

# H<sub>2</sub>O<sub>2</sub>: A Dynamic Neuromodulator

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## Abstract

Increasing evidence implicates hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) as an intracellular and intercellular signaling molecule that can influence processes from embryonic development to cell death. Most research has focused on relatively slow signaling, on the order of minutes to days, via second messenger cascades. However, H<sub>2</sub>O<sub>2</sub> can also mediate subsecond signaling via ion channel activation. This rapid signaling has been examined most thoroughly in the nigrostriatal dopamine (DA) pathway, which plays a key role in facilitating movement mediated by the basal ganglia. In DA neurons of the substantia nigra, endogenously generated H<sub>2</sub>O<sub>2</sub> activates ATP-sensitive K<sup>+</sup> (K-ATP) channels that inhibit DA neuron firing. In the striatum, H<sub>2</sub>O<sub>2</sub> generated downstream from glutamatergic AMPA receptor activation in medium spiny neurons acts as a diffusible messenger that inhibits axonal DA release, also via K-ATP channels. The source of dynamically generated H<sub>2</sub>O<sub>2</sub> is mitochondrial respiration; thus, H<sub>2</sub>O<sub>2</sub> provides a novel link between activity and metabolism via K-ATP channels. Additional targets of H<sub>2</sub>O<sub>2</sub> include transient receptor potential (TRP) channels. In contrast to the inhibitory effect of H<sub>2</sub>O<sub>2</sub> acting via K-ATP channels, TRP channel activation is excitatory. This review describes emerging roles of H<sub>2</sub>O<sub>2</sub> as a signaling agent in the nigrostriatal pathway and basal ganglia neurons.

## Keywords

basal ganglia, diffusible messenger, dopamine, hydrogen peroxide, K-ATP channels, reactive oxygen species, transient receptor potential channels

Reactive oxygen species (ROS), including superoxide (•O<sub>2</sub><sup>-</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), and the hydroxyl radical (•OH), are often viewed as toxic waste from cellular oxidative metabolism. Over the past decade, however, accumulating evidence indicates that ROS are normal components of signaling pathways. In particular, H<sub>2</sub>O<sub>2</sub> is implicated in physiological processes ranging from embryonic development to cell death (Coffman and others 2009; D'Autreaux and Toledano 2007; Gerich and others 2009; Groeger and others 2009; Miller and others 2007b; Nishida and others 2000; Rhee 2006; Rhee and others 2005; Rigoulet and others 2010; Stone and Yang 2006; Sundaresan and others 1995). In brain cells, H<sub>2</sub>O<sub>2</sub> has been implicated as an intracellular regulator of neuronal activity, growth, and organelle function (Avshalumov and others 2005; Gerich and others 2009; Lee and Rice 2008; Miller and others 2007b), as well as a diffusible messenger for neuron-glia signaling (Atkins and Sweatt 1999) and interneuronal communication, including regulation of synaptic transmission and plasticity (Auerbach and Segal 1997; Avshalumov and others 2003, 2008; Kamsler and Segal 2003; Klann and Thiels 1999; Nemoto and others 2000; Nishida and others 2000; Samanta and others 1998).

In contrast to many ROS, H<sub>2</sub>O<sub>2</sub> is neither a free radical nor an ion. These properties limit reactivity (Cohen 1994)

and increase membrane permeability (Adimora and others 2010; Bienart and others 2006, 2007; Makino and others 2004; Ramasarma 1982), so that it is well suited as both an intracellular signaling agent and a diffusible messenger. Given its low reactivity, H<sub>2</sub>O<sub>2</sub> does not readily mediate oxidative damage, unless exposed to free metal ions that can catalyze conversion of H<sub>2</sub>O<sub>2</sub> to highly reactive •OH (Cohen 1994). The ability of H<sub>2</sub>O<sub>2</sub> to diffuse away from a site of generation is a characteristic shared with other diffusible messengers, including nitric oxide, a free radical, and carbon monoxide (Dawson and Snyder 1994; Kiss and Vizi 2001). In the case of H<sub>2</sub>O<sub>2</sub>, however, cell-specific membrane permeability, as well as competing effects of the antioxidant network, may govern its efflux and entry (Adimora and others 2010; Bienart and others 2007; Makino and others 2004; Mishina and others 2010).

The first evidence that ROS could regulate neurotransmission was Terry Pellmar's (1986, 1987) finding that

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exogenously applied  $H_2O_2$  can suppress the amplitude of evoked population spikes in hippocampal slices, possibly by inhibiting transmitter release. The possibility of release inhibition was later tested directly in experiments to assess the effect of  $H_2O_2$  on evoked dopamine (DA) release in striatal slices, monitored with carbon-fiber microelectrodes and fast-scan cyclic voltammetry (Chen and others 2001). Those studies not only confirmed that exogenously applied  $H_2O_2$  could reversibly suppress axonal DA release but demonstrated for the first time that endogenously generated  $H_2O_2$  could modulate transmitter release on a sub-second timescale. Subsequent work over the next decade established how  $H_2O_2$  modulates striatal DA release, and further demonstrated that endogenous  $H_2O_2$  also regulates DA neuron activity and somatodendritic DA release in the substantia nigra pars compacta (SNc). Regulation of the nigrostriatal DA system is important because of the central role that this pathway plays in the control of movement by the basal ganglia.

This review summarizes data showing that endogenous  $H_2O_2$  is both an intracellular signal that modulates the activity of individual DA neurons in the SNc and a diffusible messenger in the striatum. The predominant effect of activity-dependent  $H_2O_2$  on the nigrostriatal pathway is inhibitory, mediated by activation of ATP-sensitive  $K^+$  (K-ATP) channels. However, emerging evidence indicates that this dynamically generated modulator can also act at a specific class of transient receptor potential (TRP) channels to have an excitatory effect on non-DA neurons in the striatum and in the substantia nigra pars reticulata (SNr). Also discussed are consequences of false  $H_2O_2$  signaling that can occur during mitochondrial dysfunction, mimicked by partial inhibition of mitochondrial complex I by rotenone. Under these conditions, activity-independent  $H_2O_2$  generation can lead to K-ATP channel opening and suppression of axonal DA release. Thus, elevated  $H_2O_2$  levels can provide both true and false signals in the brain and thereby mediate both physiological and pathophysiological signaling.

## $H_2O_2$ Generation, Regulation, and Functional Concentration

### $H_2O_2$ Generation

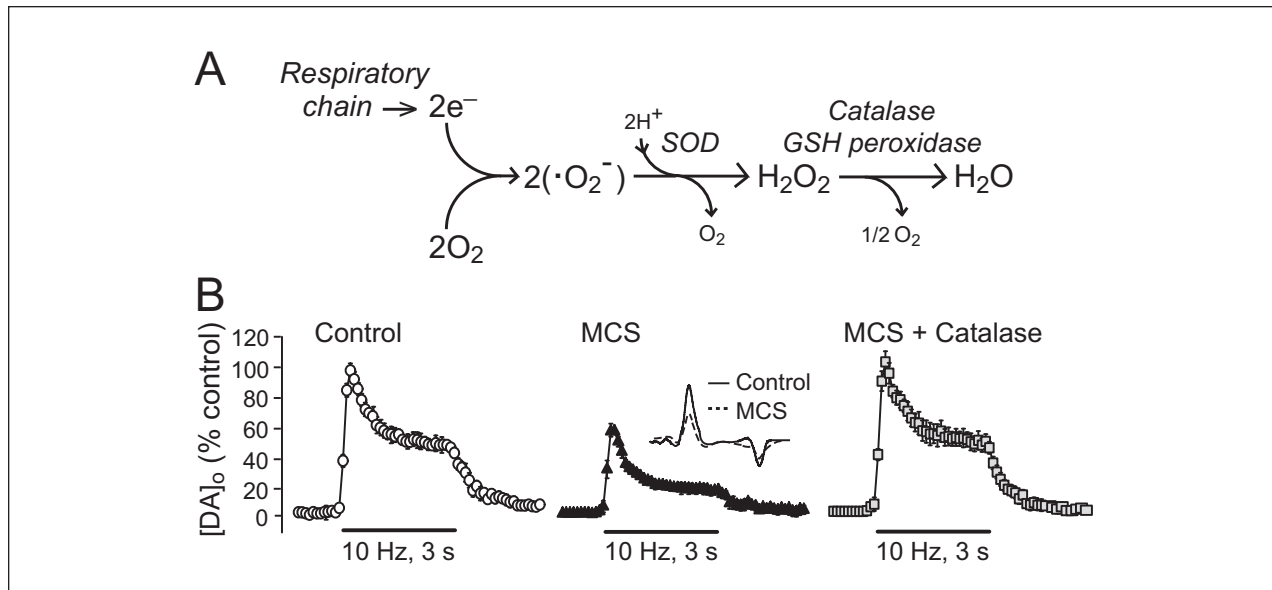
Cellular generation of ROS can occur from a variety of processes, the most ubiquitous of which is mitochondrial respiration (Adam-Vizi, 2005; Bao and others 2009; Boveris and Chance 1973; Dugan and others 1995; Kennedy and others 1992; Liu and others 2002; Peuchen and others 1997). Mitochondria produce  $\bullet O_2^-$  from the single-electron reduction of molecular oxygen (Fig. 1A). Additional sources of  $\bullet O_2^-$  include the enzymes NADPH oxidase

(Barbier 1984; Bedard and Krause 2007; Infanger and others 2006; Lambeth 2004; Rhee 2006) and monoamine oxidase (MAO; Azzaro and others 1985; Maker and others 1981). Levels of  $\bullet O_2^-$  are managed by mitochondrial and cytosolic forms of superoxide dismutase, which convert two molecules of  $\bullet O_2^-$  to  $H_2O_2$  and water (Fig. 1A). Production of  $\bullet O_2^-$  and  $H_2O_2$  is not insignificant: data from isolated brain mitochondria suggest that up to 5% of  $O_2$  consumed becomes  $H_2O_2$  (Arnaiz and others 1999), although this may depend on cell-specific levels of mitochondrial uncoupling proteins and other factors (Liss and others 2005). Generation of  $H_2O_2$  is also enhanced during periods of increased local activity, given the increased  $O_2$  consumption required to reestablish ion gradients.

### $H_2O_2$ Regulation

Absolute intracellular  $H_2O_2$  concentration—and, thus, the availability of  $H_2O_2$  to act as a diffusible messenger—is determined by the balance between activity-dependent  $H_2O_2$  generation and the competitive processes of removal by antioxidant enzymes and interactive thiols and by diffusion. The major peroxidase enzymes are glutathione (GSH) peroxidase, which is cytosolic and mitochondrial (Stults and others 1977), and catalase, which is localized in intracellular peroxisomes (Cohen 1994; Dringen and others 2005; Peuchen and others 1997) (Fig. 1A). Regulation of  $H_2O_2$  and its precursor  $\bullet O_2^-$  by antioxidant enzymes is important because interaction of either  $H_2O_2$  or  $\bullet O_2^-$  with trace metal ions, such as iron and copper, can produce the aggressive radical  $\bullet OH$ , which is managed by the low molecular weight antioxidants GSH and ascorbate (Cohen 1994). Additional regulation of  $H_2O_2$  comes from thioredoxins and other cellular thiols, as well as peroxiredoxins, which are abundant but have lower catalytic efficacy than GSH peroxidase or catalase (Adimora and others 2010; Hofmann and others 2002; Mishina and others 2010; Rhee and others 2001, 2005).

A role for  $H_2O_2$  as a neuromodulator, however, requires more subtle regulation by the antioxidant network than usually considered. This network must be structured to allow levels of  $H_2O_2$  (and other ROS) to reach functional concentrations both intracellularly and at potentially distant targets, yet prevent oxidative damage (Avshalumov and others 2004). Key features of this permissive yet protective environment include 1) the predominance of ascorbate in neurons, which provides good  $\bullet OH$  scavenging without interfering with  $H_2O_2$  signaling; 2) the predominance of cytosolic GSH peroxidase in glia; and 3) subcompartmentalization of catalase in peroxisomes in neurons and glia that help facilitate transient  $H_2O_2$  elevation and subsequent escape from the compartment of generation (Avshalumov and others 2004; Avshalumov



**Figure 1.** Factors that regulate cellular  $H_2O_2$  and consequences of manipulation of some of these on evoked striatal dopamine (DA) release. **A**) Production of  $H_2O_2$  by the mitochondrial respiratory chain and regulation by antioxidant enzymes. Mitochondria produce  $\cdot O_2^-$  from the single-electron reduction of molecular oxygen;  $H_2O_2$  is formed from  $\cdot O_2^-$  by the action of superoxide dismutase (SOD) as well as by spontaneous dismutation.  $H_2O_2$  levels are managed in part by peroxidase enzymes, glutathione (GSH) peroxidase and catalase. **B**) Modulation of striatal DA release evoked using 10 Hz, 30-pulse trains by manipulation of endogenous  $H_2O_2$  availability in guinea pig brain slices. Inhibition of GSH peroxidase by mercaptosuccinate (MCS; 1 mM) leads to suppression of evoked increases in extracellular DA concentration ( $[DA]_o$ ), monitored with carbon-fiber microelectrodes and fast-scan cyclic voltammetry (inset shows DA voltammograms under control conditions and in MCS). Application of catalase (500 IU/mL) in the continued presence of MCS reverses  $H_2O_2$ -dependent DA release suppression. Data are means  $\pm$  SEM (modified from Avshalumov et al. 2003; copyright *Journal of Neuroscience*, used with permission).

and Rice 2002; Cohen 1994; Desagher et al. 1996; Dringen and others 2005; Rice 2000; Rice and Russo-Menna 1998).

### Functional Concentrations of Modulatory $H_2O_2$

As discussed in the previous section, at any given time, cellular levels of  $H_2O_2$  reflect the balance among generation, metabolism, and  $H_2O_2$  diffusion into and out of a cell. However, there is little consensus on the range of physiologically relevant intracellular  $H_2O_2$  concentrations. Determination of absolute concentrations has been hindered by the characteristics of first-generation  $H_2O_2$ -sensitive fluorescent dyes, exemplified by dihydrodichlorofluorescein, which becomes fluorescent dichlorofluorescein (DCF) after oxidation by  $H_2O_2$  in an irreversible reaction. Dye irreversibility precludes accurate calibration, because fluorescence intensity is proportional to the number of molecules activated throughout the monitoring period rather than to concentration per se (Avshalumov and others 2007; Oyama and others 1994). Attempts to determine intracellular  $H_2O_2$  levels have been complicated further by high cellular peroxidase activity, which rapidly depletes known con-

centrations of exogenous  $H_2O_2$  (for review, see Dringen and others 2005). Nonetheless, upper limits from 100 nM to 1  $\mu$ M  $H_2O_2$  have been estimated experimentally using cultured cells or using mathematical models of contributing factors (Adimora and others 2010; Antunes and Cadenas 2000, 2001; Stone 2004). The upper limits determined experimentally are usually indicated by the onset of irreversible damage, with predictions of intracellular  $H_2O_2$  levels that are 10- to 100-fold lower than the concentrations of  $H_2O_2$  applied exogenously. Consistent with these predictions and the strong regulation by peroxidase activity they imply, endogenous peroxidase activity in guinea pig hippocampal slices is sufficient to prevent irreversible physiological damage during exposure to exogenous  $H_2O_2$  at a concentration of 1.5 mM for 15 minutes; however, damage is seen when either GSH peroxidase or catalase is inhibited pharmacologically before  $H_2O_2$  application (Avshalumov and others 2004). Other studies have shown that mitochondrial function is not impaired by mM levels of  $H_2O_2$  when the brain antioxidant network is intact (Gerich and others 2009). Strong  $H_2O_2$  regulation is also seen in the steep concentration-response for hippocampal population spike inhibition, with no suppression with 1.2 mM  $H_2O_2$ , but maximal

(80%-90%) suppression with 1.5 mM (Avshalumov and others 2000). Based on models that predict intracellular  $H_2O_2$  concentrations that are 10- to 100-fold lower than that applied exogenously, these data imply reversible efficacy at cellular levels of 15-150  $\mu M$ .

Of course, these calculations do not directly assess the range of endogenous  $H_2O_2$  fluctuations required for cell signaling. Comparisons of the effect of exogenously applied and endogenously elevated  $H_2O_2$  do begin to address this, however. The concentration of exogenous  $H_2O_2$  required for suppression of the hippocampal population spike, 1.5 mM, is also required to cause a reversible 30%-40% suppression of DA release evoked by local electrical stimulation in guinea pig striatal slices (Chen and others 2001, 2002). As in hippocampal slices, there is no evidence of oxidative damage in  $H_2O_2$ -exposed striatal slices; moreover, striatal DA content is unaltered, indicating that the observed decrease in evoked extracellular DA concentration ( $[DA]_o$ ) is not from oxidative loss of the releasable pool of DA (Chen and others 2001). Importantly, when endogenous  $H_2O_2$  signaling in the striatum is amplified by inhibition of GSH peroxidase with mercaptosuccinate (MCS; 1 mM), a 30%-40% decrease in evoked  $[DA]_o$  is also seen (Avshalumov and others 2003; Chen and others 2002); this effect is reversible by exogenous catalase, confirming that it is  $H_2O_2$  dependent (Fig. 1B). Amplification of endogenous  $H_2O_2$  with MCS causes no change in striatal DA content (Avshalumov and others 2008). As discussed below, MCS amplifies endogenous levels of  $H_2O_2$  generated during this brief, 3-second stimulation, with suppression of evoked  $[DA]_o$  seen within the first few hundred milliseconds of stimulation. This dynamic regulation is much faster than the slower processes of  $H_2O_2$  generation involved in development, for example, which occur over a time frame of minutes to hours.

The similar degree of suppression of DA release with either exogenously added or endogenously elevated  $H_2O_2$  on DA release implies that comparable  $H_2O_2$  signaling is achieved under both conditions. These data further suggest that levels of  $H_2O_2$  for physiologically relevant signaling without oxidative damage are also in the range of 15 to 150  $\mu M$ . Fortunately, the question of functional  $H_2O_2$  concentrations in the intra- and extracellular compartments of brain tissue may be resolved in the near future, given the advent of new tools for monitoring tissue levels of  $H_2O_2$ , including second-generation imaging dyes and fluorescent protein-based probes (Jin and others 2010; Meyer and Dick 2010; Miller and others 2007a; Srikun and others 2010), as well as real-time electrochemical methods (Li and others 2010; Sanford and others 2010).

## $H_2O_2$ Generation in SNc DA Neurons and Striatal Medium Spiny Neurons

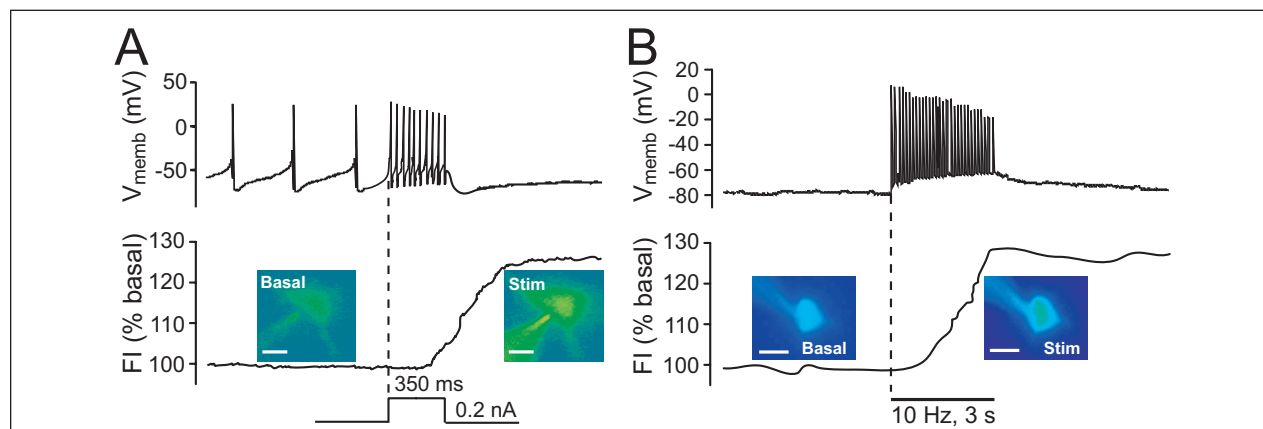
Despite the limitations of DCF fluorescence imaging for quantitative evaluation of absolute cellular  $H_2O_2$  concentrations, this dye has been used to monitor relative basal and stimulated levels of  $H_2O_2$ . Basal DCF fluorescence is detected in all SNc DA neurons examined (Fig. 2A), indicating tonic, activity-dependent  $H_2O_2$  generation during the spontaneous pacemaker firing that these cells exhibit in brain slice preparations (Avshalumov and others 2005). Basal  $H_2O_2$  levels can be amplified in a concentration-dependent manner by the GSH-peroxidase inhibitor MCS (Avshalumov and others 2005). Moreover, in the absence of MCS, cellular  $H_2O_2$  levels can be elevated dynamically when DA neuron firing rate is increased during depolarizing current injection: a 25%-30% increase in DCF fluorescence is seen with an increase in mean spike frequency from ~3 Hz to ~32 Hz (Fig. 2A) (Avshalumov and others 2005). Both basal and elevated  $H_2O_2$  levels have significant effects on the spontaneous firing of SNc DA neurons, as discussed below.

In contrast to SNc DA neurons, which are spontaneously active *in vitro*, striatal medium spiny neurons (MSNs) are typically electrically silent in brain slice preparations. Nonetheless, under control conditions, basal DCF fluorescence is also seen in striatal MSNs, reflecting a basal  $H_2O_2$  tone (Fig. 2B). During local electrical stimulation, each pulse of the stimulus train (30 pulses, 10 Hz) elicits a single action potential in MSNs (Fig. 2B). This stimulus paradigm also produces a ~30% increase in DCF fluorescence in a majority of MSNs (Avshalumov and others 2008) (Fig. 2B). Blockade of glutamatergic AMPA receptors (AMPA) prevents stimulus-induced action potentials, as well as activity-dependent  $H_2O_2$  generation in these cells. Interestingly, when 30 brief current injection pulses are used instead of local stimulation to evoke action potentials in MSNs, no increase in DCF fluorescence is seen (Avshalumov and others 2008). One explanation is that glutamate-receptor activation is an important upstream step in the generation of modulatory  $H_2O_2$  in the striatum.

## Regulation of SNc DA Neuron Activity by $H_2O_2$ and K-ATP Channels

### Basal $H_2O_2$ Levels Modulate the Firing Rate of SNc DA Neurons via K-ATP Channels

Does tonically generated  $H_2O_2$  in SNc DA neurons (Fig. 2A) influence cell activity? Yes, it does. Depletion



**Figure 2.** Basal and activity-dependent  $\text{H}_2\text{O}_2$  generation in SNc dopamine (DA) neurons and striatal medium spiny neurons (MSNs). Representative examples of simultaneous current-clamp recordings of membrane voltage ( $V_{\text{memb}}$ ) and intracellular  $\text{H}_2\text{O}_2$  indicated by changes in DCF fluorescence intensity (FI) in guinea pig striatal or midbrain slices. The time course of stimulus-induced changes in DCF FI is shown with pseudocolor images recorded under basal conditions and at the end of stimulation (scale bar = 20  $\mu\text{m}$  in DCF images). A) In all SNc DA neurons tested ( $n = 17$ ), depolarizing current injection (0.2 nA, 350 ms) induced an increase in firing rate (upper panel) accompanied by elevated  $\text{H}_2\text{O}_2$  levels ( $P < 0.01$  vs. basal FI; lower panel). Dashed vertical line indicates onset of current injection (modified from Avshalumov and others 2005; copyright *Journal of Neuroscience*, used with permission). B) In all striatal MSNs tested ( $n = 11$ ), local pulse-train stimulation (30 pulses, 10 Hz) generated a single action potential with each stimulus pulse (upper panel). In 7 of 11 MSNs, this activity was accompanied by a significant increase in DCF FI ( $P < 0.01$  vs. basal) (lower panel) (modified from Avshalumov and others 2008; copyright American Physiological Society, used with permission).

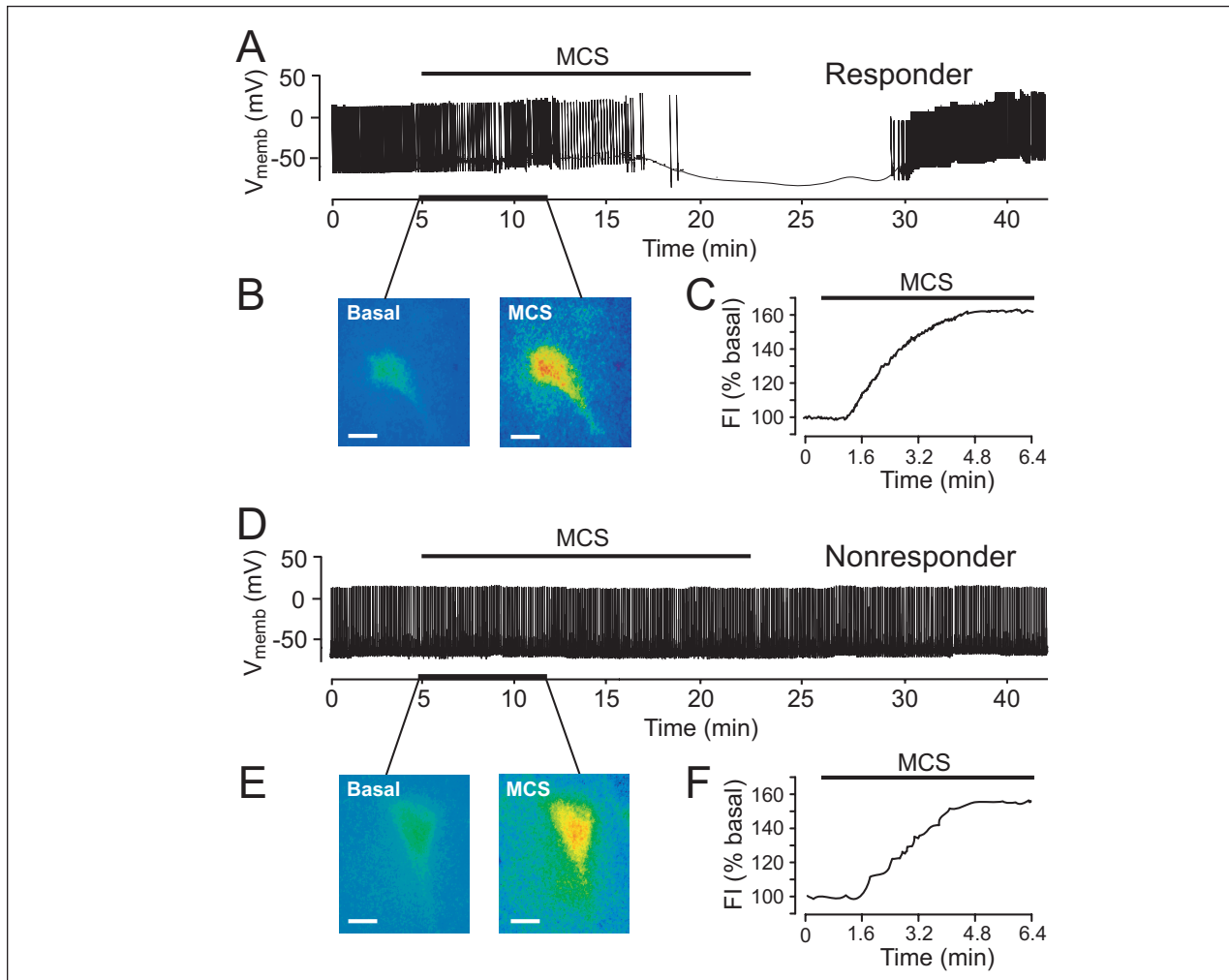
of intracellular  $\text{H}_2\text{O}_2$  by including catalase in the recording pipette used for whole-cell recording causes a ~40% increase in spontaneous firing rate in all DA neurons tested (Avshalumov and others 2005). The mechanism by which  $\text{H}_2\text{O}_2$  regulates cellular activity involves  $\text{H}_2\text{O}_2$ -dependent opening of K-ATP channels (Avshalumov and others 2005). Blocking K-ATP channels in SNc DA neurons not only causes a similar increase in spontaneous firing rate to that seen with catalase but occludes the usual increase with catalase (Avshalumov and others 2005).

K-ATP channels are octameric proteins composed of four inward rectifier  $\text{K}^+$  channel subunits that form a central pore, typically Kir6.2 in neurons and Kir6.1 in glia (Ascroft and Gribble 1998; Karschin and others 1997), and four surrounding sulfonylurea-binding subunits, either SUR1 or SUR2 (Aguilar-Bryan and others 1998; Babenko and others 1998). Channels based on SUR1 or SUR2 subunits can be distinguished by their differential sensitivity to K-ATP channel openers (Babenko and others 2000; Inagaki and others 1996). Previous physiological studies demonstrated that exogenous  $\text{H}_2\text{O}_2$  can cause membrane hyperpolarization by activating a  $\text{K}^+$  conductance in a variety of cell types, including pancreatic  $\beta$  cells (Krippel-Drews and others 1999) and CA1 hippocampal neurons (Seutin and others 1995). Studies of the nigrostriatal pathway provided the first evidence that endogenous  $\text{H}_2\text{O}_2$  can activate K-ATP channels (Avshalumov and Rice 2003; Avshalumov and others 2003, 2005).

The effect of catalase on DA neuron firing rate and the occlusion of this by a nonselective K-ATP channel blocker, glibenclamide, indicate that basal  $\text{H}_2\text{O}_2$  contributes to regulation of DA neuron excitability by maintaining a K-ATP channel tone (Avshalumov and others 2005). Note that the backfill solution in those whole-cell recording studies contained 3 mM ATP, at which concentration, K-ATP channels in DA neurons should be closed (Häusser and others 1991). 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 have shown a direct, concentration-dependent effect of  $\text{H}_2\text{O}_2$  on K-ATP channel opening by decreasing channel sensitivity to ATP (Ichinari and others 1996). Whether regulation by  $\text{H}_2\text{O}_2$  in intact cells is direct or indirect in DA neurons remains to be determined.

### *$\text{H}_2\text{O}_2$ Elevation Can Inhibit SNc DA Neuron Firing via K-ATP Channels*

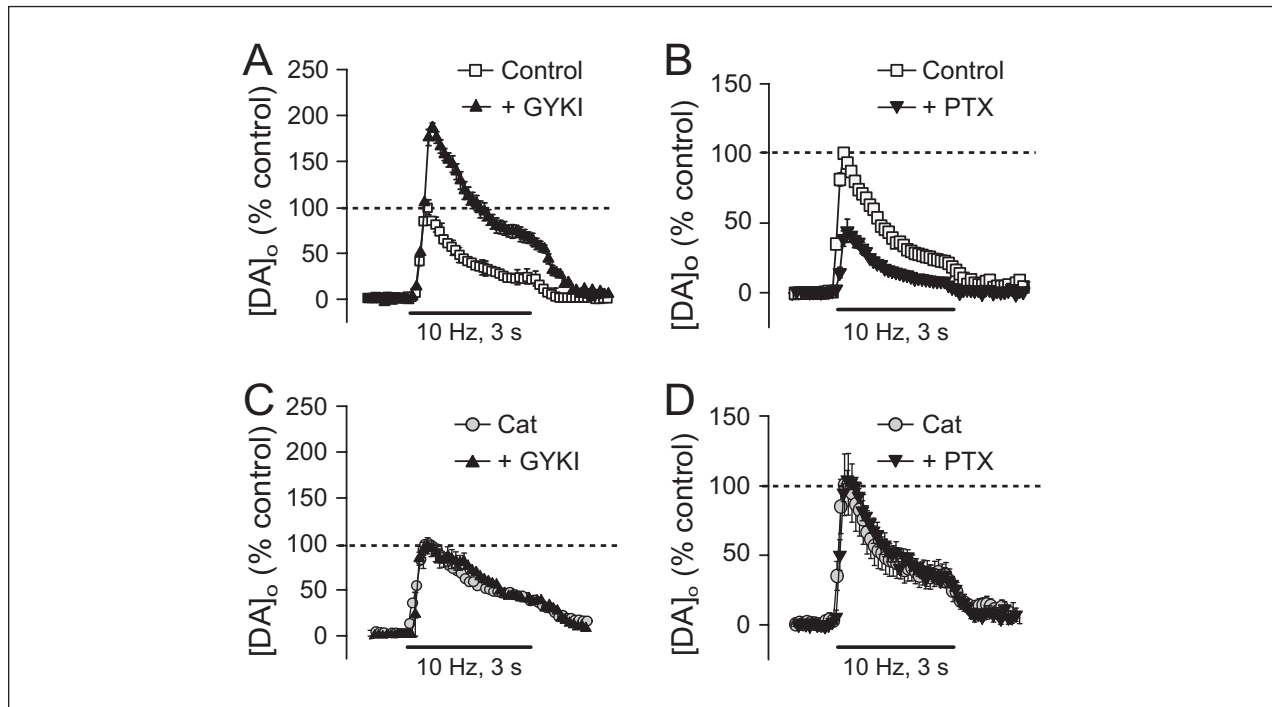
Does elevation of cellular  $\text{H}_2\text{O}_2$  above basal levels have additional effects on SNc DA neuron activity? The answer depends on the level of  $\text{H}_2\text{O}_2$  elevation. Whole-cell recording studies with simultaneous fluorescence imaging of DCF to visualize intracellular  $\text{H}_2\text{O}_2$  in DA neurons showed that moderate increases in  $\text{H}_2\text{O}_2$  ( $\leq 25\%$  increase in fluorescence intensity) during partial inhibition of GSH peroxidase by MCS (0.1-0.3 mM) has no effect on



**Figure 3.** Effects of glutathione (GSH) peroxidase inhibition on membrane properties and intracellular  $H_2O_2$  levels in SNc dopamine (DA) neurons in guinea pig midbrain slices. **A**) Inhibition of GSH peroxidase by mercaptosuccinate (MCS, 1 mM) causes hyperpolarization and cessation of spontaneous activity in one population of DA neurons (responders;  $n = 20/38$ ). **B**) Simultaneously recorded DCF fluorescence in the responder in **(A)** before (basal) and during MCS exposure; **(C)** time course of  $H_2O_2$  elevation (fluorescence intensity, FI) in this cell. **D**) MCS does not affect spontaneous activity in a second population of DA neurons (nonresponders;  $n = 18/38$ ). **E**) Simultaneously recorded DCF fluorescence images before and during MCS in the nonresponder in **(D)**; **(F)** time course of  $H_2O_2$  elevation in this cell (scale bars in DCF images = 20  $\mu m$ ) (modified from Avshalumov and others 2005; copyright *Journal of Neuroscience*, used with permission).

DA neuron firing rate. However, with greater GSH inhibition (1 mM MCS) (Fig. 3A) or application of exogenous  $H_2O_2$  (1.5 mM), 50% of recorded cells, “responders,” show K-ATP channel-dependent hyperpolarization within minutes of  $H_2O_2$  elevation (Fig. 3B,C) (Avshalumov and others 2005). The other 50% of recorded cells show no change in membrane properties with MCS (Fig. 3D), although the increase in DCF FI with MCS in “nonresponders” was indistinguishable from that in responders (Fig. 3E, 3F). This differential responsiveness is similar to that of metabolically “sensitive” and “insensitive” DA neurons described by Liss and others (1999), who showed that greater metabolic sensitivity is conveyed by SUR1- versus SUR2-based K-ATP channels.

Differential responsiveness to  $H_2O_2$  elevation also proved to be a consequence of channel subtype. Using K-ATP channel openers with some selectivity for either SUR1- or SUR2-based channels showed that  $H_2O_2$  responders hyperpolarize with SUR1-selective diazoxide but not SUR1-selective cromakalim, with the opposite pattern seen in nonresponders (Avshalumov and others 2005). These data suggest that SUR1 expression conveys enhanced sensitivity to  $H_2O_2$  elevation. Interestingly, when endogenous  $H_2O_2$  levels are increased by inhibiting catalase, the predominant peroxidase in SNc (Hung and Lee 1998), with 3-aminotriazole (ATZ), all DA neurons respond with glibenclamide-reversible hyperpolarization. Fluorescence imaging with DCF showed a more



**Figure 4.** Regulation of dopamine (DA) release in dorsal striatum by glutamate and GABA requires  $H_2O_2$ . A) AMPAR blockade by GYKI-52466 (GYKI; 50  $\mu$ M) causes a  $\sim 100\%$  increase in evoked extracellular DA concentration ( $[DA]_o$ ) in striatum ( $P < 0.001$ ,  $n = 6$ ). B) GABA<sub>A</sub> receptor blockade by picrotoxin (PTX; 100  $\mu$ M) causes a  $\sim 50\%$  decrease in evoked  $[DA]_o$  ( $P < 0.001$ ,  $n = 6$ ). C) The effect of AMPAR blockade is prevented by catalase (Cat), an  $H_2O_2$ -metabolizing enzyme. D) Catalase abolishes the effect of picrotoxin. Responses in the presence of heat-inactivated catalase were the same as control. Data are means  $\pm$  SEM, shown as percentage of same-site control (modified from Avshalumov and others 2003; copyright *Journal of Neuroscience*, used with permission).

rapid and slightly greater increase in DFC FI with ATZ than with MCS (Avshalumov and others 2005), suggesting that SUR2- as well as SUR1-based K-ATP channels can be activated by sufficiently high and/or rapid  $H_2O_2$  transients. Overall, these data show that  $H_2O_2$  has an autoregulatory role in SNc DA neurons, in which an activity-dependent  $H_2O_2$  generation suppresses neuronal activity via K-ATP channels, adding a new factor in the reciprocal relationship between metabolism and excitability in these cells.

### Modulation of DA Release in Dorsal Striatum by Glutamate and GABA Requires AMPAR-Dependent $H_2O_2$ Generation and K-ATP Channels

#### Regulation of Striatal DA Release by Glutamate and GABA Requires $H_2O_2$

Endogenous  $H_2O_2$  also provides regulation of the nigrostriatal DA pathway at the level of DA axons in the dorsal striatum. As discussed, locally evoked DA release in the striatum can be suppressed by exogenous  $H_2O_2$

or by elevation of endogenous  $H_2O_2$  during peroxidase inhibition (Avshalumov and others 2003, 2008; Chen and others 2001, 2002) (Fig. 1B). However, it is important to appreciate that dynamically generated  $H_2O_2$  modulates striatal DA release, without amplification by peroxidase inhibition. This was first seen in experiments to examine regulation of DA release by endogenous glutamate and GABA in guinea pig striatal slices. Local electrical stimulation elicits the release of these transmitters, as well as release of DA. The effect of concurrently released glutamate and GABA on  $[DA]_o$  evoked by pulse train stimulation (e.g., 10 Hz, 30 pulses) can be assessed using selective receptor antagonists. An initially surprising result in these experiments was that blockade of AMPARs by GYKI-52466 causes up to a 100% increase in evoked  $[DA]_o$ , indicating that AMPAR activation normally leads to inhibition of DA release in dorsal striatum (Avshalumov and others 2003) (Fig. 4A). The apparent absence of ionotropic glutamate receptors on DA terminals (Bernard and Bolam 1998; Chen and others 1998) suggested that any glutamatergic influence must be indirect, possibly through AMPAR-dependent regulation of GABAergic microcircuitry. However, neither GABA<sub>A</sub> receptor (GABA<sub>A</sub>R) nor GABA<sub>B</sub> receptor (GABA<sub>B</sub>R) blockade causes an increase in evoked  $[DA]_o$ . In fact, blockade of

GABA<sub>A</sub>Rs by picrotoxin causes a nearly 50% decrease in peak evoked  $[DA]_0$  (Fig. 4B), and blockade of GABA<sub>B</sub>Rs by saclofen is without effect (Avshalumov and others 2003). These data indicate that GABA<sub>A</sub>, acting at GABA<sub>A</sub>Rs, normally enhances DA release. The influence of GABA on DA release, like that of glutamate, must be indirect, since DA axons in dorsal striatum apparently do not express GABA<sub>A</sub>Rs (Fujiyama and others 2000).

Previous observations that elevation of endogenous H<sub>2</sub>O<sub>2</sub> can inhibit DA release (Chen and others 2001, 2002) and that glutamate-receptor activation can enhance mitochondrial H<sub>2</sub>O<sub>2</sub> generation (Bindokas and others 1996; Carriedo and others 2000; Dugan and others 1995; Reynolds and Hastings 1995) led to the hypothesis that AMPAR-dependent suppression of DA release might be mediated by H<sub>2</sub>O<sub>2</sub>. This proved to be true. The dramatic effect of AMPAR blockade on evoked DA release is abolished in the presence of exogenous catalase (Fig. 4C) or GSH peroxidase (Avshalumov and others 2003). The effect of GABA<sub>A</sub>R blockade by picrotoxin on evoked  $[DA]_0$  is also prevented by catalase (Fig. 4D), demonstrating that GABAergic regulation of striatal DA release requires H<sub>2</sub>O<sub>2</sub> generation as well. The additional finding that picrotoxin has no effect when AMPARs are blocked suggests that regulation of striatal DA release by glutamate and GABA depends on a common source of modulatory H<sub>2</sub>O<sub>2</sub> (Avshalumov and others 2003).

This AMPAR-dependent process appears to be the primary source of modulatory H<sub>2</sub>O<sub>2</sub> in the striatum, because the usual inhibitory effect of GSH peroxidase inhibition by MCS on DA release is absent when AMPARs are blocked by GYKI-52466 (Avshalumov and others 2003). In contrast to the tonic regulation of SNc DA neuron activity by basal H<sub>2</sub>O<sub>2</sub> levels, MCS has no effect on DA release evoked by a single stimulus pulse (Avshalumov and others 2003), implying that there is little tonic regulation of the excitability of DA axons by H<sub>2</sub>O<sub>2</sub> in striatal slices. However, suppression of evoked  $[DA]_0$  is seen within a few hundred milliseconds of the onset of pulse-train stimulation, indicating that dynamically generated H<sub>2</sub>O<sub>2</sub> downstream from AMPAR activation can rapidly suppress DA release during concurrent or subsequent stimulation of DA axons.

### Activation of K-ATP Channels Underlies H<sub>2</sub>O<sub>2</sub>-Dependent Inhibition of Striatal DA Release

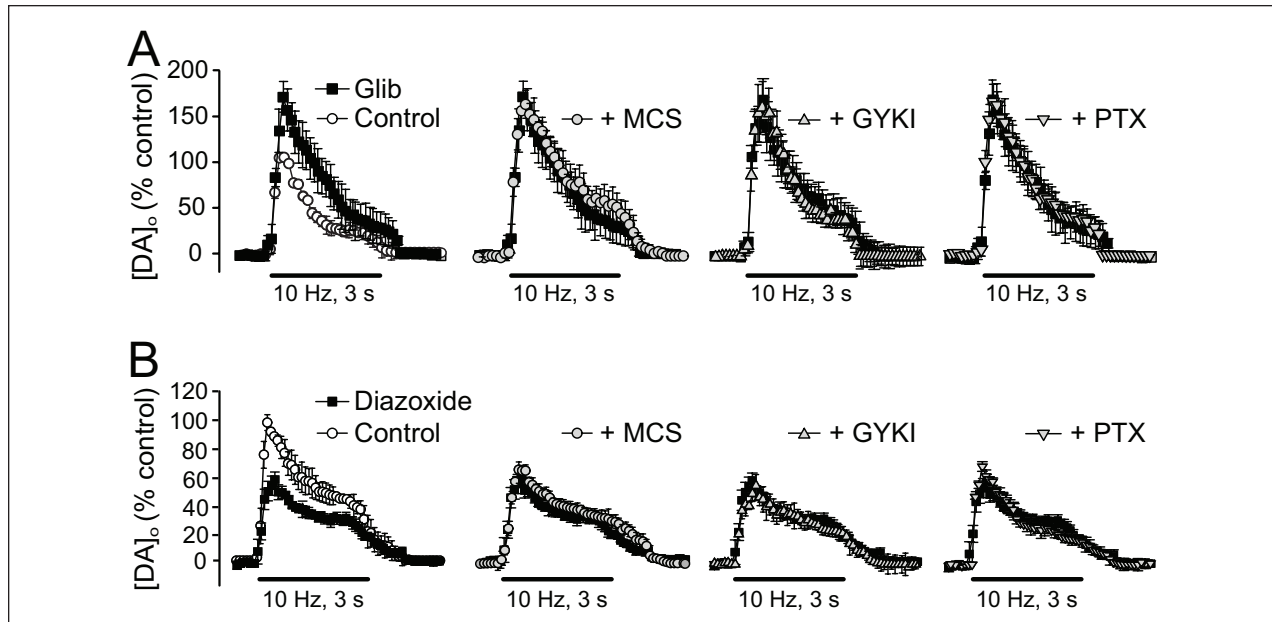
As in SNc DA neurons, elevation of H<sub>2</sub>O<sub>2</sub> in dorsal striatum leads to the opening of K-ATP channels, presumably located on DA axons, to inhibit striatal DA release (Avshalumov and others 2003, 2005; Avshalumov and

Rice 2003). Antagonism of K-ATP channels using sulfonylurea agents, either tolbutamide (Avshalumov and others 2003) or glibenclamide (Fig. 5A) (Avshalumov and Rice 2003), causes a significant increase in  $[DA]_0$  evoked by pulse-train stimulation, indicating that inhibitory K-ATP channels are normally activated during local stimulation and mediate dynamic suppression of DA release. Furthermore, when K-ATP channels are blocked, the usual effects of MCS, GYKI-52466, and picrotoxin are prevented (Fig. 5A), demonstrating that K-ATP channels are required for modulation of DA release by H<sub>2</sub>O<sub>2</sub>, glutamate, and GABA.

Are SUR1-based K-ATP channels again involved? Interestingly, activation of K-ATP channel opening by either diazoxide or cromakalim causes ~40% decrease in evoked DA release (Fig. 5B) (Avshalumov and Rice 2003). However, in the presence of diazoxide, the effects of MCS, GYKI-52466, and picrotoxin on DA release are lost (Fig. 5B), whereas these changes persist after K-ATP channel opening by SUR2-selective cromakalim, implying that glutamate-H<sub>2</sub>O<sub>2</sub>-dependent modulation of striatal DA release involves SUR1-based K-ATP channels (Avshalumov and Rice 2003). Thus, differential SUR1 versus SUR2 expression appears to be maintained throughout the nigrostriatal pathway, although the functional significance of this remains unexplored.

### Modulatory H<sub>2</sub>O<sub>2</sub> in Dorsal Striatum Is Generated in MSNs

To address the cellular source of AMPAR-dependent H<sub>2</sub>O<sub>2</sub> signaling requires a general understanding of striatal circuitry and receptor localization. The overall circuitry of the basal ganglia is relatively well established (Albin and others 1989; Kemp and Powell 1971; Smith and Bolam 1990). The dorsal striatum receive excitatory input from cortex and thalamus and provide the major inhibitory output of the basal ganglia to subcortical regions (Albin and others 1989; Smith and Bolam 1990). The principal striatal efferent cells are GABAergic MSNs (Kemp and Powell 1971), which receive synaptic glutamate input to their dendrites (Bernard and Bolam 1998; Chen and others 1998; Smith and Bolam 1990). These neurons also receive synaptic DA input from SNc DA neurons in the midbrain (Albin and others 1989; Smith and Bolam 1990). The absence of ionotropic glutamate receptors (Bernard and Bolam 1998; Chen and others 1998) and GABA<sub>A</sub>Rs (Fujiyama and others 2000) on DA axons suggests that these are not the source of glutamate-dependent H<sub>2</sub>O<sub>2</sub> generation. Prevention of the modulatory effects of endogenous glutamate and GABA by exogenously applied peroxidase enzymes, which are likely to remain in the extracellular compartment, is also consistent with a requirement for extracellular diffusion



**Figure 5.** Inhibition of striatal dopamine (DA) release by endogenous  $H_2O_2$  is mediated by K-ATP channels. **A**) K-ATP-channel blockade by glibenclamide (Glib; 3  $\mu$ M) increases evoked extracellular DA concentration ( $[DA]_o$ ) in guinea pig striatal slices ( $P < 0.01$ ,  $n = 5$ ) and prevents the usual modulation of DA release by MCS (1 mM), GYKI-52466 (GYKI; 50  $\mu$ M), and picrotoxin (PTX; 100  $\mu$ M) ( $n = 5$  for each). **B**) Diazoxide (30  $\mu$ M), a SUR1-selective  $K_{ATP}$ -channel opener, decreases evoked  $[DA]_o$  ( $P < 0.01$ , diazoxide vs. control;  $n = 5$ ) and also abolishes the effects of MCS, GYKI-52466, and PTX ( $n = 5$ ). Data are means  $\pm$  SEM (modified from Avshalumov and Rice 2003; copyright 2003 National Academy of Sciences, USA, used with permission).

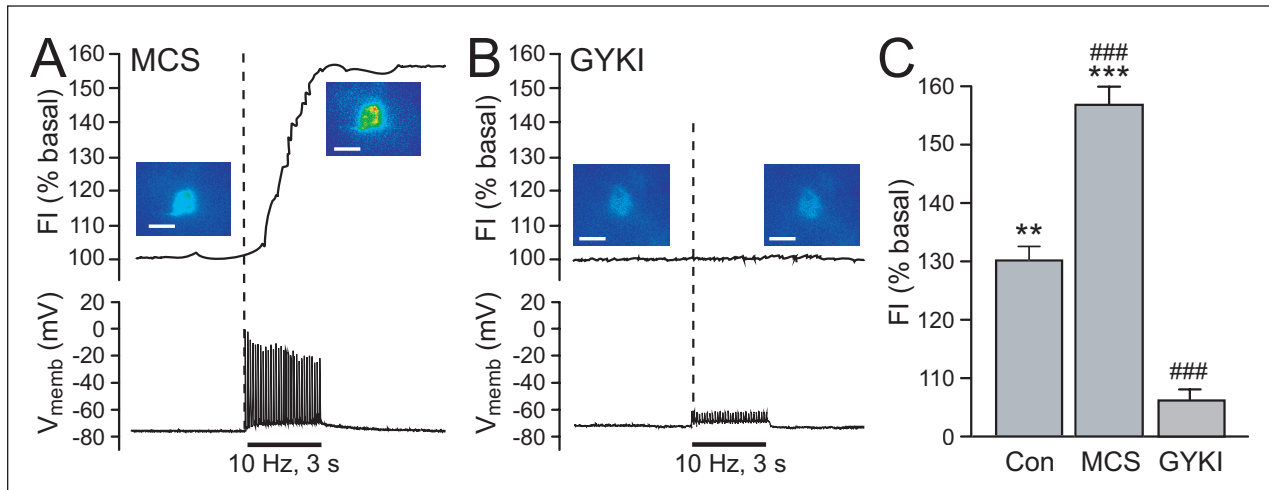
of  $H_2O_2$  to inhibit axonal DA release from presynaptic (or nonsynaptic) sites.

Current evidence implicates MSNs as the primary cellular source of modulatory  $H_2O_2$  in dorsal striatum (Avshalumov and others 2008). Not only are these the most abundant striatal neurons (90%-95% of the striatal neuron population; Kemp and Powell 1971), but the pattern of sensitivity of DA release to glutamate and GABA antagonists (Avshalumov and others 2003) mirrors the electrophysiological responsiveness of these cells (Jiang and North 1991; Kita 1996). As already discussed, local stimulation causes a  $\sim$ 30% increase over basal DCF fluorescence in a majority of MSNs (Fig. 2B); this response doubles when GSH peroxidase is inhibited by MCS, confirming amplification of  $H_2O_2$  signaling with MCS (Avshalumov and others 2008) (Fig. 6A, 6C). Conversely, AMPAR blockade by GYKI-52466 eliminates both stimulus-induced action potentials in MSNs and activity-dependent  $H_2O_2$  generation (Fig. 6B, 6C). These patterns of modulation of  $H_2O_2$  levels in MSNs are consistent with the patterns of DA release regulation seen with MCS and GYKI-52466.

The first report of DA release inhibition by endogenous  $H_2O_2$  concluded with the hypothesis that DA axons might be the primary source of activity-dependent  $H_2O_2$

(Chen and others 2001), given the abundance of axonal mitochondria within a few hundred nanometers of DA synapses (Nirenberg and others 1997). In this scenario,  $H_2O_2$  would be an autoinhibitor of DA release, in the same way that activity-dependent  $H_2O_2$  is an autoregulator of SNc DA neuron activity (Avshalumov and others 2005). However, subsequent results do not support a role for DA axons in self-generation of modulatory  $H_2O_2$ . Most obvious, the requirement for AMPAR activation (Avshalumov and others 2003) suggests that DA axons in dorsal striatum cannot be the primary source of modulatory  $H_2O_2$  because they lack AMPARs. Moreover, when AMPARs are blocked, the usual inhibition of evoked  $[DA]_o$  seen during GSH peroxidase by MCS is absent, implying that there is no  $H_2O_2$  signal to amplify (Avshalumov and others 2003).

Direct evidence for lack of DA axon involvement has come from experiments using parasagittal slices that allow distal pathway stimulation of DA axons without concurrent activation of local corticostriatal or thalamostriatal input (Avshalumov and others 2008). In those experiments, DA release was recorded from a single site during alternating local and pathway stimulation. The absence of concurrent glutamate release was indicated by a lack of effect of GYKI-52466 on pathway-evoked  $[DA]_o$  but the



**Figure 6.** Patterns of  $H_2O_2$  regulation in striatal medium spiny neurons (MSNs) mirror those predicted from modulation of evoked striatal dopamine (DA) release. **A**) Inhibition of glutathione (GSH) peroxidase by MCS (1 mM) amplifies stimulus-evoked increases in DCF FI (30 pulses, 10 Hz) (upper panel), with no effect on action potential generation in recorded MSNs (lower panel). In MCS, 7 of 7 MSNs showed a significant increase in DCF FI ( $P < 0.001$ ). **B**) The usual stimulus-induced increase in DCF FI in MSNs (Fig. 2B) is prevented by an AMPAR antagonist, GYKI-52466 (50–100  $\mu$ M) (upper panel), an AMPAR antagonist, as are stimulus-evoked action potentials monitored during simultaneous current-clamp recording (lower panel) ( $n = 7$ ;  $P > 0.05$  vs. basal) (A and B, scale bar = 20  $\mu$ m). **C.** Average stimulus-induced changes in DCF FI in  $H_2O_2$  source MSNs under control conditions (Con;  $n = 7$ ), in the presence of MCS ( $n = 7$ ), or in the presence of GYKI ( $n = 7$ ) (\*\* $P < 0.01$  vs. basal; \*\*\* $P < 0.001$  vs. basal). The increase in DCF FI in MCS is nearly twofold greater than under control conditions, whereas AMPAR blockade with GYKI markedly attenuated the usual control response (#### $P < 0.001$  vs. control) (modified from Avshalumov and others 2008; copyright American Physiological Society, used with permission).

usual enhancement during local stimulation (Fig. 7A, 7B). Confirming little, if any, direct AMPAR-independent contribution from DA axons to the generation of modulatory  $H_2O_2$ , GSH peroxidase inhibition has no effect on pathway-evoked DA release (Fig. 7C, 7D) (Avshalumov and others 2008).

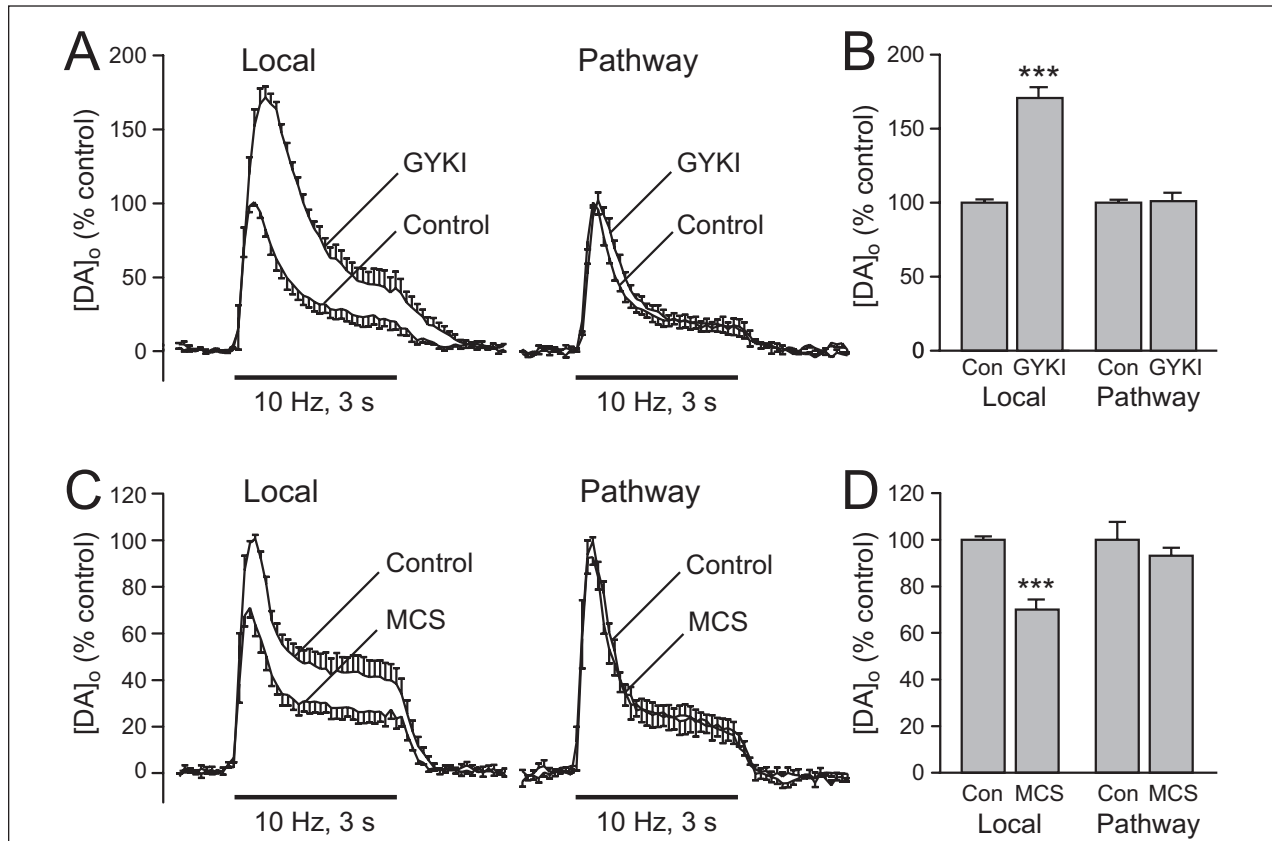
These data also imply that there are no indirect contributions to dynamically generated, modulatory  $H_2O_2$  from metabolism or autoxidation of released DA. The lack of AMPAR-dependent modulation of DA release by pathway stimulation also argues against a contribution from glutamate co-released from DA axons (Avshalumov and others 2008). Indeed, although glutamate co-release from DA axons was recently confirmed in the shell of the nucleus accumbens (ventral striatum) using optogenetic methods (Stuber and others 2010; Tecuapetla and others 2010), it was shown to be absent in dorsal striatum (Stuber and others 2010).

### Mitochondria Are the Source of AMPAR-Dependent $H_2O_2$ Signaling in Dorsal Striatum

What cellular processes generate modulatory  $H_2O_2$ ? There are three primary sources of  $H_2O_2$  in the CNS. The most ubiquitous is mitochondrial respiration, as already discussed. A second source of particular relevance in monoaminergic

regions is MAO, which catalyzes deamination of DA and other biogenic amines via a two-electron reduction of  $O_2$  to  $H_2O_2$ , with one molecule of  $H_2O_2$  produced for each molecule of DA metabolized (Cohen and others 1997; Sandri and others 1990). The isoforms of MAO are type A (MAO-A) and type B (MAO-B), with MAO-A primarily in neurons and MAO-B primarily in glia (Azarro and others 1985; Levitt and others 1985). The third source of  $H_2O_2$  is NADPH oxidase, a family of membrane-associated, multisubunit enzymes that catalyze the one-electron reduction of  $O_2$  to form  $\bullet O_2^-$  and subsequently  $H_2O_2$  (Babior 1984; Bedard and Krause 2007; Infanger and others 2006; Lambeth 2004; Rhee 2006).

The source of  $H_2O_2$  generation for rapid neuronal signaling proved to be mitochondrial respiration (Bao and others 2009). This was shown using a combination of rotenone, a complex I inhibitor, and succinate, a mitochondrial complex II substrate, which was found to limit  $H_2O_2$  production but maintain tissue ATP content (Bao and others 2009). In the presence of this mitochondrial “cocktail,” the usual effects of AMPAR blockade and GSH peroxidase inhibition on evoked  $[DA]_0$  are completely lost. These data implicate mitochondrial complex I as the primary source of modulatory  $H_2O_2$  in the striatum. Moreover, neither a combination of MAO inhibitors nor an NADPH oxidase inhibitor altered the effect of  $H_2O_2$  amplification by MCS on evoked  $[DA]_0$ . Thus, dynamic, glutamate-dependent modulation of striatal DA release



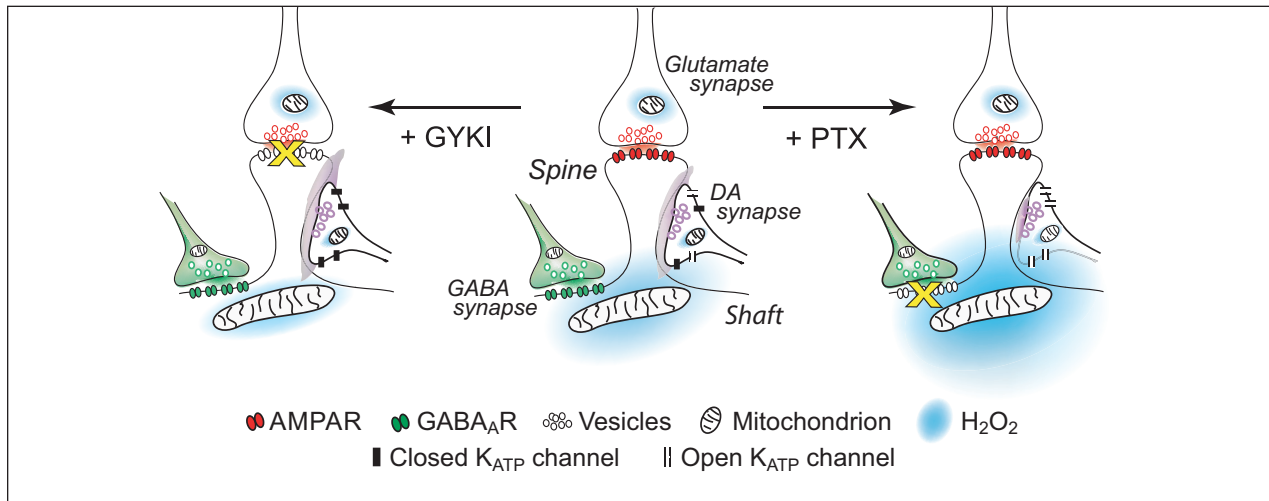
**Figure 7.** Modulatory  $H_2O_2$  is not generated in striatal dopamine (DA) axons. **A**) Average extracellular DA concentration ( $[DA]_o$ ) versus time profiles evoked at a single site by alternating local and pathway stimulation (30 pulses, 10 Hz) in the absence and presence of an AMPAR antagonist, GYKI-52466 (GYKI; 50–100  $\mu M$ ) ( $n = 6$ ). **B**) Summary of the effect of GYKI on peak  $[DA]_o$  evoked by local and pathway stimulation; control peak evoked  $[DA]_o$  for either local or DA pathway stimulation is 100%. Blockade of AMPARs causes a significant increase in  $[DA]_o$  evoked by local stimulation but has no effect on pathway-evoked DA release ( $n = 6$ ;  $***P < 0.001$  vs. local control). **C**) Average  $[DA]_o$  versus time profiles evoked at a single site by alternating local and pathway stimulation in the absence and presence of the glutathione (GSH) peroxidase inhibitor mercaptosuccinate (MCS; 1 mM) ( $n = 6$ ). **D**) Summary of the effect of MCS on peak  $[DA]_o$  at a given site evoked by local versus DA pathway stimulation; control peak evoked  $[DA]_o$  for either local or pathway stimulation was 100%. MCS causes a significant decrease in  $[DA]_o$  evoked by local stimulation but has no effect on pathway evoked  $[DA]_o$  ( $n = 6$ ;  $***P < 0.001$  vs. local control) (modified from Avshalumov and others 2008; copyright American Physiological Society, used with permission).

requires  $H_2O_2$  that originates from mitochondria, rather than MAO or NADPH oxidase. Of course, 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 participate in other aspects of neuronal regulation on a longer time scale (Brennan and others 2009; Cohen 1994; Infanger and others 2006; Kishida and Klann, 2007; Miller and others 2007b; Zekry and others 2003).

### Triad of DA, Glutamate, and GABA Synapses Bound Functionally by Diffusible $H_2O_2$

Together, the data summarized in previous sections indicate that  $H_2O_2$  must be generated in the mitochondria of

non-DA cells or processes, then diffuse from there to inhibit axonal DA release via K-ATP channel activation. Although the ability of  $H_2O_2$  to act as a diffusible messenger has been recently questioned (Mishina and others 2010), these data demonstrate that rapid cellular elevation of  $H_2O_2$  allows this ROS to escape the intracellular antioxidant network and affect adjacent targets. Notably, glutamatergic synapses are closely apposed to DA synapses on the dendritic spines of striatal MSNs (Bernard and Bolam 1998; Chen and others 1998; Smith and Bolam 1990) and are thus ideally positioned to modulate DA release via postsynaptically generated  $H_2O_2$  in these neurons (Fig. 8). Dendrites of MSNs also express GABA<sub>A</sub>Rs at sites near spines (Fujiyama and others 2000). This facilitates GABAergic inhibition that can oppose AMPAR-mediated excitation and consequent  $H_2O_2$



**Figure 8.** Triad of striatal dopamine (DA), glutamate and GABA synapses on a medium spiny neuron dendrite bound together functionally by diffusible H<sub>2</sub>O<sub>2</sub>. Generation of modulatory H<sub>2</sub>O<sub>2</sub> when GABA as well as glutamate is released (center); GABA<sub>A</sub>-receptor (GABA<sub>A</sub>R) blockade by picrotoxin (+PTX, right), and with AMPA-receptor (AMPA) blockade by GYKI-52466 (+GYKI, left) (circuitry and locations of receptors and mitochondria are from Bernard and Bolam, 1998; Chen and others 1998; Fujiyama and others 2000; Smith and Bolam, 1990) (modified from Avshalumov and others 2008; copyright American Physiological Society, used with permission).

generation from mitochondria, which are abundant in dendrites but absent from spines (Chen and others 1998; Smith and Bolam 1990). Following AMPAR activation, mitochondrial respiration would be expected to increase to meet the energy demand required for clearance of intracellular Na<sup>+</sup> and Ca<sup>2+</sup> entry via Ca<sup>2+</sup>-permeable AMPARs (Carter and Sabatini 2004), as well as via voltage-dependent Na<sup>+</sup> and Ca<sup>2+</sup> channels (Stefani and others 1998).

The resulting model of striatal DA release regulation by glutamate and GABA involves a triad of DA, glutamate, and GABA synapses, separated by a few micrometers on the dendrites of MSNs (Bernard and Bolam 1998; Chen and others 1998; Fujiyama and others 2000; Smith and Bolam 1990). In this model, mitochondrial H<sub>2</sub>O<sub>2</sub> generated in dendrites of MSNs after AMPAR activation diffuses to adjacent DA synapses where it inhibits DA release via opening of SUR1-based K-ATP channels (Fig. 8, center). By decreasing dendritic excitability, GABA attenuates AMPAR-dependent H<sub>2</sub>O<sub>2</sub> production. When GABA<sub>A</sub>Rs are blocked (+PTX) or GABA input is absent, H<sub>2</sub>O<sub>2</sub> production increases, leading to greater suppression of DA release (Fig. 8, right). Yet, when AMPARs are blocked (+GYKI) or glutamatergic input is absent, H<sub>2</sub>O<sub>2</sub> generation is minimal; DA release is no longer inhibited by H<sub>2</sub>O<sub>2</sub>-dependent K-ATP channel opening; and GABA<sub>A</sub>R-dependent regulation is lost (Fig. 8, left). Like a brake applied when a car is parked, GABA has no direct influence on DA release when MSNs are not activated by glutamate.

### False H<sub>2</sub>O<sub>2</sub> Signaling During Mitochondrial Dysfunction

The finding that mitochondria are the source of H<sub>2</sub>O<sub>2</sub> for dynamic neuronal signaling via K-ATP channel activation suggests a potential pathological role for H<sub>2</sub>O<sub>2</sub> under conditions of mitochondrial dysfunction. In isolated mitochondria, partial mitochondrial inhibition by nanomolar concentrations of rotenone alone leads to an increase in H<sub>2</sub>O<sub>2</sub> production (e.g., Votyakova and Reynolds 2001). Consistent with the expected consequences of unregulated H<sub>2</sub>O<sub>2</sub> production, exposure of striatal slices to 30-100 nM rotenone causes a concentration-dependent inhibition of [DA]<sub>o</sub> evoked by either single- or multiple-pulse stimulation that can be prevented by catalase or by K-ATP channel blockade (Bao and others 2005). Tissue ATP content was not changed under these conditions, indicating that “false” H<sub>2</sub>O<sub>2</sub> signaling, not ATP depletion, is the cause of K-ATP channel-dependent DA release suppression (Bao and others 2005). The observation that rotenone-induced suppression of DA release is seen with single-pulse stimulation indicates that enhanced H<sub>2</sub>O<sub>2</sub> generation during mitochondrial inhibition is independent of concurrent glutamate release and AMPAR activation, which are the usual triggers for “true” H<sub>2</sub>O<sub>2</sub> signaling in striatum (Bao and others 2005). These findings with rotenone have potentially important implications for Parkinson disease because mitochondrial dysfunction has been implicated as

a casual factor in nigrostriatal degeneration (Greenamyre and others 2001; Schapira and others 1990). The finding that striatal DA release can be compromised by “false”  $H_2O_2$  signaling during partial mitochondrial dysfunction suggests a mechanism for functional DA denervation that could contribute to motor deficits before structural degeneration of the nigrostriatal pathway.

### Modulation of Non-DA Neurons by $H_2O_2$ , Including Excitation via TRP Channels

One final point about dynamic neuromodulation by  $H_2O_2$  is that it is not limited to the nigrostriatal DA system. Other studies have shown 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. Indeed, instead of the usual expectation that  $H_2O_2$  is hazardous for brain health, there is evidence that elevated  $H_2O_2$  production improves spatial memory in aged mice (Kamsler and others 2007). Additionally, diffusible  $H_2O_2$  mediates 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). Recent evidence also suggests that  $H_2O_2$  may be a neuromodulator in cardiovascular regulation by the nucleus tractus solitarius (Cardoso and others 2009), with bradycardia and hypotension seen after local elevation of  $H_2O_2$  by exogenous application or following catalase inhibition. These cardiovascular effects appear to involve activation of glutamatergic neurons, but the mechanism has not yet been addressed (Cardoso and others 2009).

One mechanism by which  $H_2O_2$  can excite neurons is through activation of TRP channels. A specific TRP channel subtype implicated is TRPM2, originally named TRPC7 or LTRPC-2 (Clapham and others 2005), which exhibits activation in the presence of  $H_2O_2$  and other oxidants (McNulty and Fonfria 2005; Perraud and others 2003; Smith and others 2003). How  $H_2O_2$  activates TRPM2 channels is somewhat better understood than how it activates K-ATP channels. Although there is evidence for direct activation of TRPM2 channels by  $H_2O_2$  (Wehage and others 2002), recent data argue against this (Toth and Csanady 2010). Instead, activation may be mediated by  $H_2O_2$ -dependent elevation of ADP ribose or a synergistic action of  $H_2O_2$  and ADP ribose (Hecquet and Malik 2009; Perraud and others 2005).

Consistent with a role for TRP channels as signaling targets,  $H_2O_2$  elevation increases the excitability of striatal GABAergic MSNs through activation of a TRP channel, presumed to be TRPM2 (Bao and others 2005; Smith

and others 2003). Evidence for  $H_2O_2$ -dependent activation of TRP channels was also recently obtained from GABAergic neurons of the substantia pars reticulata (SNr) in guinea pig midbrain slices (Lee and others 2011). Elevation of endogenous  $H_2O_2$  increases the spontaneous activity of these neurons; equally important,  $H_2O_2$  depletion leads to a decrease in firing rate and regularity of SNr neurons (Lee and others 2011). This  $H_2O_2$ -dependent excitation of SNr GABAergic neurons is diametrically opposed to the inhibitory effect of both tonic and elevated  $H_2O_2$  in SNc DA neurons mediated by K-ATP channels (Avshalumov and others 2005). Importantly, much of the inhibitory input to SNc DA neurons is from axon collaterals of SNr GABAergic neurons (Lee and Tepper 2009). Thus, under conditions of  $H_2O_2$  elevation, enhanced GABAergic inhibition of SNc DA neurons with  $H_2O_2$  elevation could contribute to the previously reported inhibition of somatodendritic DA release in the SNc seen with exogenous  $H_2O_2$  or GSH peroxidase inhibition in guinea pig midbrain (Chen and others 2002).

### Summary and Conclusions

The role of  $H_2O_2$  and other ROS as signaling molecules is increasingly appreciated. Signaling by  $H_2O_2$  on time scales of minutes to hours is exemplified by its role in mediating downstream effects of growth factors that activate neuronal NADPH oxidase to generate  $H_2O_2$  (e.g., Miller and others 2007b), which then regulates the activities of intracellular kinases, phosphatases, and other enzymes. The dynamic  $H_2O_2$  signaling described in this review differs from such slow regulation in four key aspects: 1) The time scale is subsecond to seconds; 2) the source is mitochondrial respiration; 3) the targets of  $H_2O_2$  are ion channels; and 4) dynamically generated  $H_2O_2$  can act as a diffusible as well as intracellular signaling agent. In the substantia nigra,  $H_2O_2$  acts as an autoregulatory signal that modulates the activity of SNc DA neurons and SNr GABAergic neurons. In dorsal striatum,  $H_2O_2$  acts as a diffusible modulator that reverses the predicted effects of excitatory glutamate and inhibitory GABA on striatal DA release.

Somewhat surprisingly, SNc DA neurons, SNr GABAergic neurons, and striatal MSNs all express  $H_2O_2$ -sensitive K-ATP and TRP channels (Bao and others 2005; Freestone and others 2009; Häusser and others 1991; Lee and Tepper 2007; Roeper and Ashcroft 1995; Schwanztecher and Panten 1993; Smith and others 2003; Stanford and Lacey 1996). Nonetheless, population-specific responses to  $H_2O_2$  are seen. This indicates that whether  $H_2O_2$  will inhibit or excite a given neuron depends on the relative responsiveness of inhibitory K-ATP channels versus excitatory TRP channels and possibly other emerging

redox-sensitive ion channels (e.g., Tang and others 2004). Other contributing factors include the extent of  $H_2O_2$  generation and regulation in a given cell. Thus, the relative expression of  $H_2O_2$ -sensitive ion channels coupled with cell-specific  $H_2O_2$  regulation will define the specificity of dynamic neuromodulation by  $H_2O_2$ .

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