AMPAR Receptor-Dependent \( \text{H}_2\text{O}_2 \) Generation in Striatal Medium Spiny Neurons But Not Dopamine Axons: One Source of a Retrograde Signal That Can Inhibit Dopamine Release

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Avshalumov MV, Patel JC, Rice ME. AMPA receptor-dependent \( \text{H}_2\text{O}_2 \) generation in striatal medium spiny neurons but not dopamine axons: one source of a retrograde signal that can inhibit dopamine release. J Neurophysiol 100: 1590–1601, 2008. First published July 16, 2008; doi:10.1152/jn.90548.2008. Dopamine-glutamate interactions in the striatum are critical for normal basal ganglia-mediated control of movement. Although regulation of glutamatergic transmission by dopamine is increasingly well understood, regulation of dopaminergic transmission by glutamate remains uncertain given the apparent absence of ionotropic glutamate receptors on dopaminergic axons in dorsal striatum. Indirect evidence suggests glutamatergic regulation of striatal dopamine release is mediated by a diffusible messenger, hydrogen peroxide (\( \text{H}_2\text{O}_2 \)), generated downstream from glutamatergic AMPA receptors (AMPARs). The mechanism of \( \text{H}_2\text{O}_2 \)-dependent inhibition of dopamine release involves activation of ATP-sensitive \( K^+ \) (\( K_{\text{ATP}} \)) channels. However, the source of modulatory \( \text{H}_2\text{O}_2 \) is unknown. Here, we used whole cell recording, fluorescence imaging of \( \text{H}_2\text{O}_2 \), and voltammetric detection of evoked dopamine release in guinea pig striatal slices to examine contributions from medium spiny neurons (MSNs), the principal neurons of striatum, and dopamine axons to AMPAR-dependent \( \text{H}_2\text{O}_2 \) generation. Imaging studies of \( \text{H}_2\text{O}_2 \) generation in MSNs provide the first demonstration of AMPAR-dependent \( \text{H}_2\text{O}_2 \) generation in neurons in the complex brain-cell microenvironment of brain slices. Stimulation-induced increases in \( \text{H}_2\text{O}_2 \) in MSNs were prevented by an ionotropic glutamate receptor (iGluR) inhibitor. By contrast, dopamine release evoked by selective stimulation of dopamine axons was unaffected by \( \text{H}_2\text{O}_2 \)-generating agents, arguing against dopamine axons as a significant source of modulatory \( \text{H}_2\text{O}_2 \). Together, these findings suggest that glutamatergic regulation of dopamine release via AMPARs is mediated through retrograde signaling by diffusible \( \text{H}_2\text{O}_2 \) generated in striatal cells, including medium spiny neurons, rather than in dopamine axons.

INTRODUCTION

As a central component of the basal ganglia (Albin et al. 1989; Kemp and Powell 1971a), the striatum receives glutamatergic input from most of the cerebral cortex as well as from the thalamus (Berendse and Groenewegen 1990; Gerfen and Wilson 1996; Graybiel et al. 1994; Kemp and Powell 1971b; McGeorge and Faull 1989). This excitatory input forms asymmetric synapses on the heads of dendritic spines of medium spiny neurons (MSNs), which are the principal neurons of the striatum that integrate incoming cortical activity and encode striatal output (Graybiel et al. 1994; Wilson 1993). Importantly, MSNs also receive synaptic dopamine input from midbrain dopamine neurons (Freund et al. 1984; Smith and Bolam 1990). Converging glutamatergic and dopaminergic afferents control striatal network output at the level of individual spines to regulate motor and cognitive function (e.g., Cagniard et al. 2006; Costa et al. 2006). Despite the lack of direct synaptic associations between glutamatergic and dopaminergic axons (Freund et al. 1984; Smith and Bolam 1990), their apposition on MSN spines and their complementary functions suggest a reciprocal modulatory relationship.

Evidence for dopamine-dependent regulation of glutamate transmission is well established, including inhibition of glutamate release via D2 dopamine receptors on corticostriatal afferents (Bamford et al. 2004a,b; Cepeda et al. 2001). This regulation by dopamine occurs through volume transmission (Cragg and Rice 2004; Fuxe and Agnati 1991; Garris et al. 1994; Rice 2000; Rice and Cragg 2008) with synaptic spillover of dopamine facilitated by the location of dopamine transporters on dopaminergic axons and its actions mediated by predominantly extrasynaptic dopamine receptors (Cragg and Rice 2004; Nirenberg et al. 1996; Rice and Cragg 2008; Sesaek et al. 1994; Yung et al. 1995). How synaptically released glutamate regulates striatal dopamine release is much less clear. Glutamatergic transmission is primarily “hard-wired” with synapses surrounded by neuronal and glial transporters that limit glutamate spillover and ensure primary activation of subsynaptic ionotropic receptors (Barbour 2001; Bergles et al. 1999; Danbolt 2001; Galvan et al. 2006; Rusakov et al. 1999). When glutamate spillover does occur, e.g., after uptake inhibition, dopamine release can be inhibited via metabotropic glutamate receptors on dopaminergic axons (Zhang and Sulzer 2003). However, any effect of ionotropic glutamate-receptor activation on dopamine release must be indirect given that dopaminergic axons lack these receptors (Bernard and Bolam 1998; Bernard et al. 1997; Chen et al. 1998).

Indirect evidence suggests that glutamate-dependent regulation of striatal dopamine release involves a diffusible messenger, hydrogen peroxide (\( \text{H}_2\text{O}_2 \)), which is generated downstream from glutamatergic AMPA receptors (AMPARs) and which activates ATP-sensitive \( K^+ \) (\( K_{\text{ATP}} \)) channels to inhibit dopamine release (Avshalumov et al. 2003; Avshalumov and Rice 2003). The source of modulatory \( \text{H}_2\text{O}_2 \) is unknown, although the AMPAR dependence suggests that generation is unlikely to occur in dopaminergic axons. Here we tested the...
hypothesis that MSNs are an important source of modulatory H2O2 in striatum given that these cells constitute 95% of striatal neurons (Kemp and Powell 1971a) and express functional AMPARs (Bernard and Bolam 1998; Bernard et al. 1997; Carter and Sabatini 2004; Chen et al. 1998; Kita 1996). Using real-time fluorescence imaging of intracellular H2O2 in MSNs with simultaneous whole cell recording in striatal slices, we show that glutamate input to dorsolateral striatum acts via AMPARs to generate H2O2 in MSNs, which could provide a retrograde signal to inhibit axonal dopamine release. Companion ionometric and imaging data indicate that dopaminergic axons do not contribute to the generation of modulatory H2O2.

METHODS

All animal handling procedures were in accordance with the National Institutes of Health guidelines and were approved by the New York University School of Medicine Animal Care and Use Committee.

Slice preparation

Procedures for slice preparation were similar to previously published methods (Avshalumov et al. 2005; Bao et al. 2005; Koós and Tepper 1999). Young adult male guinea pigs (Hartley, 150–250 g) were deeply anesthetized with sodium pentobarbital (50 mg/kg ip) and perfused transcardially with ~30 mL of ice-cold modified artificial cerebrospinal fluid (ACSF) containing (in mM) 225 sucrose, 2.5 KCl, 0.5 CaCl2, 7 MgSO4, 28 NaHCO3, 1.25 NaH2PO4, 7 glucose, 1 ascorbate, and 3 pyruvate. After perfusion and decapitation, the brain was rapidly removed and immersed in this ice-cold modified ACSF for 1–2 min, then bisected and blocked before slicing on a Vibratome (Ted Pella, St. Louis, MO). Most whole cell recording and imaging studies were done in coronal slices. However, some slices were also cut in an angled parasaggital plane, at a 20–25° rostral-tolateral angle to the midline to preserve nigrostriatal dopaminergic fibers entering the striatum from the median forebrain bundle (see Fig. 4A). It should be noted that this angle is roughly perpendicular to incoming thalamostriatal fibers (Smeal et al. 2007), so that this slice orientation permits selective stimulation of the nigrostriatal dopamine pathway. Angled parasaggital slices were used for all dopamine recording experiments.

Visualized whole cell recording and H2O2 imaging

Striatal slices (300 μm thickness) were kept for 10 min at 34°C in a holding solution containing (in mM) 125 NaCl, 2.5 KCl, 1.0 MgCl2, 25 NaHCO3, 1.25 NaH2PO4, 25 glucose, 1 ascorbate, 3 pyruvate, and 2 CaCl2 at pH 7.3–7.4, saturated with 95% O2–5% CO2. The holding solution was then allowed to cool slowly to room temperature over ≥15 min before experimentation. For recording, slices were transferred to a submersion chamber (Warner Instruments LLC, Holliston, MA) maintained at 32°C and superfused at 1.2 mL/min with bicarbonate-buffered ACSF containing (in mM) 124 NaCl, 3.7 KCl, 26 NaHCO3, 1.3 MgSO4, 1.3 KH2PO4, 10 glucose, and 2.4 CaCl2 and saturated with 95% O2–5% CO2. MSNs in dorsolateral striatum were identified visually under infrared differential interference contrast (IR-DIC) microscopy using an Olympus BX51WI fixed-stage microscope (New York/New Jersey Scientific; Middlebush, NJ) with a ×40 water-immersion objective. Patch pipettes for whole cell recording and dye loading were pulled from borosilicate glass capillaries (1.5 mm OD, 0.86 mm ID) on a Flaming/Brown model P-97 micropipette puller (Sutter Instruments, Novato, CA). Pipettes had open tip diameters of <2 μm and resistances of 3–8 MΩ. The intracellular filling solution contained (in mM) 120 K-gluconate, 20 KCl, 2 MgCl2, 10 Na-HEPES, 10 EGTA, 2 Na2-ATP, and 0.2 GTP; pH adjusted to 7.2–7.3 with KOH, 280–290 mosm (Avshalumov et al. 2005; Bao et al. 2005; Koós and Tepper 1999). The intracellular solution also contained Alexa Red (0.1%) for cell visualization plus the fluorescent dye 2’7’ dichlorodihydrofluorescein (H2DCF) diacetate (7 μM) for H2O2 imaging (Avshalumov et al. 2005, 2007).

Imaging of intracellular H2O2 was as described previously (Avshalumov et al. 2005; Bao et al. 2005). Briefly, H2DCF diacetate was loaded into a given cell via the patch pipette used for simultaneous whole cell recording. Once a whole cell configuration was obtained, cell physiology was monitored for 15–20 min in current-clamp mode before images were taken to allow sufficient time for the dye to infiltrate the cell and cleavage by intracellular esterases to form H2DCF; H2DCF becomes fluorescent DCF after oxidation by H2O2 or other ROS (Avshalumov et al. 2005, 2007; Reynolds and Hastings 1995; Sah and Schwartz-Bloom 1999). Excitation wavelength for DCF was 488 nm with emission at 535 nm. To minimize DCF photobleaching, images were obtained at 500-ns intervals with 30-ns exposure using eight-frame averaging. Background fluorescence from an area adjacent to the recorded cell was subtracted from each averaged image for that cell. Fluorescence data are presented as [(stimulated intensity − basal)/basal] × 100%. Basal DCF fluorescence was taken as the average intensity recorded for 2 s immediately before stimulation. Stimulated intensity was determined from a 2-s average taken after the stimulus ended for comparison with the average basal fluorescence for each cell.

Activity-dependent H2O2 generation in MSNs was elicited using surface bipolar stimulating electrodes. In most experiments, local stimulation (~200 μm from a recorded cell) was used in coronal slices. However, angled parasaggital slices were used to compare the effects of local stimulation with stimulation of the nigrostriatal dopamine pathway. For these experiments, one bipolar electrode was positioned locally, then a second electrode was positioned >1.5 mm from a recorded cell with an orientation that was shown in voltammetric studies to elicit reliable dopamine release. Stimulation parameters were 30 pulses at 10 Hz with pulse duration of 100 μs and amplitude of 0.6–0.8 mA for local and 2–4 mA for pathway stimulation as optimized in studies of evoked dopamine release as described in the following text.

Voltammetric monitoring of dopamine release

All voltammetric recording of evoked dopamine release in dorsolateral striatum was done in angled parasaggital slices. Slices (400 μm) were maintained at room temperature for ≥1 h before experimentation in HEPES-buffered ACSF containing (in mM) 120 NaCl, 5 KCl, 20 NaHCO3, 6.7 HEPES acid, 3.3 HEPES salt, 2 MgSO4, 10 glucose, and 2 CaCl2 saturated with 95% O2–5% CO2, which minimizes slice edema (MacGregor et al. 2001; Rice et al. 1997). Dopamine release was examined in submerged slices maintained at 32°C and superfused with the same bicarbonate-buffered ACSF used for whole cell recording and H2O2 imaging.

Evoked extracellular dopamine concentration ([DAe]) was monitored with carbon-fiber microelectrodes (8 μm tip diameter, 30–50 μm length; made in-house using methods modified from Millar and Pelling 2001) and fast-scan cyclic voltammetry using a Millar voltameter (available from Dr. Julian Millar at Queen Mary University of London, UK). Data acquisition and analysis were as described previously (Chen et al. 2001). Carbon-fiber microelectrodes were inserted in the dorsolateral striatum with the tip 50–100 μm below the slice surface. Surface bipolar stimulating electrodes were positioned to elicit optimal [DAe] at a single recording site for both local and dopamine axon pathway stimulation (see Fig. 4). After 30-min equilibration in the slice chamber, dopamine release was evoked at 10-min intervals by alternating local and dopamine pathway stimulation (30 pulses, 10 Hz; 100-μs pulse duration). The stimulation intensity that evoked permaximal [DAe] was determined for each stimulation site; stimulus amplitude was 0.6–0.8 mA for local stimulation and 2–4 mA for dopamine pathway stimulation. Evoked [DAe] was quantified by postexperimental calibration with known concentrations of dopamine at 32°C in all media used in a given experiment.
HPLC analysis of striatal dopamine tissue content

In separate experiments, the striatal dopamine tissue content of coronal slices (400 μm) was determined using HPLC with electrochemical detection as described previously (Chen et al. 2001). Slice pairs were allowed to equilibrate for 30 min at 32°C in bicarbonate-buffered ACSF before experimentation as in dopamine release studies. One slice of each pair was then incubated for a further 30 min at 32°C in bicarbonate-buffered ACSF alone while the other was incubated in ACSF containing a glutathione (GSH) peroxidase inhibitor mercaptosuccinate (MCS, 1 mM) (Avshalumov et al. 2003) for 30 min. After incubation, a sample of striatal tissue from each slice was weighed, frozen on dry ice, and stored at −80°C for subsequent HPLC analysis. On the day of analysis, samples were diluted 100-fold with ice-cold, deoxygenated mobile-phase, sonicated, spun at 13,000 rpm for 2 min, then the supernatant (10 μL) injected directly into the HPLC system for determination of dopamine content.

Drugs and chemicals

All experimental solutions were prepared immediately before use. MCS (as mercaptosuccinic acid) and components of all ACSF solutions used were from Sigma Chemical (St. Louis, MO). Catalase was from Calbiochem (San Diego, CA). For some experiments, catalase was heat-inactivated as described previously (Avshalumov et al. 2003). Other drugs, including AP5, 1-(4-aminophenyl)-4-methyl-7,8-methyleneoxy-5H-2,3-benzodiazepine hydrochloride (GYKI-52466), and tetrodotoxin (TTX) were from Tocris Cookson (Ellisville, MO). Alexa Red and 2′,7′-H2DCF diacetate were from Invitrogen (Carlsbad, CA). Stock solutions of H2DCF diacetate were prepared in dimethylsulfoxide (DMSO) (Avshalumov et al. 2003, 2005); final DMSO levels were <0.1%, which alone had no effect on control responses.

Statistics

Data are expressed as means ± SE where n equals the number of cells for whole cell recording and imaging data or slices for dopamine release and dopamine content data. Significance of differences was assessed using paired or unpaired Student’s t-test for comparison of two groups as appropriate, one-way ANOVA for comparison of three groups, or two-way ANOVA for comparison of time versus drug-dependent effects.

RESULTS

MSN identification

Striatal MSNs were identified by their somatodendritic morphology and their electrophysiological characteristics in coronal or angled parasagittal brain slices; these characteristics were independent of plane of section. Recorded cells (n = 61) were not spontaneously active in vitro but did exhibit spike activity with depolarizing current injection as described previously (Bao et al. 2005); mean resting membrane potential was −77.0 ± 0.9 mV and input resistance was 78.1 ± 2.0 MΩ. Additionally, all neurons exhibited slow depolarization ramps in response to depolarizing currents and inward rectification with hyperpolarizing currents consistent with previously described properties of striatal MSNs (Bao et al. 2005; Kitai et al. 1979; Koós and Tepper 1999; Nisenbaum and Wilson 1995).

Stimulus-induced H2O2 generation in striatal MSNs

To monitor activity-dependent ROS generation, MSNs were loaded with H2DCF diacetate via the recording pipette; H2DCF becomes fluorescent DCF after oxidation by H2O2 or other ROS (Avshalumov et al. 2005, 2007; Reynolds and Hastings 1995; Sah and Schwartz-Bloom 1999). Although MSNs did not exhibit spontaneous activity, basal DCF fluorescence, reflecting tonic ROS production, was seen in all recorded MSNs (Fig. 1A). During local electrical stimulation, each pulse of the stimulus train (30 pulses, 10 Hz) elicited a single action potential in MSNs (Fig. 1A). This stimulus paradigm also produced a significant 25–35% increase in simultaneously recorded DCF fluorescence intensity (FI) in a majority of MSNs monitored under these conditions (P < 0.01; paired t-test vs. basal DCF FI; n = 7 of 11 recorded cells). In contrast, 4 of 11 cells showed no change in DCF FI (P > 0.05 paired t-test vs. basal) although physiological responses to stimulation were indistinguishable between these two groups as discussed further at the end of RESULTS. Interestingly, there was no intermediate response between these two extremes. When seen, increases in DCF FI were rapid, beginning after 5–10 stimulus pulses and reaching an average maximal level of 29 ± 1% (n = 7; Fig. 1, A and D). It should be noted that H2DCF is irreversibly activated by ROS, such that a plateau in DCF FI persists after stimulation, precluding evaluation of ROS clearance (e.g., Avshalumov et al. 2005).

Given that H2DCF can react with other ROS as well as H2O2, it was critical to ascertain whether or not the monitored increase in DCF FI was H2O2 dependent. Endogenous H2O2 levels are regulated primarily by the cellular enzymes GSH peroxidase and catalase (Cohen 1994; Stults et al. 1977). Therefore we examined whether stimulus-induced H2O2 generation in striatal MSNs could be modulated by manipulation of these peroxidase enzymes. When GSH peroxidase activity was inhibited by MCS (1 mM) (Avshalumov et al. 2003, 2005), the stimulus-induced increase in DCF FI was amplified to nearly twice that seen under control conditions (Fig. 1, B and D; P < 0.001 vs. control, n = 7 per group). In striking contrast to the control population, all MSNs recorded in the presence of MCS showed an increase in DCF FI during local stimulation, suggesting that the antioxidant status of individual MSNs dictates the magnitude of activity-dependent H2O2 levels. Confirming that H2O2 is the primary ROS generated in MSNs (3 of 5) showed an increase in DCF FI with stimulation in the presence of heat-inactivated catalase (26 ± 1%; P < 0.01 vs. basal; n = 3). These data not only confirmed that H2O2 was the monitored ROS in MSNs but also showed that activity-dependent H2O2 levels are governed by cellular peroxidase activity.

Stimulus-induced H2O2 generation in striatal MSNs requires AMPAR activation

In the presence of the Na+ channel blocker TTX (1 μM) (Avshalumov et al. 2005), local stimulation in dorsolateral striatum failed to elicit either action potentials or an associated increase in intracellular H2O2 in any recorded MSN (n = 5; Fig. 2, A and C), demonstrating that H2O2 generation requires action potentials. To determine whether the effect of TTX was primarily the result of inhibition of spike generation per se or inhibition of synaptic transmitter release, we compared the
effect of depolarizing current injection in individual MSNs on DCF FI with that evoked by local stimulation. Using a current injection paradigm designed to mimic the effect of local stimulation (30 pulses, 10 Hz), we found no detectable increase in endogenous intracellular H$_2$O$_2$ levels despite the induction of spike firing of each recorded MSN ($n = 5$; Fig. 2B). In the same cells, however, subsequent local stimulation caused the usual $\sim$30% increase in DCF FI (Fig. 2, B and C). Taken together, these data suggest that activity-dependent generation of modulatory H$_2$O$_2$ in striatum requires synaptic input.

Previous studies in cultured neurons indicate that intracellular ROS production increases in response to exogenous glutamate agonists (Bindokas et al. 1996; Carriedo et al. 2001; Dugan et al. 1995; Lafon-Cazal et al. 1993; Reynolds and Hastings 1995). We therefore examined whether endogenous glutamate released during local pulse-train stimulation was required for H$_2$O$_2$ generation in striatal MSNs. Striatal MSNs express both NMDA receptors (NMDARs) and AMPARs (Bernard and Bolam 1998; Chen et al. 1998). However, excitation is largely mediated by AMPARs rather than NMDARs with mild

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stimulation (Jiang and North 1991; Kita 1996; Pennartz et al.
1991). Consistent with these data, blockade of AMPARs with
GYKI-52466 (50–100 μM) (Avshalumov et al. 2003) prevented
both the generation of action potentials and stimulus-induced
increases in DCF FI in all recorded MSNs during local stimulation
(ν = 7; Fig. 3, A and C). By contrast, stimulus-induced action
potentials were unaltered by blockade of NMDARs by AP5 (100
μM) (Avshalumov et al. 2003) and an increase in DCF FI
persisted in recorded MSNs (ν = 5, Fig. 3, B and C). These data
are the first to show that endogenous glutamate acting via
AMPAReads to H2O2 generation in CNS neurons.

Does activation of dopaminergic axons produce
autoinhibitory H2O2?

Dopaminergic axons are another potential source of modu-
latory H2O2, either directly as a consequence of increased
mitochondrial oxygen consumption during action potential
generation (Boveris and Chance 1973; Kennedy et al. 1992)
and/or indirectly via dopamine metabolism or autoxidation
(Berman and Hastings 1999; Cohen 1994; Kulagina and Mi-
chael 2003) or via AMPAR activation of MSNs by glutamate
coreleased from dopaminergic axon terminals (Chuhma et al.
We examined the role of these possible contributing factors to
H2O2-dependent suppression of striatal dopamine release using
angled parasagittal slices in which dopaminergic axon tracts
within the striatum are sufficiently preserved to permit dopa-
imergic pathway stimulation 1.5–2 mm distal to a recording site.
Dopamine release evoked by alternating local and pathway
pulse-train stimulation (30 pulses, 10 Hz) was monitored
t veterometrically at a single recording site with a carbon-fiber
microelectrode (Fig. 4A) and identified by the single oxidation
and single reduction peak potentials that define the signature
current-voltammogram for dopamine (Fig. 4B, insets). Dopamine
release evoked by distal stimulation was dopamine pathway
specific; an increase in [DA]o was only seen when the path-
way-stimulating electrode was positioned ventrocaudally to the
recording electrode but not from sites that were perpendicular
to this location. The average [DA]o, evoked by local stimulation
was ∼40% higher than that evoked by dopamine pathway
stimulation (Fig. 4, C and D; P < 0.001; n = 12), reflecting the
efficacy of local stimulation to activate most, if not all, dopa-
imergic axons surrounding a recording site, contrasted with
pathway stimulation, which activates dopaminergic axons that
diverge from the stimulus site.

To elucidate whether selective activation of dopaminergic
axons generates H2O2 that might serve as an autoinhibitory signal
to regulate dopamine release, we amplified endogenous
H2O2 levels by inhibiting GSH peroxidase using MCS, then
compared the effect on [DA]o evoked locally and by pathway
stimulation. Consistent with the elevation of H2O2 levels in
MSNs during local stimulation in the presence of MCS (Fig.
1), locally evoked [DA]o was suppressed throughout the stim-
ulus after 30 min exposure to MCS (2-way ANOVA, P <
0.001 vs. same-site control, ν = 6; Fig. 5, A and B) as
described previously (Avshalumov et al. 2003). Release sup-
pression was reversible on MCS washout (not shown). In
contrast to the effect of MCS on locally evoked [DA]o, how-
ever, MCS did not alter either the peak amplitude or the time
course of [DA]o evoked at the same recording site by dopamine
pathway stimulation (2-way ANOVA, P > 0.05, ν = 6; Fig.
5, A and B). This implies the absence of modulatory H2O2
generation during selective stimulation of dopaminergic axons,
including that from dopamine metabolism or autoxidation.

The lack of effect of GSH peroxidase inhibition on dopa-
mine pathway-evoked [DA]o also argues against the possibility
that acutely elevated H2O2 levels might deplete the releasable
pool of dopamine, e.g., by direct oxidation. Separate compar-
ison studies of striatal dopamine content confirmed that dopa-
imine levels were unaltered by exposure to MCS (1 mM, 30
min) with dopamine content of 55 ± 3 nmol/g tissue wet
weight (ν = 16) in slices exposed to MCS versus 58 ± 1
nmol/g (ν = 16) in control slices (P > 0.05 MCS vs. control,
unpaired t-test).

Given evidence for co-release of glutamate and dopamine
from dopaminergic neurons in culture and from mesolimbic
dopaminergic input to ventral striatum (nucleus accumbens) in
brain slices (Chuhma et al. 2004; Kaneko et al. 1990; Sulzer et
al. 1998; Trudeau 2004), we next examined whether co-

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released glutamate during stimulation of nigrostriatal dopaminergic input to dorsolateral striatum might contribute to glutamate-dependent H$_2$O$_2$ generation and consequent dopamine release modulation. Previous studies show that striatal dopamine release evoked by local pulse-train stimulation is suppressed by concurrent glutamate release acting at AMPARs (Avshalumov et al. 2003). We therefore compared the effect of AMPAR blockade by GYKI-52466 on [DA]$_o$ evoked by local versus dopamine pathway stimulation (30 pulses, 10 Hz). As previously, GYKI-52466 caused an increase of ~70% in peak amplitude of [DA]$_o$ evoked by local stimulation (Fig. 5, C and D) with amplification of evoked [DA]$_o$ throughout the pulse train (2-way ANOVA, $P < 0.001$ vs. same-site control, $n = 6$), confirming AMPAR-dependent modulation of dopamine release. At the same recording site, however, GYKI-52466 had no effect on the amplitude of [DA]$_o$ evoked by dopamine pathway stimulation (Fig. 5, C and D; 2-way ANOVA, $P > 0.05$ vs. control, $n = 6$). These data suggest that if co-release of dopamine and glutamate from dopaminergic axons does occur in dorsolateral striatum, this source of glutamate input does not contribute to AMPAR-mediated suppression of dopamine release. These data also support the absence of concurrent activation of thalamostriatal input during dopamine pathway stimulation.

Lack of H$_2$O$_2$ generation in MSNs during dopamine pathway stimulation

To examine possible contributions from concurrent thalamostriatal activation in angled parasagittal slices directly and to test whether H$_2$O$_2$ generation consequent to stimulation of dopaminergic axons might contribute to elevated H$_2$O$_2$ levels in striatal MSNs, we compared DCF FI in MSNs during local versus dopamine pathway stimulation. Using the same orientation of stimulating and recording electrodes found to elicit reliable dopamine release (Fig. 4), dopamine pathway stimulation (30 pulses, 10 Hz) failed to evoke action potentials in any recorded MSN ($n = 13$; Fig. 6). This not only confirms the
absence of thalamostriatal pathway activation but also argues further against glutamate co-release from dopamine axons. Moreover simultaneously recorded DCF FI did not differ from basal DCF FI in these cells (n/H11005 13, P/H11022 0.05 vs. basal). The lack of detectable H2O2 elevation in MSNs, here used as “reporter cells,” suggests minimal production of diffusible H2O2 during selective dopamine axon stimulation. By contrast, subsequent local pulse-train stimulation elicited a single action potential for each stimulus pulse delivered in the same MSNs (Fig. 6), accompanied by an increase of 31/H11006 1% in DCF FI in 10 of 13 recorded cells (P/H11021 0.001 vs. basal; n/H11005 10).

H2O2 source versus nonsource MSNs

Throughout these studies, a population of MSNs showed no detectable increase in DCF FI during local stimulation under control conditions or conditions in which action potential generation was unaltered (e.g., in heat-inactivated catalase). Of 39 cells recorded under these conditions, 30 produced a detectable increase in [DA]o (77%), whereas 9 did not (23%). This implies that a majority of MSNs are H2O2 “source” cells whereas a smaller population are “nonsource” (or possibly “sink”) cells. The occurrence of nonsource cells was not simply a technical artifact from insufficient dye loading because exogenously applied H2O2

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caused an increase in DCF FI that was typically >70% of basal in these cells and was similar to that seen in source MSNs (not illustrated). A comparison of basic electrophysiological properties in subpopulations of each group indicated no difference in resting membrane potential (−77.2 ± 1.1 mV source vs. −76.2 ± 1.5 mV nonsource) or input resistance (79.9 ± 2.3 MΩ source vs. 72.9 ± 3.6 MΩ nonsource; unpaired t-test, P > 0.05 for both comparisons, n = 15 source and 5 nonsource MSNs). Excitability also appeared similar in both source and nonsource cells, given that local stimulation evoked action potentials that faithfully followed stimulation frequency in both populations. Last the viability of both populations was similar with similar recording times possible for both groups. Importantly, however, when GSH peroxidase was inhibited by MCS (e.g., Fig. 1B), all MSNs tested (7/7) showed a significant increase in DCF FI, as noted in the preceding text. This implies that differences in peroxidase enzyme activity in MSN subpopulations determine intracellular H₂O₂ levels in a given cell and, therefore whether the cell will be a source of glutamate-dependent, modulatory H₂O₂.

**DISCUSSION**

How synaptically released transmitters affect adjacent release sites that lack discrete point-to-point synaptic connections, like dopaminergic and glutamatergic inputs to striatal MSNs, is a long-standing question. In the case of dopamine, the answer is that it acts by volume transmission (Cragg and Rice 2004; Fuxe and Agnati 1991; Garris et al. 1994; Rice 2000; Rice and Cragg 2008). However, glutamate acts primarily within a synapse (Barbour 2001; Rusakov et al. 1999) with spillover limited by avid uptake. Thus whether synaptically released glutamate acting at ionotropic glutamate receptors regulates dopamine release is unresolved. We show here that glutamatergic excitation of AMPARs in striatal MSNs increases generation of the diffusible signaling molecule, H₂O₂, in a large population of these cells. This is the first direct demonstration of H₂O₂ generation in neurons by *endogenous* glutamate. By contrast, we found no evidence for generation of modulatory H₂O₂ by dopamine axons or released dopamine. Together, these data support a model of glutamatergic regulation of striatal dopamine release that is mediated in large part by AMPAR-dependent H₂O₂ generated as a retrograde messenger in striatal neurons, including MSNs, rather than dopamine axons.

**H₂O₂ as a signaling molecule**

In contrast to other ROS, H₂O₂ is neither a free radical nor an ion, which limits its reactivity (Cohen 1994) and increases its membrane permeability (Bienart et al. 2006, 2007; Makino et al. 2004; Ramasarma 1982), so that it is well-suited as a diffusible messenger. Recent evidence suggests that cell-specific membrane permeability to H₂O₂ governs its efflux and entry (Bienart et al. 2007; Makino et al. 2004). Cellular levels, therefore, reflect the balance among generation, primarily by mitochondrial respiration (Boveris and Chance 1973; Dugan et al. 1995; Kennedy et al. 1992; Liu et al. 2002), metabolism, primarily by GSH peroxidase and catalase (Cohen 1994; Stults et al. 1977) but also peroxiredoxins (Hofmann et al. 2002; Rhee et al. 2001), and H₂O₂ diffusion into and out of cells (Makino et al. 2004). The role of peroxide metabolism in managing cellular H₂O₂ levels in MSNs was shown by the marked amplification of activity-dependent H₂O₂ levels in all recorded MSNs after GSH peroxidase inhibition (Fig. 1B and D). The finding that all recorded MSNs showed a stimulation-induced increase in H₂O₂, in the presence of MCS also suggests that GSH peroxidase helps determine whether individual MSNs are H₂O₂-source or nonsource cells. Importantly, demonstration that AMPAR-dependent generation of modulatory H₂O₂ occurs in MSNs (Fig. 3A), but not dopaminergic axons (Fig. 5), further indicates that H₂O₂ must diffuse from an external cellular source to inhibit axonal dopamine release.

**No generation of modulatory H₂O₂ by dopamine axons**

In our initial report showing that endogenously produced H₂O₂ inhibited dopamine release, we suggested that dopamine axons might be the primary source of activity-dependent H₂O₂ (Chen et al. 2001), given the abundance of mitochondria within a few hundred nanometers of dopamine axon terminals (Ni- renberg et al. 1997). In that case, H₂O₂ would be an autoinhibitor of dopamine release as it is in dopamine cell bodies (Avshalumov et al. 2005). However, the hypothesis of direct generation of modulatory H₂O₂ by dopamine axons is not supported by our subsequent work, including the present studies. Most obviously, we found that dynamic regulation of dopamine release by endogenous H₂O₂ requires AMPAR activation (Avshalumov et al. 2003). As noted in the introduction, this implies that dopamine axons cannot be the primary source of modulatory H₂O₂ because they lack AMPARs (Bernard and Bolam 1998; Bernard et al. 1997; Chen et al. 1998).

Indeed GSH peroxidase inhibition by MCS has no effect on locally evoked [DA]₀ when AMPARs are blocked (Avshalumov et al. 2003), indicating that there is no remaining H₂O₂ signal to amplify. The present finding that MCS has no effect on pathway-evoked [DA]₀ (Fig. 5A) confirms that there is little, if any, direct AMPAR-independent contribution from dopamine axons to the generation of modulatory H₂O₂.

These and other data also imply that there are no indirect contributions to dynamically generated H₂O₂ from metabolism or autoxidation of released dopamine (Berman and Hastings 1999; Cohen 1994; Kulagina and Michael 2003) or from glutamate co-released from dopamine axons (Chuhma et al. 2001; Kaneko et al. 1990; Sulzer et al. 1998; Trudeau 2004). The findings that MCS has no effect on pathway-evoked dopamine release (Fig. 5A) or on locally evoked release when AMPARs are blocked and evoked [DA]₀ is nearly twofold higher than control (Avshalumov et al. 2003) argue against a modulatory role for H₂O₂ formed from released dopamine. Indirect support for this argument comes from the absence of detectable elevation in DCF FI in MSNs during selective dopamine pathway stimulation, which could have reflected H₂O₂ diffusion from dopamine axons (Fig. 6). The inability of dopamine pathway stimulation to increase DCF FI or to alter MSN physiology (Fig. 6) coupled with the absence of an effect of AMPAR blockade on dopamine pathway-evoked [DA]₀ (Fig. 5C) further shows that glutamate co-released from dopamine axons does not contribute to AMPAR-dependent generation of modulatory H₂O₂.

**Glutamate-dependent generation of H₂O₂ in MSNs**

In contrast to the lack of evidence for self-regulation of dopamine release by H₂O₂ generated in dopamine axons, the
present studies clearly demonstrate glutamate-dependent H$_2$O$_2$ generation in MSNs. The findings and approaches used differ in three main ways from earlier work on glutamate-receptor agonist-induced increases in H$_2$O$_2$ or other ROS levels in cultured neurons (e.g., Bindokas et al. 1996; Carriedo et al. 2001; Dugan et al. 1995; Lafon-Cazal et al. 1993; Reynolds and Hastings 1995). First, in previous studies, exogenous glutamate agonists were used to elicit increases in ROS, whereas here endogenously released glutamate was the trigger. Second, the earlier focus was on glutamate neurotoxicity, whereas the present studies demonstrate that AMPAR-dependent H$_2$O$_2$ generation is a component of normal glutamate signaling in striatum. Third, because CNS neurons in culture lack glia to provide glutamate uptake and limit excitotoxicity, as well as the glial antioxidant network that limits oxidative damage (e.g., Avshalumov et al. 2004), it is surprising and significant that increases in intracellular H$_2$O$_2$ in MSNs also occur in the complex microenvironment of brain slices during AMPAR activation by endogenous glutamate.

It is also notable that action potential generation alone in MSNs was not sufficient to generate H$_2$O$_2$. Although the factors underlying this difference are not yet known, a requirement for glutamatergic input has been reported previously for another class of diffusible messengers, the endocannabinoids. Kreitzer and Malenka (2005) found that detectable endocannabinoid release from MSNs when postsynaptic depolarization was paired with presynaptic stimulation but not with postsynaptic depolarization was paired with presynaptic stimulation but not with postsynaptic depolarization alone.

Are MSNs the only source of dynamic, modulatory H$_2$O$_2$ generation? The role of other striatal cells has not yet been examined; however, it is likely that MSNs are an important source of modulatory H$_2$O$_2$ given that MSNs constitute 90–95% of the neuron population of the striatum (Kemp and Powell 1971a). Moreover, glutamate synapses are closely opposed to dopaminergic synapses on the dendrites of MSNs (Freund et al. 1984; Smith and Bolam 1990) and are therefore ideally positioned to modulate dopamine release via postsynaptically generated H$_2$O$_2$. Consistent with this anatomical evidence, stimulated H$_2$O$_2$ generation in MSNs is dependent on AMPAR activation, amplified by inhibition of GSH peroxidase, and eliminated by catalase (Figs. 1 and 3) in a pattern consistent with the previously reported consequences of these manipulations on dopamine release (Avshalumov et al. 2003). Thus H$_2$O$_2$ produced in MSNs alone could be sufficient to mediate glutamate-dependent regulation of dopamine release. Of course, contributions from the remaining 5–10% of striatal neurons could also occur as most of these express AMPAR subunits (Deng et al. 2007). Striatal glia might contribute, as well, with the caveat that although cultured striatal astrocytes express AMPAR protein (Fan et al. 1999), there are no reports of functional consequences of AMPAR activation in these cells in striatum.

How does H$_2$O$_2$ inhibit dopamine release? We have reported previously that H$_2$O$_2$ signaling in the striatum requires the activation of K$_{ATP}$ channels (Avshalumov and Rice 2003; Avshalumov et al. 2003), which are found throughout the nigrostriatal dopamine pathway (Avshalumov et al. 2003, 2005; Liss et al. 1999; Mourre et al. 1989; Xia and Haddad 1991; Zini et al. 1993). Although other H$_2$O$_2$ sensing systems from phosphatases and kinases to transcription factors can also regulate cell function (Finkel et al. 2003; Kamsler and Segal 2004; Kishida and Klann 2007; Rhee 2006; Rhee et al. 2005; Veal et al. 2007), these processes are very slow (minutes to hours) compared with the rapid, dynamic H$_2$O$_2$ regulation of striatal dopamine release which occurs on a subsecond time scale.

Based on these findings, we propose a model of glutamate-dependent modulation of axonal dopamine by AMPAR-dependent H$_2$O$_2$ generation in MSNs in which AMPAR activation of dendrites and soma of these cells leads to generation of H$_2$O$_2$ that diffuses to adjacent dopamine axons and inhibits dopamine release via opening of K$_{ATP}$ channels (Fig. 7, center). When AMPARs are blocked (+GYKI), H$_2$O$_2$-dependent regulation of dopamine release via K$_{ATP}$ channels is lost (Avshalumov and Rice 2003), and dopamine release is enhanced (Fig. 7, left). Conversely, when activity-dependent levels of H$_2$O$_2$ are amplified by inhibiting GSH peroxidase with MCS, this leads to enhanced H$_2$O$_2$-dependent K$_{ATP}$-channel activation (Avshalumov and Rice 2003), and further suppression of dopamine release (Fig. 7, right).

Conclusions and implications

What are the implications of diffusible H$_2$O$_2$ as the mediator of striatal glutamate-dopamine interactions? As an inhibitory intermediate, endogenously generated H$_2$O$_2$ reverses conventional glutamatergic excitation and leads to inhibition of dopamine release via H$_2$O$_2$-sensitive K$_{ATP}$ channels (Avshalumov

![FIG. 7. Model of axonal dopamine release regulation by glutamate acting via AMPARs and generation of diffusible H$_2$O$_2$ in striatal MSNs. Center: activation of AMPARs on MSN dendrites generates H$_2$O$_2$ that diffuses to adjacent dopamine axons and inhibits dopamine release via opening of K$_{ATP}$ channels. Left: when AMPARs are blocked (+GYKI), H$_2$O$_2$-dependent regulation of dopamine release via K$_{ATP}$ channels is lost and dopamine release is enhanced. Right: when activity-dependent levels of H$_2$O$_2$ are amplified by inhibiting GSH peroxidase with MCS, this leads to enhanced H$_2$O$_2$-dependent K$_{ATP}$-channel activation and further suppression of dopamine release.](http://jn.physiology.org/)}
and Rice 2003). Generation of detectable levels of H$_2$O$_2$ in a majority of MSNs following AMPAR activation would necessarily contribute to dopamine release inhibition given that this diffusible messenger will readily leave the cell in which it is produced. It would be expected that H$_2$O$_2$ is produced in all MSNs, and potentially all AMPAR-expressing cells, during glutamatergic activation. Whether a particular MSN or other cell is an H$_2$O$_2$ source, however, apparently depends largely on the peroxidase activity in that cell, inasmuch that all recorded MSNs showed an increase in DCF FI when GSH peroxidase was inhibited, whereas stimulus-evoked H$_2$O$_2$ levels were kept below detection limits in ~25% of recorded MSNs under control conditions. Does detection indicate functionally relevant levels of H$_2$O$_2$? Our previous studies of the effect of endogenous H$_2$O$_2$ on the physiology of dopaminergic neurons in the substantia nigra indicate that the answer is yes. In those studies, we compared increases in DCF FI when endogenous H$_2$O$_2$ was amplified by increasing concentrations of the GSH peroxidase inhibitor, MCS. Low concentration of MCS, even H$_2$O$_2$ was amplified by increasing concentrations of the GSH peroxidase activity in that cell, inasmuch that all recorded MSNs showed an increase in DCF FI when GSH peroxidase was inhibited, whereas stimulus-evoked H$_2$O$_2$ levels were kept below detection limits in ~25% of recorded MSNs under control conditions. Does detection indicate functionally relevant levels of H$_2$O$_2$? Our previous studies of the effect of endogenous H$_2$O$_2$ on the physiology of dopaminergic neurons in the substantia nigra indicate that the answer is yes. In those studies, we compared increases in DCF FI when endogenous H$_2$O$_2$ was amplified by increasing concentrations of the GSH peroxidase inhibitor, MCS. Low concentration of MCS, even those sufficient to cause a significant increase in DCF FI, had no effect on K$_{ATP}$ channels in dopamine neurons, implying that there is a threshold for K$_{ATP}$ channel modulation (i.e., a functional H$_2$O$_2$ concentration) that is above DCF detection limits (Avshalumov et al. 2005). Thus DCF sensitivity to H$_2$O$_2$ appears to be even greater than the sensitivity of K$_{ATP}$ channels to endogenous H$_2$O$_2$ levels. These data therefore imply that glutamatergic activation of source MSNs could produce levels of H$_2$O$_2$ sufficient to inhibit nigrostriatal dopamine input to those cells (Fig. 7), whereas nonsource MSNs lack this capacity. This would also hold true for other striatal cells that might serve as sources of H$_2$O$_2$.

Differential regulation of H$_2$O$_2$ generation in MSNs could be important physiologically, given differential dopaminergic regulation of striatonigral versus striatopallidal MSNs (Surmeier et al. 2007). For example, in striatonigral MSNs in which D1 dopamine-receptor activation enhances responsiveness to glutamatergic input (Albin et al. 1989; Surmeier et al. 2007), a consequent increase in H$_2$O$_2$ generation would provide an inhibitory signal to decrease dopamine input, which would then reverse D1-enhanced excitability in a reciprocal glutamate-dopamine feedback loop. By contrast, in striatopallidal MSNs, D2-receptor activation decreases excitability. A consequent decrease in H$_2$O$_2$ generation would lead to enhanced local dopamine release, resulting in further D2-receptor-mediated inhibition of MSNs excitability. The absence of activity-dependent H$_2$O$_2$ elevation in those MSNs, for example, would avoid an endless negative feedback process.

Last, glutamate-dopamine dysregulation has been implicated as a causal factor in Parkinson’s disease (Bevan et al. 2006; Chase and Oh 2000), Huntington’s disease (Di Filippo et al. 2007), schizophrenia (Sawa and Snyder 2002; Thompson et al. 2004), and addiction (Hyman and Malenka 2001; Kelley 2004). Oxidative stress is also linked causally to several of these disorders (Browne and Beal 2006; Orth and Schapira 2002; Klein and Schlossmacher 2006; Tosic et al. 2006). The finding that AMPAR activation in MSNs by endogenously release glutamate leads to enhanced H$_2$O$_2$ generation provides a new starting point for understanding glutamate-dopamine function and dysfunction.

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