible messenger, nitric oxide ($NO^{\bullet}$). Moreover, H_2O_2 has no unpaired electrons and is therefore not a free radical, in contrast to superoxide ($^{\bullet}O_2^-$), the hydroxyl radical ($^{\bullet}OH$), and even $NO^{\bullet}$, and so does not readily cause oxidative damage (43).

Research in our laboratory over the past decade has focused on two primary topics. The first is the neuroprotective role of low molecular weight antioxidants, including ascorbate; the second is regulation of dopamine (DA) release by endogenous neurotransmitters and neuromodulators. These topics converged when we began to examine DA release regulation under conditions of oxidative stress.

Dopaminergic transmission is important in a wide range of brain functions, including learning and cognition (32), control of movement (3, 30, 59), and mediation of desire and re-

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ward (27). Consequently, *dysfunction* of dopaminergic transmission has been implicated in several significant brain disorders, including the psychoses of schizophrenia, the movement deficits of Parkinson's disease, and addiction to substances including cocaine and amphetamine. Our recent discovery that endogenously generated H_2O_2 regulates DA transmission in the nigrostriatal pathway via activation of ATP-sensitive potassium (K_{ATP}) channels, therefore, has implications for both normal and pathophysiological conditions. Our evidence thus far suggests that K_{ATP}-channel opening in DA neurons is a direct effect of H_2O_2 (14), consistent with findings from previous electrophysiological studies of the effect of H_2O_2 at K_{ATP} channels in isolated membrane patches from cardiac myocytes (75,132). This contrasts sharply with previously recognized mechanisms of H_2O_2 signaling that involve intracellular signaling cascades (for review, see Refs. 78, 110, 128).

In this review, we summarize our recent studies showing that H_2O_2 is a diffusible and intracellular messenger in the nigrostriatal DA pathway. We will then discuss characteristics of the neuronal and glia antioxidant networks that permit H_2O_2 signaling, yet prevent oxidative damage. Lastly, we will consider several methodological issues inherent in the examination of H_2O_2 as a neuromodulator, including estimates of physiologically relevant concentrations of H_2O_2 and characteristics of available and emerging fluorescent dyes for H_2O_2 imaging. Together, the studies presented illustrate how the use of a combination of methods, including voltammetric detection of DA release, whole-cell recording, and ROS imaging in brain slices, together with pharmacological manipulation of tissue peroxidase activity, can provide new insights into the roles of H_2O_2 in the CNS.

Most experimental data presented were obtained *in vitro* using brain slices. Brain slice preparations offer three main advantages for the kinds of questions we have addressed. First, local brain microcircuitry, including synaptic connections and neuron–glial interactions (13), is preserved in slices. Second, the three-dimensional cytoarchitecture of the tissue is maintained, so that normal intra- and extracellular compartments are intact (112). Third, the use of *in vitro* slices facilitates accurate electrode placement, ease of combining imaging methods with other techniques, and ready application of pharmacological agents at known concentrations. Reflecting these advantages, a number of mechanistic insights into the regulation of DA release have been obtained using brain slices, which allow voltammetric detection of DA release in discrete brain regions, without complicating factors inherent to *in vivo* studies, including animal behavior, anesthesia, and indirect effects from distant structures via long pathways (for review, see Ref. 101).

H_2O_2 AS A SIGNALING AGENT IN THE NIGROSTRIATAL PATHWAY

Generation of ROS occurs during a variety of metabolic processes, the most important of which is mitochondrial respiration, which produces $^{\bullet}O_2^-$ from a single-electron reduction of molecular oxygen (25, 89, 106). Additional sources of ROS include enzymes such as monoamine oxidase, which produced H_2O_2 during deamination of DA and other monoamines (43, 116), and NADPH oxidase (117, 120), which produce $^{\bullet}O_2^-$ that can participate in various signaling cascades. Levels of $^{\bullet}O_2^-$ are managed by mitochondrial and cytosolic forms of superoxide dismutase; dismutation of $^{\bullet}O_2^-$ produces H_2O_2 that is regulated by glutathione (GSH) peroxidase, which is found in mitochondria and in the cytosol, and by catalase, which is localized in peroxisomes (42, 52, 129). Interaction of either $^{\bullet}O_2^-$ or H_2O_2 with trace metal ions, including iron and copper, can produce the aggressive radical, $^{\bullet}OH$, which is neutralized primarily by the low molecular weight antioxidants GSH and ascorbate (42). Thus, both enzymes and low molecular weight antioxidants work together to regulate ROS and prevent oxidative damage. A role for H_2O_2 and other ROS as neuromodulators requires that oxidant regulation must be more subtle than previously thought, however.

Our initial studies of the effect of H_2O_2 on neurotransmission were based on previous work by Terry Pellmar (103–105), who had shown that exogenous H_2O_2 could suppress the amplitude of evoked population spikes in guinea pig hippocampal slices, possibly by inhibiting transmitter release. We tested the effect of H_2O_2 on transmitter release directly in guinea pig striatal slices using real-time monitoring of evoked extracellular DA concentration ($[DA]_o$) with carbon-fiber microelectrodes and fast-scan cyclic voltammetry. Consistent with Pellmar's hypothesis, exogenous H_2O_2 (1.5 m*M*, 15 min) causes a reversible 30–40% decrease in pulse-train evoked $[DA]_o$ that is not accompanied by loss of tissue DA content or evidence of oxidative damage (38). It should also be noted that 1.2 m*M* H_2O_2 has no effect, demonstrating the marked efficacy of brain slice preparations in handling excess H_2O_2 (see also Ref. 13).

In the course of our experiments, we also obtained data suggesting that *endogenous* H_2O_2 might have a physiologically relevant role in the regulation of axonal DA release in striatum. These findings shifted our focus from viewing H_2O_2 as a mediator of oxidative stress, to viewing it as a neuromodulator. We explored the potential modulatory role for endogenously generated H_2O_2 by manipulating slice peroxidase activity (12, 38, 39). Amplification of endogenous H_2O_2 levels by inhibiting GSH peroxidase with mercaptosuccinate (MCS) also causes a 30–40% decrease in pulse-train evoked $[DA]_o$ in dorsolateral striatum similar to that seen with exogenously applied H_2O_2 (12, 39). Again, no change in DA content is seen and DA release suppression is fully reversible after MCS washout or when the slice is superfused with catalase in the continued presence of MCS (12). Similar suppression of evoked $[DA]_o$ is seen in the presence of MCS in the shell of the nucleus accumbens and in the substantia nigra pars compacta (SNc), consistent with a modulatory role for H_2O_2 in those regions, as well (39).

Modulatory H_2O_2 in striatum is generated downstream from glutamatergic AMPARs

Importantly, further studies showed that MCS has no effect on $[DA]_o$ evoked by a single stimulus pulse (12), implying that modulatory H_2O_2 is generated *dynamically* during the

initial pulse(s) of a train, then inhibits DA release during subsequent pulses. This dynamic regulation is rapid, with DA release suppression seen within a few hundred milliseconds after initiation of a 10 Hz pulse train (12). The requirement for multiple-pulse stimulation also implied the involvement of striatal circuitry. Given that previous studies in cultured neurons had shown that glutamate receptor activation can enhance mitochondrial H_2O_2 generation (23, 31, 55, 109), we reasoned that locally evoked glutamate release from the corticostriatal pathway might contribute to generation of modulatory H_2O_2. Consistent with normal glutamate-dependent suppression of striatal DA release, blockade of glutamatergic AMPA receptors (AMPARs) by the selective antagonist, GYKI-52466 causes up to a 100% increase in evoked $[DA]_o$ versus control (11, 12) (Fig. 1A). Additionally, the apparent absence of presynaptic ionotropic glutamate receptors on DA terminals (22, 40) suggests that any glutamatergic influence must be indirect. The involvement of a diffusible inhibitory intermediate like H_2O_2 would provide a link between glutamatergic and dopaminergic synapses in the absence of direct synaptic contact.

The central role of H_2O_2 in AMPAR-dependent inhibition of DA release was demonstrated by the complete prevention of the effect of AMPAR blockade in the presence of exogenous catalase (Fig. 1B) or GSH peroxidase (12). Is AMPAR-dependent H_2O_2 generation the primary source of modulatory H_2O_2 in the striatum? The answer appears to be yes because the usual suppression of evoked $[DA]_o$ when GSH peroxidase is inhibited by MCS is abolished when AMPARs are blocked by GYKI-52466 (12).

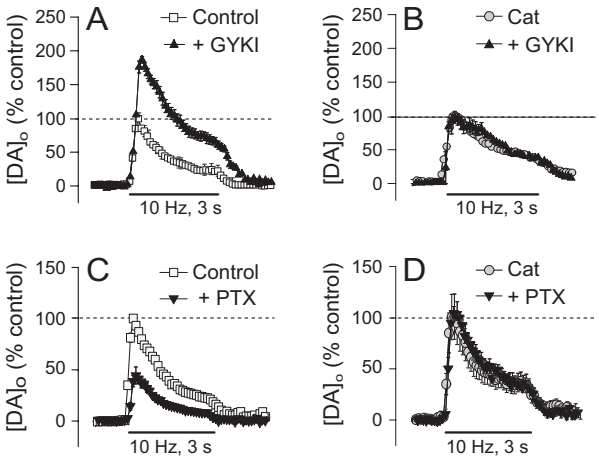

FIG. 1. Regulation of striatal DA release by glutamate and GABA requires H_2O_2. (**A**) AMPAR blockade by GYKI-52466 (GYKI; 50 μ*M*) causes a ~100% increase in evoked $[DA]_o$ in striatum ($p < 0.001$, $n = 6$). (**B**) The effect of AMPAR blockade is prevented by catalase (Cat, 500 U/mL). (**C**) $GABA_A$R blockade by picrotoxin (PTX; 100 μ*M*) causes a ~50% decrease in evoked $[DA]_o$ ($p < 0.001$, $n = 6$). (**D**) Catalase abolishes the effect of picrotoxin. Responses in the presence of heat-inactivated catalase were the same as control. Data are means ± SEM, illustrated as percentage of same-site control (modified from Ref. 12).

Interestingly, GABA-dependent regulation of DA release in dorsal striatum also involves modulatory H_2O_2. Under control conditions, blockade of $GABA_A$ receptors ($GABA_A$Rs) by picrotoxin causes a ~50% decrease in evoked $[DA]_o$, indicating that $GABA_A$R activation normally *enhances* DA release (Fig. 1C). The influence of GABA on DA release, like that of glutamate, must be indirect, since DA axons also do not express $GABA_A$Rs (63). Importantly, the effect of picrotoxin is completely prevented by catalase (Fig. 1D), implicating H_2O_2 in this process. Moreover, $GABA_A$R blockade has no effect when AMPARs are blocked. Together, these data indicate that glutamate and GABA act on the same pool of modulatory H_2O_2 that is generated downstream from AMPARs.

K_{ATP} channels mediate H_2O_2-dependent inhibition of striatal DA release

The findings discussed thus far show that endogenous H_2O_2 is an inhibitory messenger that mediates the effects of glutamate and GABA on axonal DA release in dorsal striatum. How does H_2O_2 inhibit DA release? The answer is that H_2O_2 generation leads to the opening of K_{ATP} channels (11, 12, 14). Previous physiological studies demonstrated that *exogenous* H_2O_2 can cause membrane hyperpolarization and decreased excitation by activating a K^+ conductance in a variety of cell types, including hippocampal CA1 neurons, cardiac myocytes, and pancreatic β-cells (84, 75, 122, 132). Our studies of DA release in striatal slices provided the first evidence that endogenous H_2O_2 causes *functionally relevant* activation of K_{ATP} channels. Blockade of K_{ATP}-channels with either tolbutamide or glibenclamide causes a significant increase in $[DA]_o$ versus control during local pulse-train stimulation, indicating that K_{ATP} channels are activated under these conditions and that these channels inhibit DA release (11, 12). Blockade of K_{ATP} channels also prevents the inhibitory effect of MCS on DA release, as well as the usual effects of AMPAR blockade by GYKI-52466 and $GABA_A$R blockade by picrotoxin, confirming that K_{ATP} channels are *required* for modulation of DA release by H_2O_2, glutamate, and GABA.

We then examined which subtype of K_{ATP} channels mediates H_2O_2 sensitivity. These channels are octameric proteins composed of four inwardly rectifying pore-forming units, typically Kir6.2 in neurons (7, 79), and four sulfonylurea receptor sites (SUR1 or SUR2) (2). SUR1- and SUR2-based channels can be distinguished by their differential sensitivity to K_{ATP}-channel openers, with diazoxide preferentially acting at SUR1-based channels and cromakalim at SUR2-based channels (15, 76). In dorsal striatum, K_{ATP} channel opening by either diazoxide or cromakalim causes a 30–40% suppression of DA release; however, SUR1-selective diazoxide prevents the usual pattern of H_2O_2-dependent modulation by MCS, GYKI-52466, and picrotoxin, whereas these effects persist in the presence of SUR2-selective cromakalim (11). These data imply that when SUR1-based K_{ATP} channels have already been opened by diazoxide, this occludes normal K_{ATP}-channel dependent modulation by H_2O_2.

Modulatory H_2O_2 in striatum is generated in medium spiny neurons

Increasing evidence implicates medium spiny neurons as the primary cellular source of modulatory H_2O_2 in dorsal striatum. Not only are these the most abundant striatal neurons (90–95%) (80), but they also express both AMPARs and $GABA_A$Rs, in contrast to DA axons in dorsal striatum, which apparently express neither (22, 40, 63). Recent fluorescence imaging studies in our laboratory support the hypothesis that medium spiny neurons are a key cellular source of modulatory H_2O_2 in dorsal striatum (102). In these studies, dihydrodichlorofluorescein (H_2DCF) diacetate (7 μ*M*) was loaded into individual medium spiny neurons via the patch pipette used for simultaneous whole-cell recording (for methods, see Refs. 14 and 19). Cleavage of the diacetate group by intracellular esterases leaves H_2DCF trapped in the cell for subsequent oxidation to fluorescent DCF. Basal DCF fluorescence is seen in all striatal spiny neurons, reflecting a tonic level of H_2O_2 and other ROS (19). Local stimulation (with the same 30 pulse, 10 Hz pulse trains that we typically use to elicit concurrent release of DA and glutamate) causes a ~30% increase in DCF fluorescence in these cells, which is further enhanced when GSH peroxidase is inhibited by MCS. Strikingly, AMPAR blockade by GYKI-52466 prevents this stimulus-activated H_2O_2 generation in medium spiny neurons, confirming glutamate-dependent H_2O_2 generation (102).

Our working model of axonal DA release regulation by glutamate, therefore, is that H_2O_2 is generated in the dendrites of medium spiny neurons after glutamate release and AMPAR activation. Modulatory H_2O_2 then diffuses to adjacent DA synapses, where it inhibits DA release via opening of SUR1-containing K_{ATP}-channels. By decreasing dendritic excitability, GABA acting via $GABA_A$Rs lessens H_2O_2 production in medium spiny neurons; conversely, when $GABA_A$Rs are blocked, activity-dependent H_2O_2 production would be increased, leading to greater suppression of DA release. By contrast, when AMPARs are blocked, H_2O_2 generation is minimal, DA release increases, and $GABA_A$R-dependent regulation is lost (12).

Endogenous H_2O_2 acts via K_{ATP} channels to regulate DA neuron excitability in SNc

Consistent with the abundant expression of K_{ATP} channels throughout the nigrostriatal pathway (56, 95, 138), the activity of DA neurons in the SNc is also regulated by H_2O_2 acting via K_{ATP} channels (14). Both basal and elevated H_2O_2 levels have significant effects on DA cell membrane properties. In midbrain slice preparations, DA neurons exhibit spontaneous pacemaker activity. Under these conditions, basal DCF fluorescence is readily detected in DA neurons loaded with H_2DCF diacetate, consistent with tonic H_2O_2 generation in these cells (14). Strikingly, this basal H_2O_2 tone has a significant effect on DA cell excitability: depletion of intracellular H_2O_2 by including catalase in the patch-pipette or blockade of K_{ATP} channels by glibenclamide causes a ~40% increase in spontaneous firing rate in all DA neurons tested (Fig. 2). Moreover, catalase has no effect in cells patched in the presence of glibenclamide (Fig. 2). Importantly, the backfill solution in these studies contained 3 m*M* ATP, at which concentration K_{ATP} channels should be closed (70). Thus, it is unlikely that the resting K_{ATP} channel tone in DA neurons is caused by low ATP. Indeed, previous studies using inside-out membrane patches from cardiac cells suggest that H_2O_2 can have a direct effect on K_{ATP}-channel opening by decreasing channel sensitivity to ATP (75, 132). This is likely to involve one or more redox-sensitive sites, although it should be noted that sulfhydryl complexing agents cause channel closure, rather than opening (41).

In companion studies using DCF fluorescence to visualize intracellular H_2O_2, we found that moderate increases in H_2O_2 (≤25% increase in fluorescence intensity) during partial inhibition of GSH peroxidase by MCS (0.1–0.3 m*M*) (Fig. 3) has no effect on DA neuron firing rate. However, with greater GSH peroxidase inhibition (1 m*M* MCS) or application of exogenous H_2O_2 (1.5 m*M*), 50% of recorded cells, 'responders', show K_{ATP}-channel dependent hyperpolarization, whereas 50%, 'nonresponders', do not (14). This distribution is similar to that of metabolically sensitive and insensitive DA neurons reported previously by Liss *et al*. (88), in which greater metabolic sensitivity was found to be mediated by SUR1-based K_{ATP} channels. Indeed, we found that H_2O_2-responders also hyperpolarize with SUR1-selective diazoxide, but not with SUR2-selective cromakalim, showing that SUR1 expression conveys sensitivity to elevated H_2O_2 (14), as described above for striatum. When endogenous H_2O_2 levels are increased by inhibiting the predominant peroxidase in SNc (72), catalase, with 3-aminotriazole (ATZ), however, all DA neurons respond with glibenclamide-reversible hyperpolarization. DCF imaging data indicates that ATZ rapidly amplifies intracellular H_2O_2, whereas MCS causes a slower in-

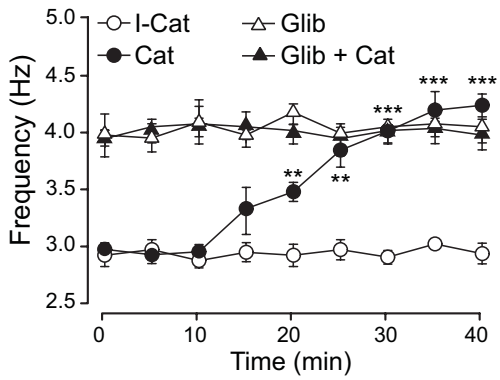

FIG. 2. Regulation of spontaneous activity in DA neurons by H_2O_2 and K_{ATP} channels. Pacemaker activity in DA neurons in the SNc increases progressively when catalase (Cat; 500 U/mL) included in the patch-pipette backfill solution (n = 7); heat-inactivated catalase (I-Cat) does not alter spontaneous activity (n = 6). Blockade of K_{ATP} channels with glibenclamide (Glib, 100 n*M*) 15 min before patching increases firing rate (n = 5; $p < 0.01$) and prevents the Cat-induced increases in firing rate (n = 5). Data are means ± SEM; $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs.* control frequency after 10 min recording (modified from Ref. 14).

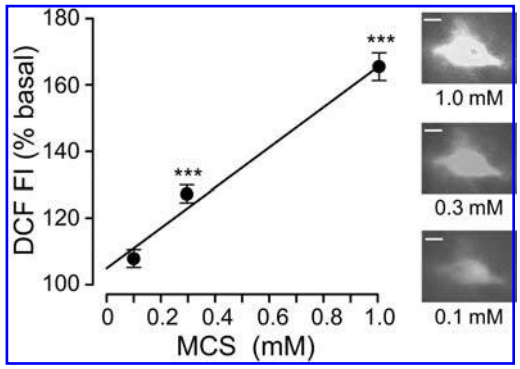

FIG. 3. Concentration-dependent effect of GSH peroxidase inhibition by MCS on H_2O_2 amplification. Inhibition of GSH peroxidase by MCS (0.1–1 m*M*) causes a progressive increase in DCF fluorescence intensity (FI), indicating increasing H_2O_2 levels ($R^2 = 0.946$). All concentrations were tested in each cell ($n = 7$; ***$p < 0.001$ vs. basal). Images are representative examples of DCF fluorescence for each concentration of MCS tested in a single DA neuron (*scale bars* = 20 μm) (modified from Ref. 14, including conversion of images to *grayscale*).

crease (14). Thus, SUR2-based K_{ATP} channels can also be activated by sufficiently *high* and/or *rapid* increases in H_2O_2. Overall, these data show that H_2O_2 serves an autoregulatory role in SNc DA neurons, in which activity-dependent H_2O_2 generation leads to suppression of neuronal activity via K_{ATP} channels, thus enhancing the reciprocal relationship between metabolism and excitability.

TRUE AND FALSE SIGNALING BY MITOCHONDRIAL H_2O_2 IN THE STRIATUM

Dynamic H_2O_2 signaling originates in mitochondria

We have examined three possible subcellular sources of H_2O_2 that might contribute to dynamic, glutamate-dependent regulation of axonal DA release in dorsal striatum (18). The first is mitochondrial respiration. Of the four mitochondrial complexes, complexes I and III are the primary sources of $^{\bullet}O_2^-$ and H_2O_2 during O_2 consumption (1, 35, 85). The second potential source of H_2O_2 is monoamine oxidase (MAO), which catalyzes DA deamination via a two-electron reduction of O_2 to H_2O_2 (43, 116). There are two isoforms MAO, type A (MAO-A) and type B (MAO-B); DA is a good substrate for both (123). The third possible source of H_2O_2 is NADPH oxidase, a family of membrane-associated, multi-subunit enzymes that catalyze the one-electron reduction of O_2 to form $^{\bullet}O_2^-$ and H_2O_2 (16, 17). In the CNS, NADPH oxidase is expressed predominately in microglial cells and to a lesser extent in neurons (143), including the recent identification of a functional form in hippocampal synapses (131).

To evaluate the role of mitochondria in dynamic generation of modulatory H_2O_2, we used a cocktail of rotenone (50 n*M*), a complex I inhibitor, and succinate (5 m*M*), a mitochondrial complex II substrate, which should limit H_2O_2 production, but maintain tissue ATP levels (18). Although slice ATP content falls after 90 min in rotenone alone, companion slices in the rotenone-succinate cocktail show no drop in ATP, validating our rationale for the use of this cocktail. Strikingly, the effects of both GYKI-52466 and MCS on evoked $[DA]_o$ is lost in the presence of the rotenone-succinate cocktail, implicating mitochondrial complex I as the primary source of modulatory H_2O_2 in the striatum (18). By contrast, the effect of MCS on evoked $[DA]_o$ is unaffected by either a cocktail of MAO inhibitors, clorgyline (MAO-A) and pargyline (MAO-B), or an NADPH oxidase inhibitor, phenylarsine oxide. It should be noted that our choice of NADPH oxidase inhibitors was limited to phenylarsine oxide, because it was the only one of several inhibitors examined that did not interfere with voltammetric detection of DA release.

These data indicate that dynamic, glutamate-dependent modulation of striatal DA release requires H_2O_2 that originates from mitochondria, rather than MAO or NADPH oxidase. This does not exclude a role for MAO-dependent DA metabolism or NADPH oxidase as sources of H_2O_2 and/or $^{\bullet}O_2^-$ that might participate in other aspects of neuronal function. For example, Kulagina and Michael (86) using amperometric microsensors to detect extracellular H_2O_2 in the striatum *in vivo* found an initial increase in H_2O_2 during electrical stimulation of the dopaminergic median forebrain bundle followed by a prolonged increase in extracellular H_2O_2. The initial peak is not altered in animals administered an MAO inhibitor; however the prolonged increase is largely prevented, indicating that MAO metabolism can be an important source of H_2O_2 on a timescale of minutes. Similarly, NADPH oxidase has been shown to be a key source of $^{\bullet}O_2^-$ that is required for some aspects of NMDA receptor signaling in the hippocampus (81), including regulation of long-term potentiation (82).

False H_2O_2 signaling during mitochondrial dysfunction

Importantly, suppression of striatal DA release also occurs during unregulated generation of H_2O_2 when mitochondrial complex I is partially inhibited by nanomolar concentrations of rotenone (19), which are sufficient to increase H_2O_2 production in isolated mitochondria (137). After 30 min in 50 n*M* rotenone, evoked $[DA]_o$ falls by ~30%; release suppression can be prevented by catalase or by K_{ATP}-channel blockade, with no change in striatal ATP content *versus* control (19). Together, these data confirm H_2O_2-dependent K_{ATP} channel activation in the effects of rotenone on DA release. It should also be noted that rotenone-induced suppression of DA release is seen with single-pulse, as well as multiple-pulse stimulation, indicating that enhanced H_2O_2 generation during mitochondrial inhibition does not require AMPAR activation, but rather reflects a direct effect on mitochondrial activity (19). Importantly, decreased activity of mitochondrial complex I is seen in the SNc in *postmortem* samples from individuals with Parkinson's disease (119), leading to the hypothesis that mitochondrial dysfunction is a contributing fac-

tor to DA neuron degeneration in Parkinson's (61, 66, 98). Our findings add a new perspective to the understanding of consequences of mitochondrial dysfunction, whereby 'false' H_2O_2 signaling contributes to *functional* DA denervation by inhibiting DA release, as well as to anatomical degeneration of the nigrostriatal DA pathway.

THE BRAIN ANTIOXIDANT NETWORK: PERMISSIVE, YET PROTECTIVE

As discussed above, oxidative damage by endogenous ROS is prevented by the brain antioxidant network, which includes low molecular weight antioxidants, enzymes, and repair systems (42, 44, 52, 93, 111, 142). Intriguingly, however, antioxidant regulation differs between neurons and glia, with higher levels of GSH and GSH-related enzymes in glia than in neurons (45, 50, 90, 106, 108, 113, 124, 135), but higher levels of ascorbate and the ascorbate transporter, SVCT2, in neurons than in glia (21, 113, 136). This differentiation could reflect the need for ROS signaling in neurons, with additional protection from oxidative damage provided by surrounding glia, as discussed further below. Consistent with this hypothesis, evidence from cultured cells suggest that glia play a critical role in protecting neurons from oxidative stress (45, 51, 53, 54, 130). Moreover, the ability of glia to protect neurons in culture is abolished by inhibition of GSH synthesis (53), as well as inhibition of GSH peroxidase or the major cellular peroxidase, catalase (45, 49).

We recently examined the neuroprotective role of glial antioxidants in a more nearly intact neuronal–glial microenvironment than possible in cell culture by comparing the consequences of oxidative stress caused by elevated H_2O_2 in guinea pig and rat brain slices (13). This comparison was based on the rationale that the ratio of glia to neurons is higher in guinea pig brain than in rat brain, given the lower neuron density of guinea pig versus rat brain (133) and the relative constancy of glial density across species (20, 62, 69, 113, 134). We found initially that pathophysiological consequences of H_2O_2 exposure seen in rat brain slices, including hyperexcitability, indicated by epileptiform activity in the hippocampus (10), and edema (26), are absent in guinea pig slices (13). The higher tolerance of guinea pig brain versus rat brain tissue to H_2O_2 exposure implies that the higher glia-to-neuron ratio in this species provides additional antioxidant protection, particularly from glia.

We therefore examined the contributions of GSH synthesis, GSH peroxidase, and catalase to the resistance of guinea pig brain tissue to H_2O_2 challenge and whether exogenous ascorbate could compensate for the loss of any of these components of the antioxidant network. After inhibition of any one of these antioxidant enzymes in guinea pig brain, H_2O_2 exposure causes the pathological consequences typically seen in rat slices (13). For example, after GSH peroxidase inhibition by MCS or catalase inhibition by ATZ in guinea pig hippocampal slices, recovery of evoked population spike amplitude during washout of H_2O_2 is delayed compared to that in control slices and is accompanied by epileptiform activity. However, when ascorbate is included at its normal extracellular concentration of 400 μ*M* (111) during H_2O_2 washout, these pathophysiological consequences of exogenous H_2O_2 exposure are prevented in peroxidase-inhibited guinea pig hippocampal slices (13). Inclusion of ascorbate during H_2O_2 washout also prevents H_2O_2-induced epileptiform activity in rat hippocampal slices (10).

In contrast to the efficacy of ascorbate in preventing pathological consequences of *exogenous* H_2O_2, this antioxidant and $^{\bullet}OH$ scavenger has no effect on the modulation of striatal DA release by *endogenously generated* H_2O_2 (12). This important observation indicates not only that ascorbate permits H_2O_2-dependent signaling in striatum, but also that inhibition of DA release is a direct effect of H_2O_2, rather than $^{\bullet}OH$. Thus, ascorbate is ideally suited as a key neuronal antioxidant 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.

WHAT ARE PHYSIOLOGICALLY RELEVANT CONCENTRATIONS OF INTRACELLULAR H_2O_2?

Studies in isolated brain mitochondria suggest that the amount of H_2O_2 produced during oxidative phosphorylation can reach 5% of the amount of O_2 consumed (6). Given that the rate of O_2 consumption in gray matter is 2–5 μmol/g tissue wet weight per min (68, 91), or 2–5 m*M* (assuming 1 g = 1 mL), up to 250 μ*M* H_2O_2 could be generated every minute within brain neuropil. Activity-dependent H_2O_2 generation would be expected to be localized to neurons rather than glia, because of the roughly 10-fold higher rate of neuronal O_2 consumption (121). Absolute concentration of H_2O_2 in a given cell at any time will depend not only on the activity of sources of H_2O_2, but also on the activity of the local antioxidant network, especially GSH peroxidase and catalase (42).

At the present time, there is little consensus on the normal range of intracellular H_2O_2 concentration in brain cells. Accurate assessment of brain cell concentration has been hindered in part by the characteristics of first-generation H_2O_2-sensitive fluorescent dyes, including H_2DCF, which becomes fluorescent DCF after oxidation by H_2O_2 in an irreversible reaction that precludes accurate calibration (14, 100). Direct dye calibration is further complicated by the typically high cellular activities of GSH peroxidase and catalase in brain tissue, which rapidly deplete initial levels of exogenously applied H_2O_2 (see Ref. 52 for review). As discussed further below, confirmation of actual H_2O_2 levels required for signaling awaits a new generation of fluorescent dyes or other detection methods that will allow quantitative assessment of intracellular H_2O_2 concentration.

Nonetheless, there have been a number of attempts to estimate normal intracellular H_2O_2 concentration using biochemical methods. For example, it has been estimated that during steady-state exposure of a non-neuronal cell line, Jurkat T-cells, to micromolar levels of exogenous H_2O_2, intracellular H_2O_2 concentration is maintained at a level that is sevenfold lower than that in the medium, because of intracellular peroxidase activity (4). These data were subsequently used to make

the argument that normal intracellular H_2O_2 concentration cannot exceed ~1 μ*M*, because the threshold for initiation of apoptosis in Jurkat T-cells was between 5 and 10 μ*M* (5, 128).

However, our own data from guinea pig brain slices suggest that *transient* intracellular H_2O_2 elevation can far exceed 1 μ*M* without irreversible damage. As discussed above, inhibition of endogenous GSH peroxidase with MCS leads to enhanced DCF fluorescence in DA neurons of the SNc, with consequent K_{ATP}-channel dependent hyperpolarization in ~50% of recorded cells (14). Application of *exogenous* H_2O_2 causes a similar response in these H_2O_2-responsive neurons, when applied at a concentration of 1.5 m*M*. Similarly, as discussed above, DA release elicited by local pulse-train stimulation (30 pulses, 10 Hz) in the striatum is suppressed by 30–40% when GSH peroxidase is inhibited or when 1.5 m*M* H_2O_2 is applied (12, 38, 39). If a sevenfold extra- to intracellular gradient in H_2O_2 is assumed for conditions of exogenous H_2O_2 application, it would suggest that intracellular levels reached during GSH peroxidase inhibition by MCS might transiently exceed 200 μ*M*. Importantly, the effects of exogenous H_2O_2 and MCS in both midbrain and striatum are reversible and are not accompanied by loss of tissue DA content or evidence of oxidative damage (12, 38). Moreover, as already noted, MCS-induced suppression of striatal DA release does not involve formation of toxic ·OH (12), in contrast to induction of apoptosis in Jurkat T-cells, which is ·OH-dependent (5).

TECHNICAL ISSUES IN H_2O_2 DETECTION: EMPHASIS ON DCF IMAGING

A variety of experimental tools is available to address the biological function of H_2O_2, with known caveats and limitations for the use of each of these. Most of the caveats are related to the specificity of available agents, from catalase, which can metabolize other hydroperoxides in addition to H_2O_2, to H_2DCF, which can be oxidized to fluorescent DCF by many ROS, as discussed further below. Consequently, no single agent or technique can provide conclusive evidence of H_2O_2 involvement; however, a *combination* of methods can, as we have demonstrated in our studies of H_2O_2 in the nigrostriatal pathway (12, 14, 19).

Fluorescence imaging with DCF and other indicator dyes

Fluorescence imaging using H_2DCF has been used for over 30 years to detect increased production of H_2O_2 and other ROS (14, 33, 71, 109, 114, 115). Previous examination of the chemistry of this dye indicates that H_2O_2, a mild oxidant, is less effective than stronger oxidants, like ·OH, in oxidizing H_2DCF to form fluorescent DCF. For example, LeBel *et al*. (87) reported that the increase in DCF fluorescence in the presence of exogenous H_2O_2 is attenuated by ~90% when H_2O_2 was applied along with a metal ion chelator, deferoxamine, indicating that the primary oxidant is ·OH. LeBel *et al*. also found that DCF fluorescence intensity is amplified when H_2O_2 was applied with added iron (Fe^{2+}) (87). However, two aspects of these studies are often overlooked. First, at least 10% of the H_2O_2-dependent increase in DCF fluorescence persists in the presence of deferoxamine, suggesting that H_2O_2 can indeed form fluorescent DCF directly. Second, when H_2O_2 concentration is increased in the presence of Fe^{2+}, DCF fluorescence increases with a strict linear dependence on H_2O_2 concentration (Fig. 4; from 87), demonstrating a clear H_2O_2-dependent response. These findings indicate that DCF fluorescence can be 'H_2O_2-sensitive,' even when activation is indirect (*i.e.*, via ·OH).

Our own studies of H_2O_2-dependent regulation of DA neuron activity support this view (14). We examined the concentration–response of DCF fluorescence intensity in SNc DA neurons with increasing concentrations of the GSH peroxidase inhibitor MCS (Fig. 3). Because it is not possible to calibrate fluorescence intensity of DCF (or other currently available ROS-sensitive dyes), DCF cannot be used to quantify ROS. However, fluorescence intensity can be used as an index of relative changes in the extent and rate of ROS generation when a single variable is changed, as in our studies of progressive GSH peroxidase inhibition in tonically active DA neurons. Under these conditions, DCF fluorescence increases linearly with increasing MCS concentration [*i.e.*, increasing GSH peroxidase inhibition (Fig. 3)], consistent with an H_2O_2-dependent signal (14).

Of course, peroxidase inhibition should produce a relatively 'clean' increase in H_2O_2, whereas most other conditions would not. Such studies mandate corroboration of DCF imaging data with other evidence to confirm H_2O_2 involvement. For example, in our studies of false H_2O_2 signaling in the striatum under conditions of rotenone-induced mitochondrial dysfunction, we used DCF imaging to reveal the time course of ROS generation in medium spiny neurons (19). Two additional sets of data helped confirm that the observed rotenone-induced increased in DCF fluorescence is largely H_2O_2 dependent. First, the increase in fluorescence is markedly attenuated in the presence of exogenous catalase (Fig. 5). Catalase also prevents rotenone-induced effects on DA release and medium spiny neuron membrane properties, further

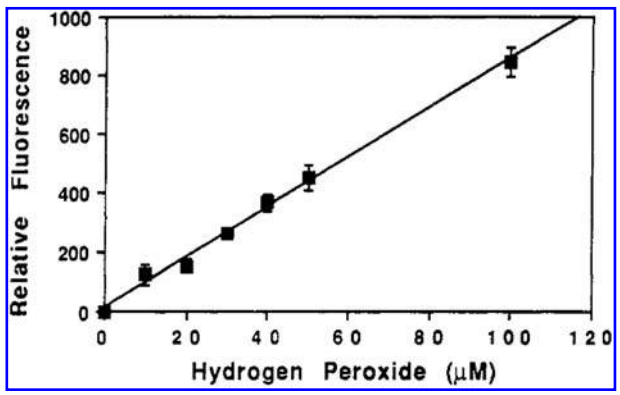

FIG. 4. Concentration–response curve of H_2DCF oxidation in the presence of H_2O_2. "The Fe^{2+} concentration was held constant at 10 μ*M*, and all incubations were performed at 37°C for 5 min. The data were obtained from three independent experiments and are expressed as the means ± SEM." (Figure and legend from Ref. 87.)

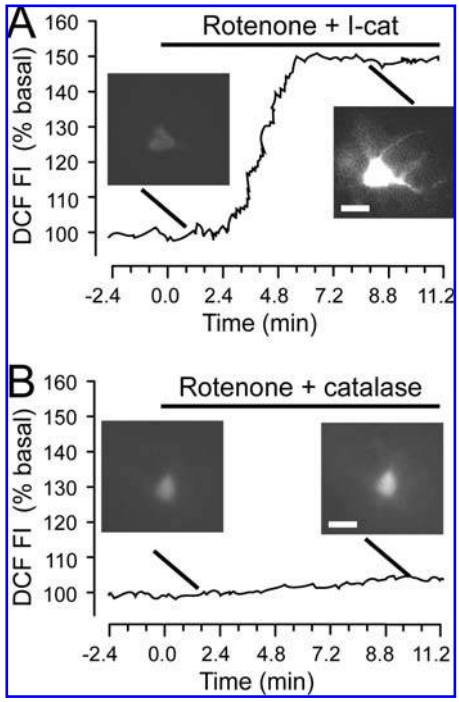

FIG. 5. Rotenone-induced increase in DCF fluorescence intensity (FI) is H_2O_2 dependent. Representative time course of DCF FI changes; a modified grayscale was used to enhance the contrast between basal and rotenone-induced DCF images. (A) Under control conditions (heat-inactivated catalase, I-cat), rotenone (50 n*M*) causes a ~50% increase in DCF FI in a medium spiny neuron in a striatal slice. (B) Rotenone-enhanced FI is prevented by active catalase (500 U/mL; $n = 3$), confirming the primary dependence of the DCF signal on H_2O_2 (data from Ref. 19).

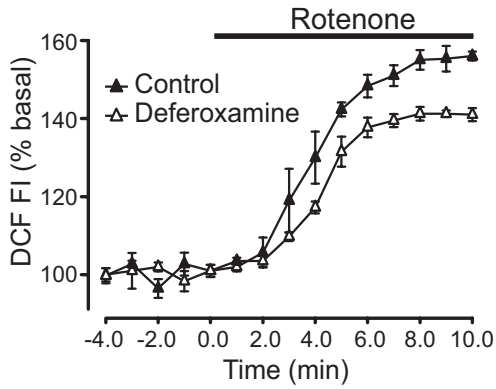

FIG. 6. Effect of metal ion chelation by deferoxamine on rotenone-induced DCF fluorescence intensity (FI). Under control conditions, rotenone (50 n*M*) causes the usual increase in DCF FI. However, when deferoxamine, a metal ion chelator, is included in the backfill solution of the patch-pipette used for simultaneous whole-cell recording, DCF FI is significantly lower than under control conditions ($n = 4$; $p < 0.001$, ANOVA), indicating some contribution of $^{\bullet}OH^{-}$ to the DCF signal seen during partial mitochondrial inhibition by rotenone.

supporting a role for *functionally relevant* H_2O_2 generation (19). Second, to address possible contributions from metal ion-dependent $^{\bullet}OH$, we examined whether the rotenone-induced increases in DCF fluorescence persists in the presence of deferoxamine. With 1 m*M* deferoxamine in both the patch-pipette backfill solution and in the superfusing ACSF, the DCF signal was attenuated by ~30% versus control ($p < 0.001$, $n = 3$) (Fig. 6), suggesting a ~30% contribution of $^{\bullet}OH$ to the DCF signal. Together, these data indicate that, the DCF signal under these conditions is predominantly H_2O_2 dependent and confirm the value of DCF imaging as a companion technique in studies of H_2O_2-dependent neuromodulation.

Other methods for in situ H_2O_2 detection

One other currently available dye for H_2O_2 imaging is Amplex Red (145), which has been used successfully in studies of H_2O_2 generation in isolated mitochondria (*e.g.*, 137). Like the oxidation of H_2DCF to DCF, however, the oxidation of Amplex Red to its fluorescent product is irreversible, also precluding dye calibration. Irreversible activation also means that neither DCF nor Amplex Red imaging can be used to assess the time course of H_2O_2 clearance after elevation. A further drawback of Amplex Red is that its activation apparently requires exogenous peroxidase activity (145). Although there is endogenous GSH peroxidase and catalase in neurons, we have not had success in H_2O_2 detection with Amplex Red loaded into single neurons.

Given the limitations of these currently available dyes, there is increasing interest in the development of new imaging methods to study H_2O_2. Among the most promising are boronate-based dyes, including Peroxyfluor-1 (PF1), that are cell permeable and have excellent selectivity for H_2O_2 (36, 94). The specificity of PF1 and related dyes for H_2O_2 comes from chemoselective boronate deprotection, rather than nonspecific oxidation. Limitations include relatively slow activation and that these first generation dyes cannot readily be trapped in cells. Nonetheless, their introduction heralds an exciting new direction in dye development.

Other methods that have been used with increasing success in cultured cells, including organotypic cultures, are based on expression of redox-sensitive variants of green fluorescent proteins (ro-GFPs) (24, 28, 48, 99). In these proteins, surface-exposed cysteine pairs are introduced that allow disulfide bond formation, making the interaction with H_2O_2 or other ROS a reversible process, which is an advantage over other currently available imaging agents. These probes can be targeted to specific cells after transfection in culture or *in vivo*. At present, however, the response time of these probes is very slow, on the order of minutes. Moreover, because disulfide bond formation required to activate ro-GFPs can occur in the presence of many oxidants, evidence for an H_2O_2-dependent signal must be confirmed using other methods, as described earlier for identification of the source of DCF signals.

Lastly, electrochemical biosensors can be used to detect H_2O_2 in biological systems, including in the extracellular fluid of brain tissue *in vivo* (86). Amperometric H_2O_2 sensors provide the basis for detection of nonelectroactive substances, including glutamate (92) and choline (64), when coupled to selective enzymes that produce detectable H_2O_2. The

extracellular concentration of H_2O_2 detected in striatum during electrical stimulation of the median forebrain bundle is 1–3 μ*M* (86). Given the relative small striatal volume taken up by DA axons, this means that the H_2O_2 concentration attained within these activated axons would necessarily be several-fold higher. The authors suggest that detected H_2O_2 may be derived from the oxidation of released DA in the extracellular space (86). However, this would be surprising, given that the usual homeostatically regulated concentration of ascorbate in the extracellular fluid is ~400 μ*M* (see Ref. 111 for review), which should be sufficient to protect the low micromolar levels of $[DA]_o$ achieved under these conditions. Our *in vitro* brain slice data also argue against DA oxidation as a primary source of activity-dependent H_2O_2. Blockade of AMPARs can cause a doubling of evoked $[DA]_o$ (12), yet under these same conditions, the usual stimulation-induced increase in DCF fluorescence monitored in medium spiny neurons is virtually abolished (102). These findings suggest that there is little contribution from DA oxidation to H_2O_2 generation, even in brain slice preparations, in which there is little residual extracellular ascorbate (111).

SUMMARY AND IMPLICATIONS

In our first report of DA release modulation by endogenous H_2O_2 in striatum (38), we suggested that H_2O_2 might be generated presynaptically at DA synapses to serve as an autoinhibitory signal that would limit DA release after axonal activation, given the close apposition of mitochondria to presynaptic sites in DA axons (*e.g.*, 96). However, our subsequent studies showed that generation of modulatory H_2O_2 requires AMPAR activation and can be fine-tuned by $GABA_AR$ activation. These findings argue against primary H_2O_2 generation in DA axons, since they lack AMPARs and $GABA_ARs$ (22, 40, 63). Rather, we found that generation of modulatory H_2O_2 must occur in non-DA cells or processes then diffuse to DA axons to inhibit DA release. Our data suggest that regulation of striatal DA release by glutamate and GABA involves a triad of DA, glutamate, and GABA synapses, separated by a few micrometers on the dendrites of medium spiny neurons (22, 40, 63, 125), and bound together *functionally* by diffusible H_2O_2. Regardless of the source, endogenously generated H_2O_2 reverses conventional glutamatergic excitation by opening SUR1-based K_{ATP} channels to inhibit striatal DA release. This discovery represents a new and potentially important mechanism of external regulation of DA release that establishes a formerly 'missing link' in the reciprocal relationship between DA and glutamate in striatum. Moreover, because DA-glutamate dysfunction has been implicated as a causal factor in Parkinson disease (37, 65, 97), schizophrenia (46, 118), and addiction (73, 83), exploration of this process may also suggest novel pathways through which dysfunction could occur.

Thus, neuromodulation by H_2O_2 can be a double-edged sword: although activity-dependent H_2O_2 generation may provide important regulation of DA release, an imbalance between H_2O_2 generation and metabolism could result in oxidative stress, which has been implicated in nigrostriatal degeneration in Parkinson's disease (42, 43, 97, 126, 139) and, more recently, as a causal factor in schizophrenia (47, 141). Thus, loss of normal H_2O_2 regulation might contribute to DA-system pathology. It is relevant to note, therefore, that inhibition of GSH peroxidase by MCS leads to DA-neuron hyperpolarization (14) and suppression of somatodendritic DA release in the SNc (39); however, MCS does not inhibit DA release in the adjacent ventral tegmental area (VTA) (39). This difference between SNc and VTA is potentially important, because DA neurons of the SNc degenerate in Parkinson's whereas those in the VTA are relatively spared (60, 140).

Lastly, it should be noted that neuromodulation by H_2O_2 is not limited to the nigrostriatal system. Other studies have shown that H_2O_2 influences characteristics of long-term potentiation in the hippocampus (9, 77, 78), 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 (8). These data, as well as our own findings (12, 14), demonstrate that H_2O_2 can act as an intracellular signaling agent *and* as a diffusible messenger. For H_2O_2 to act at both intracellular and potentially distant targets requires that the brain antioxidant network is structured to allow functional levels of H_2O_2 and other ROS, while at the same time preventing oxidative stress. These requirements add a previously unrecognized dimension to oxidant management. We have proposed (13) that key features of such a permissive environment are the predominance of cytosolic GSH peroxidase activity in glia, with subcompartmentalization of catalase in peroxisomes in both glia and neurons, and the predominance of ·OH-scavenging ascorbate in neurons (42, 52, 111, 113).

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ABBREVIATIONS

ACSF, artificial cerebrospinal fluid; AMPAR, AMPA receptor; ATZ, 3-aminotriazole; CNS, central nervous system; DA, dopamine; $[DA]_o$, extracellular dopamine concentration; DCF, dichlorofuorescein; $GABA_AR$, $GABA_A$ receptor; GSH, glutathione; H_2DCF, 2,7 dichlorodihydrofluorescein; K_{ATP} channels, ATP-sensitive K^+ channels' MCS, mercaptosuccinate; PF1, Peroxyfluor-1; roGFP, redox-sensitive green fluorescent protein; ROS, reactive oxygen species; SNc, substantia nigra pars compacta; SUR, sulfonylurea receptor site; VTA, ventral tegmental area.

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