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SYMPOSIUM REVIEW

Inhibitory and excitatory neuromodulation by hydrogen peroxide: translating energetics to information

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Abstract Historically, brain neurochemicals have been broadly classified as energetic or informational. However, increasing evidence implicates metabolic substrates and byproducts as signalling agents, which blurs the boundary between energy and information, and suggests the introduction of a new category for 'translational' substances that convey changes in energy state to information. One intriguing example is hydrogen peroxide (H_2O_2), which is a small, readily diffusible molecule. Produced during mitochondrial respiration, this reactive oxygen species, can mediate dynamic regulation of neuronal activity and transmitter release by activating inhibitory ATP-sensitive K^+ (K_{ATP}) channels, as well as a class of excitatory non-selective cation channels, TRPM2. Studies using *ex vivo* guinea pig brain slices have revealed that activity-generated H_2O_2 can act via K_{ATP} channels to inhibit dopamine release in dorsal striatum and dopamine neuron activity in the substantia nigra pars compacta. In sharp contrast, endogenously generated H_2O_2 enhances the excitability of GABAergic projection neurons in the dorsal striatum and substantia nigra pars reticulata by activating TRPM2 channels. These studies suggest that the balance of excitation *vs.* inhibition produced in a given cell by metabolically generated H_2O_2 will be dictated by the relative abundance of H_2O_2-sensitive ion channel targets that receive this translational signal.

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Abbreviations AMPAR, AMPA receptor; DA, dopamine; DCF, dichlorofluorescein; FFA, flufenamic acid; FI, fluorescence intensity; GSH, glutathione; K_{ATP} channel, ATP-sensitive K^+ channel; MAO, monoamine oxidase; MCS, mercaptosuccinate; MSN, medium spiny neuron; NOX, NADPH oxidase; RF1, Redoxfluor-1; ROS, reactive oxygen species; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; TRP channel, transient receptor potential channel .

Introduction

F. O. Schmitt and Fred Samson introduced the notion of 'informational' substances to describe a neurochemical class that is distinct from 'energetic' substances (Schmitt, 1984, 1985). In this classification scheme, informational substances include neurotransmitters, exemplified by glutamate and GABA, whereas energetic substances include metabolic substrates, like glucose and oxygen.

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Increasingly, however, the line between these broad categories has blurred. Glutamate is not only the primary excitatory transmitter in the CNS, but also an important metabolic substrate (e.g. Dienel, 2013), and glucose not only provides energy for cells, but also conveys information that modulates the activity of specific glucose-sensing neurons (Routh, 2010; Thorens, 2012). Adding further complexity to the delineation between information and energy is that oxygen consumption during mitochondrial respiration, an energetic process, generates reactive oxygen species (ROS), including superoxide ($\bullet O_2^-$) and hydrogen peroxide (H_2O_2) (Boveris & Chance, 1973; Dugan *et al.* 1995; Liu *et al.* 2002; Bao *et al.* 2009; Rigoulet *et al.* 2011; Mailloux *et al.* 2013) that can act as signalling molecules (Sundaresan *et al.* 1995; Kamsler & Segal, 2004; Rhee, 2006; Stone & Yang, 2006; Avshalumov *et al.* 2007; Kishida & Klann, 2007; Miller *et al.* 2007*b*; Gerich & Funke, 2009; Groeger *et al.* 2009; Rigoulet *et al.* 2011; Rice, 2011; Jeong *et al.* 2012; Sies, 2014).

Molecules like ROS are at the interface between energy and information, and merit a class of their own. Indeed, they could be viewed as 'translational' substances that provide an interpretation of cellular activity within a cell or to neighbouring cells. In the case of ROS in the CNS, increased neuronal activity drives energy demand, which drives metabolism, and thereby generates signalling molecules that can act both within individual neurons (as intracellular signals) and between neurons (as diffusible messengers) to give immediate modulatory feedback about local activity. Other molecules in this class might include ATP, adenosine and glucose.

A particularly strong candidate as a 'translational' substance is H_2O_2. During mitochondrial electron transport, oxygen is first reduced to the free radical $\bullet O_2^-$, up to 5% of which leaves the respiratory process (Arnaiz *et al.* 1999), and can be converted to H_2O_2 by the enzyme superoxide dismutase or by spontaneous dismutation (see Peuchen *et al.* 1997). Oxygen consumption is proportional to local activity (Kennedy *et al.* 1992), with the greatest demand to support ATP-dependent signalling, e.g. informational processes (Engl & Attwell, 2015). The H_2O_2 produced in this process is well positioned to serve as a dynamic reporter of neuronal activity. Other slower metabolic processes also generate H_2O_2, including NADPH oxidases (NOXs) that produce $\bullet O_2^-$, and thus H_2O_2 (Babior, 1984; Lambeth, 2004; Infanger *et al.* 2006; Rhee, 2006; Bedard & Krause, 2007), and monoamine oxidases (MAOs), which produce one molecule of H_2O_2 for each biogenic amine molecule metabolized (Maker *et al.* 1981; Azzaro *et al.* 1985; Cohen, 1994). These additional sources allow a range of timescales for H_2O_2 signalling that extend from a rapid, subsecond level for mitochondrial H_2O_2 generation to slower regulation by growth factor activation of NOX, for example, which can proceed over hours, days, or even longer (Miller *et al.* 2007*b*).

Among the best-studied intracellular targets for slow regulation by H_2O_2 are redox-sensitive phosphatases that are inactivated by H_2O_2 and kinases that are activated by this ROS (Klann & Thiels, 1999; Rhee *et al.* 2005; Woolley *et al.* 2013). Modulation of phosphatase and kinase activities regulates signal propagation downstream of receptors, so that H_2O_2 helps fine-tune transmission to further downstream targets, including transcription factors like p53, NF-κB and AP-1, which are also H_2O_2 sensitive (Groeger *et al.* 2009; Woolley *et al.* 2013). Through these processes, H_2O_2 plays key roles in cell growth and survival. The primary source of H_2O_2 for these regulatory processes is NOX, which is activated by growth factors and other receptor activators (Rhee *et al.* 2005; Miller *et al.* 2007*b*, 2010; Groeger *et al.* 2009; Woolley *et al.* 2013).

In contrast to slow signalling mediated by NOX- and MAO-generated H_2O_2 that can regulate intracellular signalling cascades, rapid, subsecond signalling by H_2O_2 originates in mitochondria (Bao *et al.* 2009) and acts on cellular ion channels (Seutin *et al.* 1995; Krippeit-Drews *et al.* 1999; Avshalumov *et al.* 2003, 2003; Avshalumov & Rice, 2003; Bao *et al.* 2005; Patel *et al.* 2011; Lee *et al.* 2011, 2013). Early physiological studies showed that application of *exogenous* H_2O_2 can cause membrane hyperpolarization by activating a K^+ conductance in various cell types, including CA1 hippocampal neurons (Seutin *et al.* 1995) and pancreatic β-cells (Krippeit-Drews *et al.* 1999). Our laboratory subsequently identified ATP-sensitive K^+ (K_{ATP}) channels as key inhibitory targets of *endogenous*, as well as exogenous H_2O_2 (Avshalumov *et al.* 2003, 2005; Avshalumov & Rice, 2003; Patel *et al.* 2011). More recently, we identified a subclass of transient receptor potential (TRP) channels in basal ganglia neurons that are dynamically regulated by H_2O_2 (Bao *et al.* 2005; Lee *et al.* 2011, 2013). Work in our laboratory has focused on rapid signalling by H_2O_2 in regions of the basal ganglia, particularly the striatum and the substantia nigra pars compacta (SNc) and pars reticulata (SNr). Our cellular focus has been on dopaminergic (DAergic) neurons of the SNc that project to the dorsal striatum, forming the nigrostriatal pathway that plays critical roles in movement and motor learning (Carlsson, 2002; Redgrave *et al.* 2011), and on GABAergic neurons of the SNr, which are basal ganglia output neurons (Zhou & Lee, 2011).

This review describes how H_2O_2 rapidly translates a dynamic change in cellular metabolism, specifically mitochondrial oxygen consumption, to a salient signal. The targets of this signal are H_2O_2-sensitive ion channels that can influence the excitability of the neuron in which H_2O_2 is generated, as well as neighbouring cellular elements. Most data presented were obtained using *ex vivo* brain slices prepared from adult male guinea pigs or mice after inducing deep anaesthesia (50 mg kg^{-1} sodium pentobarbital, I.P.). Methods include fast-scan

cyclic voltammetry (FCV) to detect DA release, whole-cell recording to monitor neuronal activity, and fluorescence imaging to indicate H_2O_2 generation (e.g. Avshalumov *et al.* 2003, 2005, 2008; Bao *et al.* 2009; Lee *et al.* 2011, 2013; Patel & Rice, 2013).

Cellular regulation of H_2O_2

The previous section discussed sources of H_2O_2, but it is important to recognize that sinks, including metabolism and diffusion, are critical in shaping patterns of H_2O_2 signalling, as well. Thus, cellular H_2O_2 regulation must not only prevent oxidative damage from H_2O_2 elevation, but also allow intracellular and extracellular concentrations sufficient for a signalling effect to be achieved (Avshalumov *et al.* 2004; Murphy *et al.* 2011; Rice, 2011). Absolute basal and dynamic intracellular concentrations of H_2O_2 remain a matter of debate; the technical and conceptual issues involved have been reviewed elsewhere (Adimora *et al.* 2010; Murphy *et al.* 2011; Rice, 2011).

Peroxidase enzymes that metabolize H_2O_2 include glutathione (GSH) peroxidase, which is found in the cytosol and in mitochondria (Stults *et al.* 1977), and catalase, which is localized to intracellular peroxisomes (Cohen, 1994; Peuchen *et al.* 1997; Dringen *et al.* 2005). Dynamic H_2O_2 regulation is also provided by peroxiredoxins and thioredoxins, which act in a complicated dance that not only buffers H_2O_2 levels to prevent potentially toxic consequences of oxidative stress, but also facilitates the elevation of intracellular H_2O_2 to levels sufficient for signalling (Rhee *et al.* 2005; Adimora *et al.* 2010; Rhee & Woo, 2011; Jeong *et al.* 2012).

Complementing H_2O_2 regulation by cellular peroxidase enzymes are roles played by the low molecular weight antioxidants GSH and ascorbate. These antioxidants protect against possible pathological consequences of H_2O_2 elevation through their actions as scavengers of the aggressive hydroxyl radical (•OH) produced from the interaction of H_2O_2 or $\bullet O_2^-$ with trace metal ions (Cohen, 1994). Additionally, GSH is an essential cofactor for GSH peroxidase activity, and with other cellular thiols provides critical regulation of peroxiredoxins and thioredoxins through reduction of disulfide bonds involved in activation and inactivation (Stults *et al.* 1977; Rhee & Woo, 2011; Mailloux *et al.* 2013). Ascorbate, by contrast, has a higher redox potential than GSH and so cannot reduce oxidized thiols or break disulfide bonds, and does not interact with H_2O_2 directly (Rice, 2000; Avshalumov *et al.* 2004; Rhee & Woo, 2011). Notably, ascorbate is the primary low molecular weight antioxidant in neurons (with an intracellular concentration of 10 mM) (Rice & Russo-Menna, 1998), so that it is in a prime position to permit neuronal H_2O_2 signalling, yet prevent pathological consequences that could occur from unregulated H_2O_2 generation and •OH production (Rice, 2012).

One final point about H_2O_2 regulation is the extent to which H_2O_2 can diffuse from a site of generation to act at targets in the same or neighbouring cells. In contrast to other ROS, H_2O_2 is not a free radical and not an ion. These properties not only limit H_2O_2 reactivity and extend its lifetime (Cohen, 1994), but also increase its membrane permeability (Ramasarma, 1982; Bienert *et al.* 2007; Adimora *et al.* 2010). However, increasing evidence indicates that net cellular H_2O_2 efflux and entry is governed by cell-specific membrane permeability factors coupled with competing effects of the antioxidant network (Makino *et al.* 2004; Bienert *et al.* 2007; Adimora *et al.* 2010; Miller *et al.* 2010; Mishina *et al.* 2011; Bienert & Chaumont, 2014). In particular, the cellular expression of specific aquaporin isoforms, including aquaporins 3 and 8 (Miller *et al.* 2010; Bienert & Chaumont, 2014), has been shown to facilitate the passive diffusion of H_2O_2 across membranes, and thereby influence its efficacy as an intracellular signalling agent, as well as a diffusible messenger.

Endogenous H_2O_2 inhibits axonal and somatodendritic DA release

Regulation of dopamine release from DAergic axons or cell bodies and dendrites (somatodendritic release) can be studied readily in *ex vivo* brain slices using carbon-fibre microelectrodes with FCV (Rice *et al.* 2011; Patel & Rice, 2013). For such studies, local electrical stimulation is used to elicit an increase in extracellular DA concentration ($[DA]_o$), which reflects the net influence of DA release and uptake. The use of pulse-train stimulation allows the influence of concurrently released transmitters and local activity changes on $[DA]_o$ as the train progresses. For the studies summarized here, stimulation parameters were commonly 30 pulses at 10 Hz, although trains of as few as 7 pulses at 10 Hz are sufficient to reveal modulation by endogenously generated H_2O_2 in guinea pig dorsal striatum (K. A. Moran & M. E. Rice, unpublished observations).

Using these methods, we found that exogenous H_2O_2 causes a reversible, 30% suppression of evoked $[DA]_o$ in dorsal striatum that is not accompanied by a change in tissue DA content or signs of oxidative damage (Chen *et al.* 2001). We then examined a role for endogenous H_2O_2 in DA release regulation by inhibiting GSH peroxidase to amplify levels of dynamically generated H_2O_2 levels using mercaptosuccinate (MCS). Exposure to MCS causes similar reversible decreases in evoked $[DA]_o$ in dorsal and ventral striatum to those seen with exogenous H_2O_2, again with no change in tissue DA content (Chen *et al.* 2002; Avshalumov *et al.* 2008). Significantly, DA release suppression in the dorsal striatum

can be reversed by the H_2O_2 metabolizing enzyme catalase in the continued presence of MCS, confirming H_2O_2 involvement (Avshalumov *et al.* 2003). Suppression of evoked $[DA]_o$ also persists when DA uptake is inhibited, indicating an effect on DA *release*, not uptake (Avshalumov *et al.* 2003).

Although exogenous H_2O_2 causes suppression of somatodendritic DA release in the SNc and in the adjacent ventral tegmental area (VTA), GSH peroxidase inhibition decreases evoked $[DA]_o$ in SNc, but not VTA (Chen *et al.* 2002). This difference between SNc and VTA is potentially important, because DAergic neurons of the SNc degenerate in Parkinson's disease and in animal models of Parkinson's, whereas those in the VTA are relatively spared (Yamada *et al.* 1990; Fearnley & Lees, 1991; Betarbet *et al.* 2000).

Endogenous H_2O_2 in dorsal striatum is generated downstream of glutamatergic AMPA receptor activation

Local electrical stimulation evokes release of glutamate, GABA and other transmitters, as well as DA. In striatum, GSH peroxidase inhibition by MCS has no effect on $[DA]_o$ evoked by a *single* stimulus pulse (Avshalumov *et al.* 2003), indicating that modulatory H_2O_2 must be generated dynamically during initial and subsequent pulses of a stimulus train to inhibit on-going DA release. The generation of this modulatory H_2O_2 proved to require activation of AMPA receptors (AMPARs), as AMPAR blockade causes a marked increase in pulse-train-evoked $[DA]_o$ in guinea pig dorsal striatum (Avshalumov *et al.* 2003), indicating that physiological glutamate release inhibits axonal DA release in dorsal striatum via AMPARs. An essential role for inhibitory H_2O_2 in mediating this effect, as well as in mediating the opposing effect on DA release by GABA acting at $GABA_A$ receptors, was subsequently shown by the loss of regulation in the presence of exogenous catalase or GSH peroxidase (Avshalumov *et al.* 2003).

As reviewed elsewhere, the absence of AMPARs and $GABA_A$Rs on DAergic axons in dorsal striatum led us to postulate that the cellular sources of glutamate-dependent H_2O_2 generation were not DAergic axons, but rather other striatal neurons, including the predominant striatal neurons, GABAergic medium spiny neurons (MSNs) (Avshalumov *et al.* 2007, 2008; Rice *et al.* 2011; Rice, 2011; Patel & Rice, 2012). We tested this hypothesis using single-cell fluorescence imaging of H_2O_2, in which dihydro-dichlorofluorescein diacetate (H_2DCF-diacetate) is loaded into cells via a patch pipette used for whole-cell recording (Avshalumov *et al.* 2005, 2008). This dye is cleaved by intracellular esterases to form H_2DCF, which becomes fluorescent DCF when oxidized. Local stimulation in guinea pig striatal slices activates a single action potential with each stimulus pulse in recorded MSNs (Fig. 1*A*, lower panel). Concurrent imaging of DCF fluorescence confirmed a significant increase in DCF fluorescence intensity (FI) evoked using the same pulse-train stimulation parameters as in DA release studies (Fig. 1*A*, upper panel). Activity-dependent increases in DCF FI are enhanced when GSH peroxidase is inhibited by MCS, and abolished by the addition of exogenous catalase, confirming H_2O_2 detection (Avshalumov *et al.* 2008). Consistent with a requirement for glutamate-dependent AMPAR activation in the generation of modulatory H_2O_2 in dorsal striatum, antagonism of AMPARs prevents stimulus-induced action potentials and H_2O_2 generation in MSNs (Fig. 1*B*) (Avshalumov *et al.* 2008). These and other data support a role for dynamically generated H_2O_2 as a diffusible messenger that is generated in striatal MSNs (and possibly other local neurons), and diffuses to adjacent DAergic axons to inhibit DA release (Avshalumov *et al.* 2008; Rice, 2011).

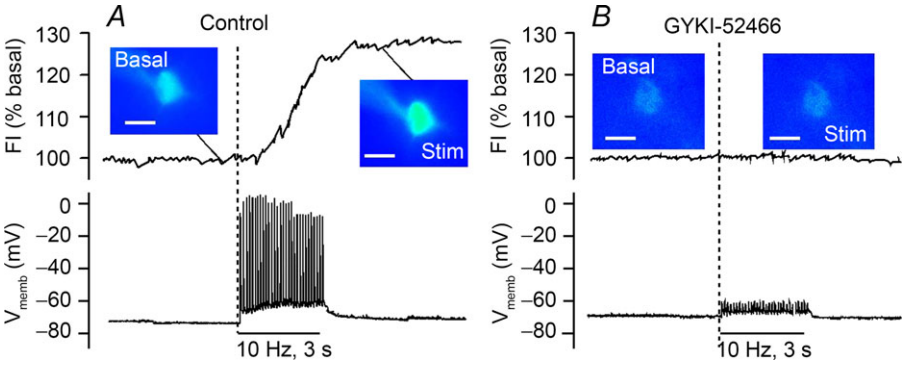

Figure 1. Modulatory H_2O_2 generation in guinea pig striatal medium spiny neurons (MSNs) requires AMPAR activation
A, local electrical stimulation (30 pulses at 10 Hz; 100 μs, 0.5 mA) causes tetrodotoxin-sensitive action potentials and increased DCF fluorescence intensity (FI) in MSNs in *ex vivo* slices of guinea pig dorsal striatum. *B*, AMPAR blockade with GYKI-52466 (50 μM) prevents stimulus-induced spikes and H_2O_2 generation (modified from Avshalumov *et al.* 2008).

H_2O_2 acts via K_{ATP} channels in DAergic axons

The targets at which H_2O_2 acts to regulate nigrostriatal DA release are K_{ATP} channels (Avshalumov *et al.* 2003; Avshalumov & Rice, 2003). Blocking K_{ATP} channels with a sulfonylurea receptor antagonist not only enhances pulse-train-evoked $[DA]_o$ in striatum, but also prevents the usual H_2O_2-dependent inhibition of DA release by MCS, as well as the patterns of DA release regulation by AMPAR and $GABA_AR$ activation (Avshalumov *et al.* 2003; Avshalumov & Rice, 2003; Rice, 2011). These data show that K_{ATP} channels are *required* for DA release modulation

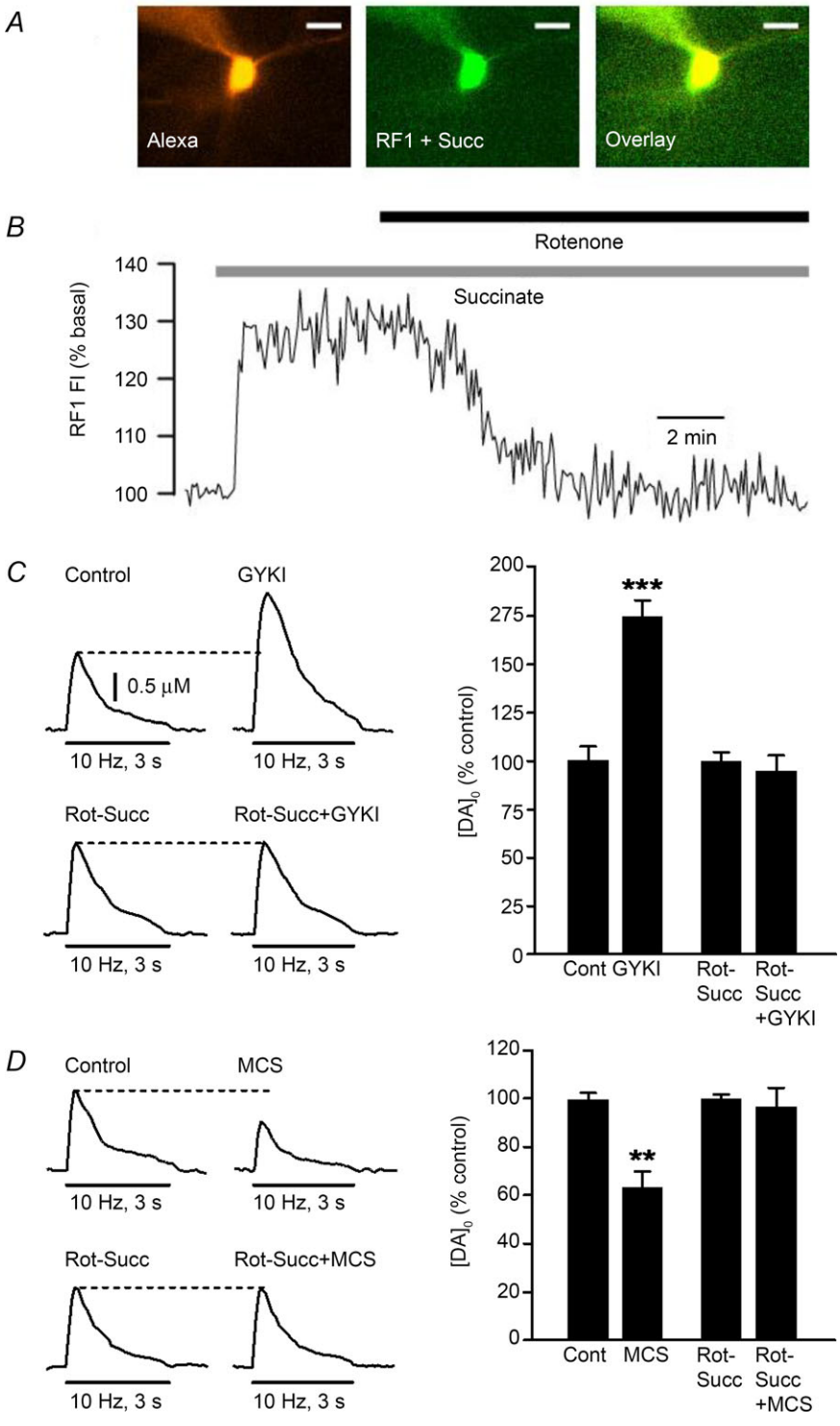

Figure 2. Role of mitochondrial H_2O_2 in dynamic modulation of striatal DA release
A, superfusion of succinate (5 mM) causes an increase in fluorescence intensity (FI) of the reversible H_2O_2-sensitive dye RF1 in a medium spiny neuron (MSN) in a guinea pig striatal slice; Alexa Red was included in the pipette solution to allow morphological identification of the imaged MSN (scale bar = 20 μm). *B*, time course of the increase in RF1 FI in the MSN in *A* during succinate exposure; the increase in FI was reversed by rotenone, at a concentration (50 nM) that leads to partial inhibition of mitochondrial complex I, in the continued presence of succinate ($n = 7$). *C*, representative pulse-train-evoked $[DA]_o$ (30 pulses, 10 Hz) in artificial cerebrospinal fluid (aCSF) alone (Control) or in the presence of rotenone + succinate (Rot + Succ), before and after the addition of an AMPAR antagonist, GYKI-52466 (GYKI, 50 μM). Bar graphs show average evoked $[DA]_o$ normalized to the starting condition for each experiment; GYKI caused a significant increase in evoked $[DA]_o$ ($^{***}P < 0.001$ *vs.* same-site control; $n = 5$), which was prevented by the rotenone–succinate cocktail ($P > 0.05$ *vs.* same-site Rot-Succ; $n = 6$). *D*, representative pulse-train-evoked $[DA]_o$ in aCSF alone or in the presence of Rot-Succ before and after the addition of a GSH peroxidase inhibitor, MCS (1 mM). Bar graphs show average evoked $[DA]_o$ normalized to the starting condition for each experiment; MCS caused a significant decrease in evoked $[DA]_o$ ($^{**}P < 0.01$ *vs.* same-site control; $n = 5$), which was prevented by the rotenone-succinate cocktail ($P > 0.05$ *vs.* same-site Rot-Suc; $n = 5$).

by H_2O_2, glutamate and GABA (Avshalumov *et al.* 2003; Avshalumov & Rice, 2003). Consequently, striatal DA release is decreased by K_{ATP} channel openers, whether evoked with single-pulse or pulse-train stimulation (Avshalumov & Rice, 2003; Patel *et al.* 2011). Although release suppression is seen with an opener selective for either SUR1- or SUR2-subunit-containing K_{ATP} channels, only a SUR1-selective opener occludes the effects of MCS, as well as the effects of AMPAR and $GABA_AR$ antagonism, on evoked $[DA]_o$ showing that DA release regulation by glutamate-dependent H_2O_2 requires SUR1-based K_{ATP} channels (Avshalumov & Rice, 2003).

A key aspect of our hypothesis that H_2O_2 is a diffusible messenger in the striatum is that K_{ATP} channels are located directly on DAergic axons. Our observation that single-pulse-evoked $[DA]_o$ is suppressed by K_{ATP} channel openers supports direct localization, which was confirmed using immunohistochemical methods showing striatal colocalization of a K_{ATP} channel subunit with tyrosine hydroxylase, an enzyme required for DA synthesis (Patel *et al.* 2011; Patel & Rice, 2012). Together with our previous work, these data indicate that H_2O_2 is a diffusible messenger, which is generated in striatal MSNs, but acts at K_{ATP} channels on DA axons.

It should be emphasized at this point that H_2O_2-dependent signalling via ion channel activation is fast and transient, with a subsecond to second time scale (Patel *et al.* 2011; Patel & Rice, 2012). This was assessed using paired-pulse stimulation to evoke $[DA]_o$ with pharmacological blockade of K_{ATP} channels or amplification of H_2O_2 levels using GSH peroxidase inhibition. Maximal H_2O_2-/K_{ATP} channel-dependent suppression of DA release is seen 500 ms after an initiating stimulus, with a significant influence persisting until ~1000 ms (Patel *et al.* 2011).

Mitochondria are the subcellular source of H_2O_2 for dynamic striatal signalling

Studies in isolated mitochondria indicate that succinate, a substrate of mitochondrial complex II, drives H_2O_2 production by back-flow of electrons to complex I, which is prevented by partial complex I inhibition by rotenone (Votyakova & Reynolds, 2001; Liu *et al.* 2002; Gyulkhandanyan & Pennefather, 2004). We demonstrated that this regulation also occurs in whole cells in brain slices using a reversible H_2O_2-sensitive dye Redoxfluor-1 (RF1) (Miller *et al.* 2007*a*). Succinate caused a rapid increase in RF1 FI in striatal MSNs (Fig. 2*A* and *B*), which was reversed by co-application of rotenone at a concentration that leads to partial complex I inhibition (50 nM) in the continued presence of succinate (Fig. 2*B*) (Bao *et al.* 2009). We then examined pulse-train-evoked DA release in this rotenone + succinate cocktail to assess whether the *sub*cellular source of modulatory H_2O_2 was mitochondrial respiration. Consistent with the expected effect of enhanced H_2O_2 generation, succinate alone suppressed evoked $[DA]_o$; suppression of DA release was prevented by catalase, confirming H_2O_2 involvement, and was reversed by rotenone (Bao *et al.* 2009). Most importantly, rotenone + succinate prevents the usual increase in evoked $[DA]_o$ with AMPAR blockade (Fig. 2*C*) *and* the decrease that usually accompanies GSH peroxidase inhibition by MCS (Fig. 2*D*). By contrast, inhibition of either NADPH oxidase or MAO had no effect on pulse-train-evoked $[DA]_o$ or on the usual suppression of DA release seen when GSH peroxidase is inhibited by MCS (Bao *et al.* 2005). These data show that mitochondrial respiration is the source of H_2O_2 that can then *translate* the significance of a glutamate-activated increase in cellular activity and mitochondrial metabolism in MSNs to a signal at DAergic axons to dynamically regulate DAergic transmission.

H_2O_2 regulates SNc DA neuron activity via K_{ATP} channels

DCF imaging in SNc DAergic neurons in guinea pig midbrain slices revealed tonic and activity-dependent H_2O_2 generation in these spontaneously active cells (Avshalumov *et al.* 2005). Notably, tonically generated H_2O_2 has a significant effect on DA cell excitability: depletion of intracellular H_2O_2 by including catalase in the pipette solution or blockade of K_{ATP} channels causes a significant increase in spontaneous firing rate in all DAergic neurons tested. Moreover, catalase in the pipette has no effect when K_{ATP} channels are blocked,

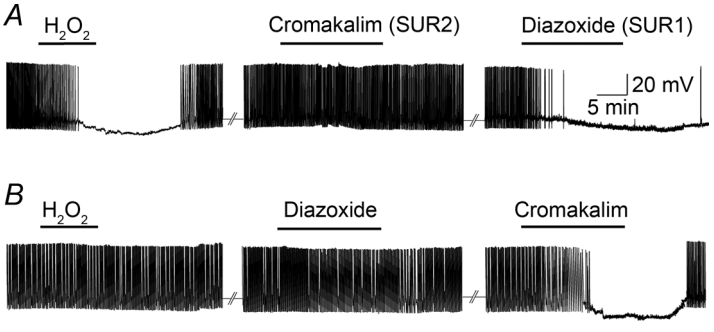

Figure 3. SUR1-containing K_{ATP} channels convey enhanced sensitivity to H_2O_2 elevation in guinea pig SNc DAergic neurons
A, exogenous H_2O_2 (1.5 mM) (or MCS, 1 mM) causes hyperpolarization in 'responders'; this is mimicked by SUR1-selective K_{ATP}-channel opener, diazoxide (60 μM), whereas a SUR2-selective opener, cromakalim (60 μM), has no effect ($n = 6$). *B*, a second population of SNc DAergic neurons does not respond to H_2O_2, MCS, or diazoxide, but hyperpolarizes with cromakalim ($n = 6$) (modified from Avshalumov *et al.* 2005).

demonstrating that tonically generated H_2O_2 regulates DAergic cell activity via K_{ATP} channels (Avshalumov *et al.* 2005). Notably, the pipette backfill solution typically contains 3 mM ATP, which should close K_{ATP} channels (Häusser *et al.* 1991), suggesting that H_2O_2 may act by decreasing channel sensitivity to ATP; the mechanism of regulation has yet to be elucidated, however (for discussion, see Patel & Rice 2012).

Conversely, elevation of H_2O_2 by exogenous application or GSH peroxidase inhibition causes neuronal hyperpolarization in ~50% of SNc DAergic neurons examined in guinea pig midbrain. Neurons in this sensitive population, which we call 'responders', are also hyperpolarized by an SUR1-subunit-selective K_{ATP} channel opener whereas those that are insensitive to H_2O_2 ('non-responders') are hyperpolarized by an SUR2-subunit-selective opener (Fig. 3) (Avshalumov *et al.* 2005). These data indicate that SUR1 expression in SNc DA neurons conveys sensitivity to elevated H_2O_2, as seen for striatal DA axons (Avshalumov & Rice, 2003; Patel *et al.* 2011). Overall, these data show that H_2O_2 plays an auto-regulatory role in SNc DA neurons via the activation of inhibitory K_{ATP} channels.

Activation of TRPM2 channels by H_2O_2 in GABAergic neurons in the basal ganglia

Increasing evidence points to an additional ion channel target for H_2O_2, which is a subclass of TRP channels that provides regulation complementary to that provided by K_{ATP} channels. Although a variety of TRP channels are expressed in the brain, one subclass, TRPM2 (TRP melastatin 2), is uniquely sensitive to activation by H_2O_2 (Fleig & Penner, 2004). Activation of these channels by H_2O_2 leads to an increase in neuronal excitability (Bao *et al.* 2005; Lee *et al.* 2011, 2013), rather than the decrease that accompanies activation of K_{ATP} channels. How H_2O_2 activates TRPM2 channels is somewhat better understood than its mechanism at K_{ATP} channels, but not without debate. Although there is evidence for direct activation of TRPM2 channels by H_2O_2 (Wehage *et al.* 2002), other data argue against this (Tóth & Csanády, 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 (Perraud *et al.* 2005; Lange *et al.* 2008).

The first evidence for H_2O_2-dependent regulation of neuronal excitability by TRP channels emerged in the course of studies to investigate the role of mitochondria as a source of modulatory H_2O_2 (Bao *et al.* 2005, 2009). In these studies we found that partial mitochondrial complex I inhibition by nanomolar concentrations of rotenone leads to unregulated generation of H_2O_2, indicated by single-cell DCF imaging in MSNs in guinea pig dorsal striatum (Fig. 4*A*) (Bao *et al.* 2005; Avshalumov *et al.* 2007). Unsurprisingly, this increase in H_2O_2 leads to suppression of evoked DA release, which is prevented by catalase or by K_{ATP} channel blockade (Fig. 4*B*), demonstrating H_2O_2 and K_{ATP} channel involvement. What was more surprising at the time, however, was that simultaneous current-clamp recording in MSNs showed a depolarization and an increase in excitability

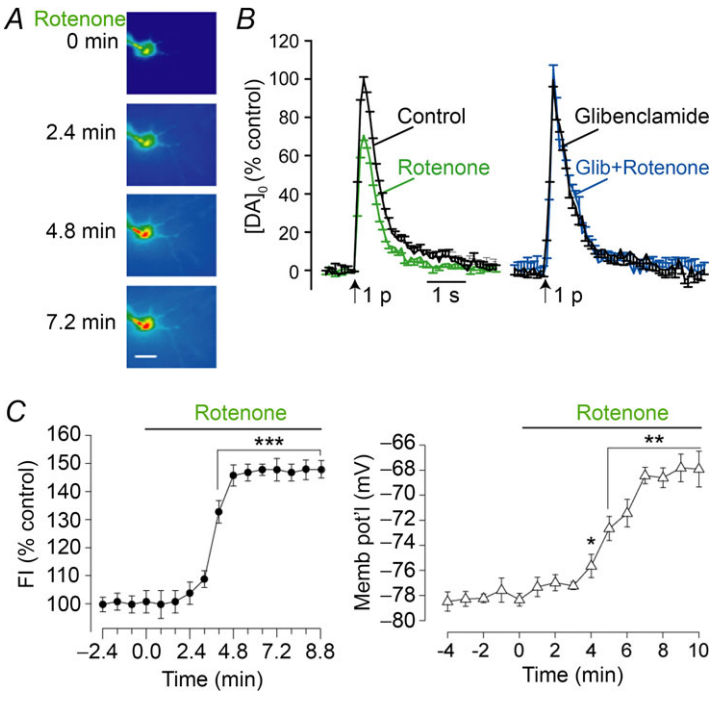

Figure 4. Unregulated H_2O_2 generation during partial mitochondrial complex I inhibition by rotenone inhibits striatal DA release via K_{ATP} and excites MSNs via TRP channels
A, DCF fluorescence intensity (FI) in a MSN in a guinea pig striatal slice under control conditions ($t = 0$) and during exposure to rotenone (50 nM); scale bar is 20 μm. *B*, average DA release records after single-pulse stimulation elicited at 5 min intervals under control conditions and after 30 min exposure to rotenone (50 nM; $n = 7$) compared with release in the presence of glibenclamide (Glib; 3 μM) and glibenclamide plus rotenone ($n = 5$). Data are normalized, with maximum $[DA]_o$ under control conditions for each slice taken as 100%. *C*, average time course of H_2O_2 generation (DCF FI) recorded in single MSNs during rotenone exposure ($n = 13$; $^{***}P < 0.001$ rotenone *vs.* basal FI; ANOVA) compared to the time course of rotenone-induced changes in membrane potential (Memb pot'l) in these same MSNs ($n = 10$; $^{*}P < 0.05$; $^{**}P < 0.01$ rotenone *vs.* control; ANOVA) (modified from Bao *et al.* 2005).

of those neurons, with a time course that paralleled the increase in DCF FI (Fig. 4*C*) (Bao *et al.* 2005). Changes in MSN membrane properties were prevented by catalase, confirming H_2O_2 dependence. Moreover, the increase in excitability was also prevented by flufenamic acid (FFA), a non-selective TRP channel antagonist, implicating H_2O_2-dependent TRP channel activation in these GABAergic projection neurons (Bao *et al.* 2005). Although FFA can inhibit several TRP subtypes, as well as other ion channels (Guinamard *et al.* 2013), recognized targets include H_2O_2-sensitive TRPM2 channels (Hill *et al.* 2004). As discussed further below, TRPM2 channels have been identified as the target for H_2O_2-dependent modulation of SNr GABAergic projection neurons (Lee *et al.* 2013). Given that TRPM2 channels are expressed in striatal MSNs, it is likely that they also contribute to H_2O_2 modulation of MSNs (Hill *et al.* 2006). Blocking K_{ATP} channels led to enhanced MSN depolarization during rotenone exposure, indicating that concurrently activated K_{ATP} channels in these cells serves to counterbalance the predominant TRP-dependent effects of H_2O_2 elevation.

We then turned our attention to spontaneously active GABAergic projection neurons in the SNr (Lee *et al.* 2011, 2013). Consistent with opposing regulation of cellular activity by H_2O_2 acting at TRP *vs.* K_{ATP} channels, exogenous catalase causes a *decrease* in firing rate of

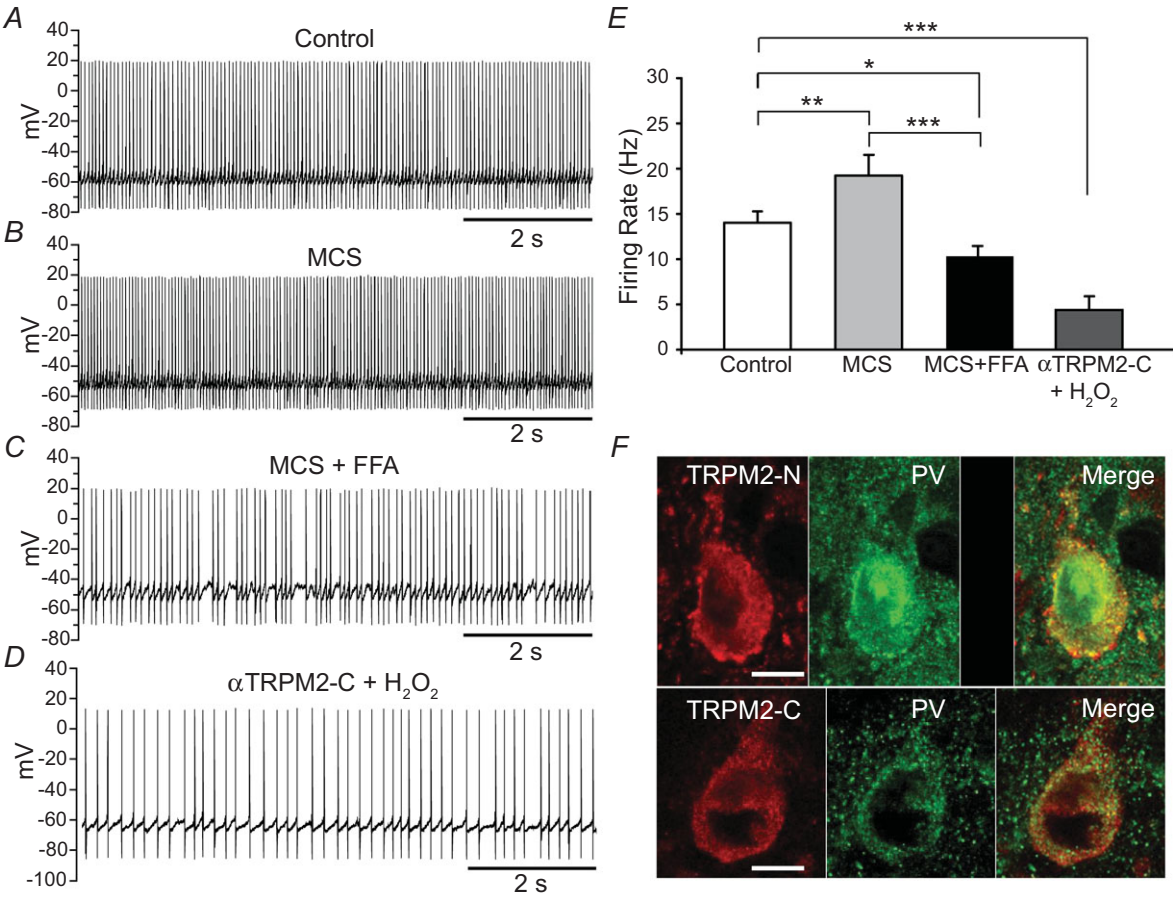

Figure 5. TRPM2 channel blockade reverses H_2O_2-dependent increases in firing rate in guinea pig SNr GABAergic neurons
A, spontaneous activity of a SNr GABAergic neuron under control conditions. *B*, amplification of endogenous H_2O_2 levels by GSH peroxidase inhibition with MCS increases spontaneous firing rate. **C**, in the presence of a non-specific TRP channel blocker, flufenamic acid (FFA), H_2O_2 causes a decrease, rather than increase in SNr GABAergic neuron firing rate. *D*, the effect of elevated H_2O_2 is also converted to a suppression of spontaneous activity when TRPM2 channels are blocked by including an antibody to the C-terminus of TRMP2 channels (α-TRPM2-C) in the pipette solution. *E*, summary of the influence of elevated H_2O_2 on spontaneous SNr neuron firing rate. Increases in firing rate by H_2O_2 are reversed and suppressed below control levels by FFA or by α-TRPM2-C (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$) (modified from Lee *et al.* 2011, 2013). *F*, immunohistochemical labelling of guinea pig SNr neurons with parvalbumin (PV), a marker for GABAergic neurons, and an antibody to the N- or C-terminus of TRPM2, confirming TRPM2 expression in these cells. Staining is eliminated following pre-adsorption of the primary antibody with its immunogenic peptide (top row, third panel from left); scale bar, 10 μm. (modified from Lee *et al.* 2013).

SNr GABAergic neurons in guinea pig midbrain slices, indicating maintenance of excitability by basal levels of H_2O_2 (Lee *et al.* 2011). In sharp contrast to the inhibitory effect of H_2O_2 elevation on SNc DAergic neurons (Fig. 3) (Avshalumov *et al.* 2005), elevation of H_2O_2 levels causes an *increase* in the firing rate of SNr GABAergic neurons, whether through amplification of endogenous levels by GSH peroxidase inhibition (Fig. 5*A*, *B* and *E*) or exposure to exogenous H_2O_2 (Lee *et al.* 2011). Implicating H_2O_2-sensitive TRPM2 channels in this process, these increases in SNr neuron firing rates are blocked by FFA (Fig. 5*C* and *E*), as well as when an antibody to the C-terminus of TRPM2 channels is included in the pipette solution (Fig. 5*D* and *F*). In addition to these studies of functional TRPM2 expression, immunohistochemistry (Fig. 5*F*) and *in situ* hybridization studies provide anatomical confirmation of TRPM2 channels in guinea pig SNr GABAergic neurons (Lee *et al.* 2013). Companion immunohistochemical studies of guinea pig midbrain also demonstrated expression of TRPM2 channels in SNc DAergic neurons (Fig. 6) (Lee *et al.* 2013), consistent with other studies showing a functional role for these ion channels in SNc DAergic neurons in rats (Chung *et al.* 2011), as well as evidence for the presence of TRPM2 in DAergic neurons in mice (Mrejeru *et al.* 2011).

In addition to regulating the spontaneous firing rate of SN neurons, TRPM2 channels are required for NMDA-induced burst firing in SNr GABAergic neurons (Lee *et al.* 2013). Notably, we found that H_2O_2 modulates NMDA-induced burst firing in these cells, leading to an increase in burst duration and a decrease in burst frequency (Fig. 7). These findings reveal another modulatory role for H_2O_2 that could be especially relevant in Parkinson's disease, in which increased burst firing in SNr neurons and increases in ROS both occur (see Lee *et al.* 2013).

Species similarities and differences

Most studies of H_2O_2 as a dynamic neuromodulator have been conducted using *ex vivo* brain slices from guinea pigs. Initial experiments suggested species independence of the basic inhibitory effect of H_2O_2 elevation on pulse-train-evoked DA release in dorsal striatum, with a similar reversible suppression of pulse-train-evoked $[DA]_o$ in *ex vivo* striatal slices from rat, guinea pig and marmoset when GSH peroxidase was inhibited by MCS (Rice *et al.* 2002; Avshalumov *et al.* 2003). The Sombers group has further demonstrated H_2O_2-dependent suppression of DA release in rat striatum *in vivo* (Spanos *et al.* 2013). Recent studies have confirmed DA release suppression when endogenous H_2O_2 is elevated in mouse striatal slices, as well (B. O'Neill, R. Asri, J. C. Patel & M. E. Rice, unpublished observations). However, additional evidence suggests that dynamic regulation of DA release by glutamate, GABA and H_2O_2 may differ in mouse dorsal striatum with possible H_2O_2-independent regulation of DA release by these transmitters.

As noted above, studies in *ex vivo* slices from young adult guinea pig brain show greater sensitivity of SUR1- *vs.* SUR2-based K_{ATP} channels to activation by H_2O_2 in striatal DAergic axons (Avshalumov & Rice, 2003; Patel *et al.* 2011) as well as SNc DAergic neurons (Avshalumov *et al.* 2005). These findings are consistent with the greater metabolic sensitivity of SUR1- *vs.* SUR2-expressing SNc DAergic neurons in slices from neonatal mice (Liss *et al.* 1999), although adult mice appear to express only SUR1-based K_{ATP} channels (Liss *et al.* 2005). Rat SNc DAergic neurons also show K_{ATP} channel-dependent

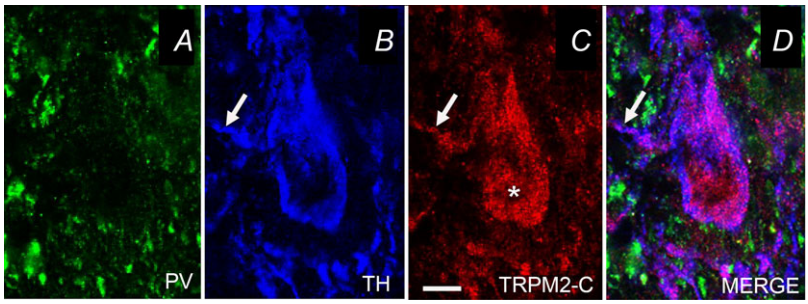

Figure 6. TRPM2 channel expression in guinea pig SNc DAergic neurons
A, Immunohistochemical labelling of guinea pig SNc shows little somatic staining with an antibody to parvalbumin (PV), a marker for GABAergic neurons, but *B*, abundant staining of most SNc neurons with an antibody to tyrosine hydroxylase (TH), confirming their identity as DAergic neurons. *C*, TH-immunopositive neurons are also immunopositive for TRPM2, indicated using an antibody to the C-terminus of TRPM2 (TRPM2-C). *D*, Merged image of PV, TH, and TRPM2-C. Arrows point to TRPM2 in DAergic dendrites; scale bar, 20 μm (modified from Lee *et al.* 2013).

hyperpolarization with exogenous H_2O_2 application, with equal sensitivity of all DAergic cells, at least under the conditions tested (Geracitano *et al.* 2005). Interestingly, in these same studies, hypoxia-induced K_{ATP} channel activation was reversed by H_2O_2, which served as a source of molecular oxygen, as shown previously (Walton & Fulton, 1983).

Another species difference in H_2O_2-dependent modulation of neuronal activity is that H_2O_2 elevation in mouse midbrain slices leads to inhibition of SNr GABAergic neurons via predominant K_{ATP} channel activation, as opposed to the TRPM2-dependent excitation seen with H_2O_2 elevation in guinea pig SNr neurons (Lee *et al.* 2011). Differences in the functional activation of H_2O_2-dependent K_{ATP} and TRPM2 channels between guinea pigs and mice suggest divergent roles for this regulatory process across species. The need for neuronal regulation by a metabolic signal like H_2O_2 might depend on unique behavioural demands across species that require differential patterns of ion channel expression. Other possible factors include species differences in H_2O_2 generation or metabolism. For example, H_2O_2 metabolism by the glial antioxidant network differs between species, with stronger control in guinea pig (or human) *vs.* mouse (or rat), because of higher glia-to-neuron ratio of guinea pig brain (Avshalumov *et al.* 2004). Regardless of these differences, however, responsiveness to H_2O_2 across diverse species supports the idea that this molecule is an important regulator of neuronal function.

Opposing effects of H_2O_2 via K_{ATP} and TRPM2 channels in specific neuron populations

A seemingly paradoxical factor in H_2O_2-mediated signalling is that many target neurons express both K_{ATP} and TRPM2 channels that have opposing effects when activated by H_2O_2. For example, in addition to TRPM2 channels (Lee *et al.* 2013), GABAergic SNr neurons also express K_{ATP} channels (Schwanstecher & Panten, 1993; Stanford & Lacey, 1996; Lutas *et al.* 2014) that lead to H_2O_2-activated hyperpolarization of guinea pig SNr GABAergic neurons when TRPM2 channels are blocked (Fig. 5*C–E*; Lee *et al.* 2011). Conversely, an inward current is observed in SNc

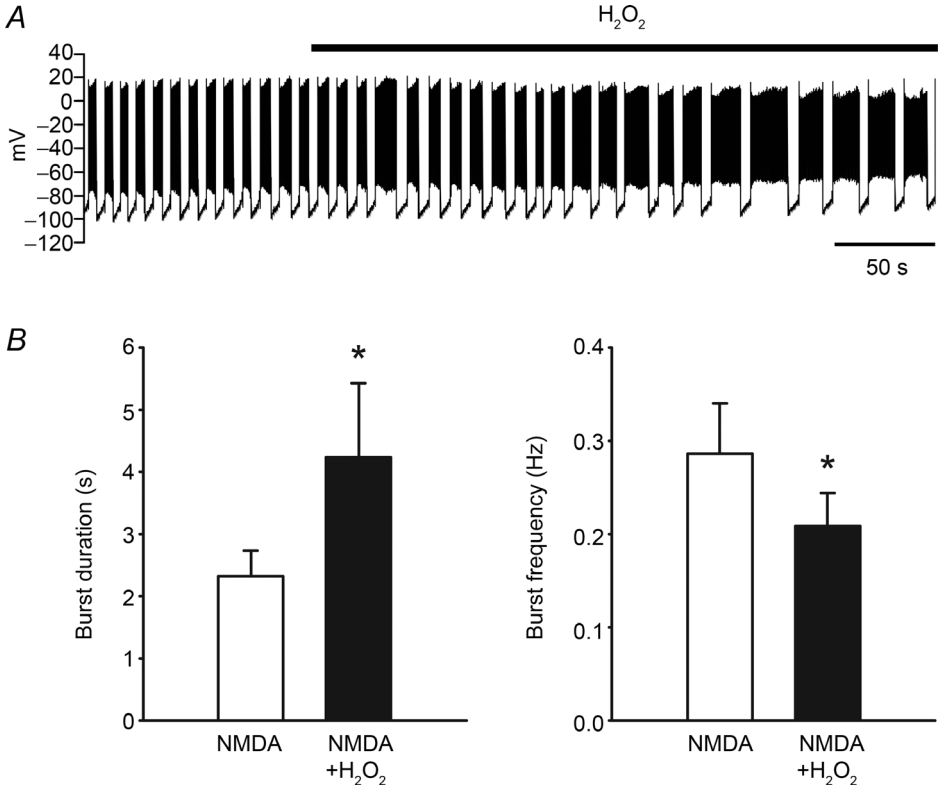

Figure 7. H_2O_2 modulates NMDA-induced burst firing in SNr GABAergic neurons
A, activity of a SNr GABAergic neuron exhibiting NMDA-induced (30 μM) burst firing before and after addition of exogenous H_2O_2 (1.5 mM). *B*, both exogenous H_2O_2 and amplified endogenous H_2O_2 induced by inhibiting GSH peroxidase with MCS (1 mM) caused an increase in burst duration and a decrease in burst frequency (*$P < 0.05$) (modified from Lee *et al.* 2013).

DAergic neurons in response to H_2O_2 as opposed to the usual outward current and hyperpolarization when K_{ATP} and other potassium channels are blocked (Avshalumov *et al.* 2005; Geracitano *et al.* 2005; Chung *et al.* 2011). This raises the interesting possibility that the effect of H_2O_2 on target neurons is defined by the abundance and/or relative activity of these opposing channels and suggests that ATP and H_2O_2 are involved in a dynamic interplay regulating neuronal excitability, as discussed further below (Fig. 8).

There is a rich literature linking K_{ATP} channel activity to metabolic state in basal ganglia structures, with ATP identified as the primary signalling molecule. Factors that influence ATP (and ATP/ADP), such as oxygenation, influence K_{ATP} channel status, with anoxic conditions leading to K_{ATP} channel opening (Amoroso *et al.* 1990; Murphy & Greenfield, 1992; Jiang *et al.* 1994; Guatteo *et al.* 1998). Glucose concentration can also influence K_{ATP} channel activity with lower glucose concentrations favouring K_{ATP} channel opening, presumably through effects on intracellular ATP concentration (Amoroso *et al.* 1990; During *et al.* 1995; Marinelli *et al.* 2000). However, we have also found a relationship between glucose concentration and mitochondrial H_2O_2 production with lower glucose concentrations promoting mitochondrial activity and H_2O_2 production in striatal MSNs (L. Bao, C. R. Lee & M. E. Rice, unpublished observations), further linking metabolic activity to signalling by H_2O_2.

Less is known about the physiological processes that regulate TRPM2 channels in SNc and SNr neurons. However, several metabolic and activity-dependent mechanisms have been implicated, including activity-dependent increases in intracellular calcium, as well as conditions that elevate H_2O_2 and other ROS (Lee & Tepper, 2007; Freestone *et al.* 2009; Chung *et al.* 2011; Mrejeru *et al.* 2011; Lee *et al.* 2013).

Overall, H_2O_2 can have an excitatory or inhibitory effect on an individual neuron that is apparently based

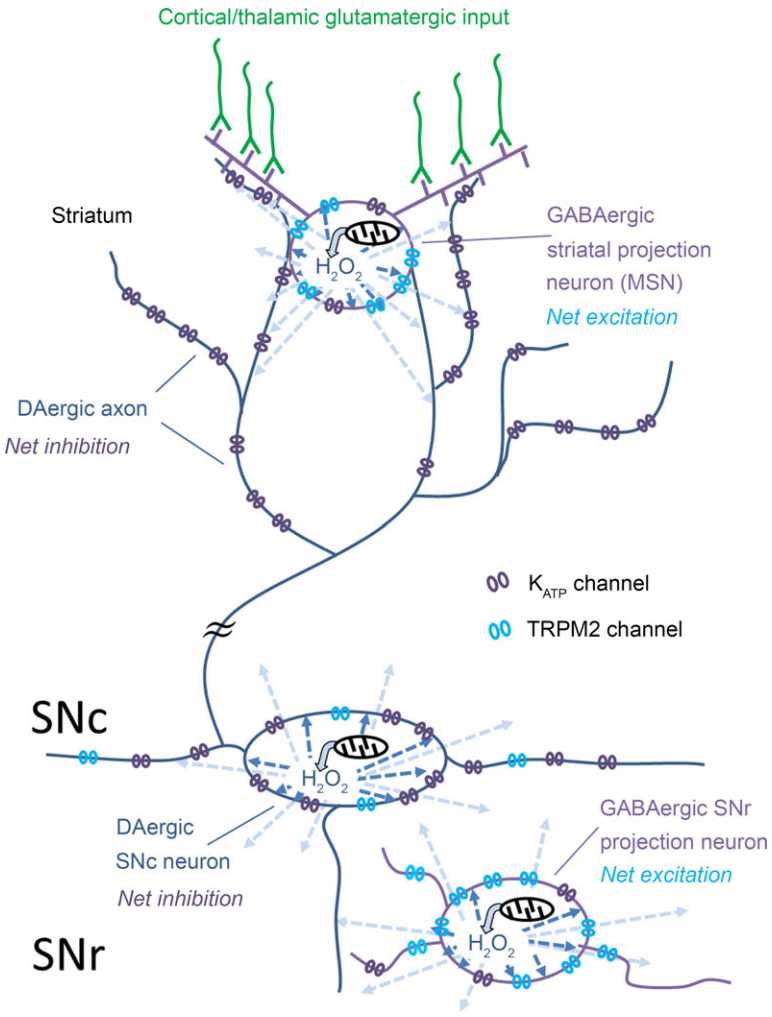

Figure 8. Net influence of mitochondrially generated H_2O_2 on DA release and on the excitability of guinea pig basal ganglia neurons mediated by K_{ATP} and TRPM2 channels
Axonal DA release in striatum and somatodendritic DA release in SNc are suppressed by the net inhibitory effect of elevated H_2O_2 acting at K_{ATP} channels in DAergic neurons. In striatum, H_2O_2 generated in MSNs acts as a diffusible messenger (light blue arrows) to inhibit DA release by activating K_{ATP} channels on neighbouring DAergic axons. The source of dynamically generated H_2O_2 is mitochondrial respiration. The net effect of H_2O_2 generated within spontaneously active SNc DAergic neurons (dark blue arrows) is also inhibitory via K_{ATP} channel activation. Although striatal MSNs, which are GABAergic projection neurons, and SNr GABAergic projection neurons express both K_{ATP} and TRPM2 channels, the predominant effect of H_2O_2 on these cells is excitatory via TRPM2 channels. It should be noted that glutamatergic drive is required for action potential-dependent generation of H_2O_2 in MSNs, although partial mitochondrial inhibition by rotenone can lead to unregulated H_2O_2 generation. By contrast, sufficient H_2O_2 is generated tonically in spontaneously active SNr and SNc neurons to modulate firing rate independent of synaptic input.

on the relative activities of K_{ATP} and TRPM2 channels in that cell. This working model is supported by evidence from our studies of H_2O_2-dependent modulation of DA release and basal ganglia neuron excitability in *ex vivo* guinea pig brain slices, reviewed here (Fig. 8). Specifically, the net effect of H_2O_2 elevation on striatal DA release is inhibitory via K_{ATP} channels (Avshalumov & Rice, 2003; Bao *et al.* 2005; Patel *et al.* 2011). Similarly, the predominant influence of K_{ATP} channels on SNc DAergic neuron excitability is also reflected in the net hyperpolarization seen with H_2O_2 elevation in these midbrain projection neurons (Avshalumov *et al.* 2005) (Fig. 8). In contrast, the net influence of H_2O_2 is to increase the excitability of GABAergic striatal MSNs (Bao *et al.* 2005) and GABAergic SNr neurons (Lee *et al.* 2011, 2013), which would enhance the output of these inhibitory projection neurons, presumably by the predominant influence of TRPM2 channels in both neuron populations (Fig. 8). The source of modulatory H_2O_2 that can influence transmitter release and neuronal activity on a subsecond time scale is presumed to be mitochondrial respiration, as shown for striatal DA release regulation by H_2O_2-dependent activation of K_{ATP} channels (Bao *et al.* 2009; Patel *et al.* 2011). Notably, basal H_2O_2 levels in midbrain SN DAergic and GABAergic neurons generated during spontaneous firing activity in these cells is sufficient to provide a modulatory tone that is mildly inhibitory in SNc DAergic neurons (Avshalumov *et al.* 2005) and mildly excitatory in SNr GABAergic cells (Lee *et al.* 2011). In the striatum, however, dynamically generated H_2O_2 in MSNs requires activation of AMPARs by glutamatergic input and consequent action potential generation (Avshalumov *et al.* 2003, 2008).

Conclusions

The findings summarized here reveal an exquisite interaction between mitochondrial respiration and neuronal excitability, bridged by H_2O_2, which acts as a 'translational' substance that communicates the increase in metabolism to neuronal membranes via activation of K_{ATP} and TRPM2 channels. In its translational role, H_2O_2 generated within a given neuron can mediate autoinhibition and/or autoexcitation, but can act as a diffusible messenger to influence the activity of neighbouring cells (Fig. 8). Actions of H_2O_2 at K_{ATP} and TRPM2 channels indicate that the net effect of H_2O_2 on a given cell or transmitter release site will reflect the balance of activity between H_2O_2-sensitive target channels expressed and thereby provide cell-type-specific patterns of modulation. These patterns of regulation have implications not only for normal regulation of basal ganglia transmitters and neuronal activity, but also for pathological conditions like Parkinson's disease, in which oxidative stress has been identified as a potential underlying factor in SNc DAergic neuron degeneration (e.g. Obeso *et al.* 2010). It should be noted that even without DAergic neuron loss, elevated levels of H_2O_2 would be expected to cause a net decrease in DAergic transmission. Increased H_2O_2 generation (or impaired metabolism) could lead to suppression of axonal DA release via K_{ATP} channel activation, which would be compounded by H_2O_2-dependent inhibition of SNc DAergic neuron excitability, resulting in *functional* DA denervation of target regions, like dorsal striatum (Bao *et al.* 2005; Avshalumov *et al.* 2005) (Fig. 8). At the same time, the increased excitability of SNr GABAergic output neurons via H_2O_2 and TRPM2 channels (as seen in guinea pig SNr) would further exaggerate motor inhibition (Fig. 8).

Two final points about H_2O_2-dependent regulation of neuronal signalling are that: (1) K_{ATP} and TRPM2 channels are expressed by many neurons in addition to those discussed here, so that modulation by H_2O_2 is likely to be widespread; and (2) additional targets for H_2O_2-dependent regulation are emerging, including GABA receptors that mediate inhibitory synaptic transmission (Accardi *et al.* 2014; Penna *et al.* 2014). In this light, it is also likely that dynamic cellular modulation by H_2O_2 is not limited to the CNS. For example, there are established functional roles for K_{ATP} channels in pancreatic β-cells, cardiac myocytes and muscle (McTaggart *et al.* 2010; Flagg *et al.* 2010; Coetzee, 2013), with emerging evidence for TRPM2 channels in β-cell function, as well (Uchida & Tominaga, 2014). Moreover, K_{ATP} channels in β-cells and cardiac myocytes are sensitive to exogenous H_2O_2 (Ichinari *et al.* 1996; Tokube *et al.* 1998; Krippeit-Drews *et al.* 1999), supporting the idea that metabolically generated H_2O_2 may be poised to provide modulatory signals in excitable cells throughout the body.

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Additional information

Competing interests

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