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Nitric Oxide Donors Enhance the Frequency Dependence of Dopamine Release in Nucleus Accumbens

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Dopamine (DA) neurotransmission in the nucleus accumbens (NAc) is critically involved in normal as well as maladaptive motivated behaviors including drug addiction. Whether the striatal neuromodulator nitric oxide (NO) influences DA release in NAc is unknown. We investigated whether exogenous NO modulates DA transmission in NAc core and how this interaction varies depending on the frequency of presynaptic activation. We detected DA with cyclic voltammetry at carbon-fiber microelectrodes in mouse NAc in slices following stimuli spanning a full range of DA neuron firing frequencies (1–100 Hz). NO donors 3-morpholinosydnonimine hydrochloride (SIN-1) or *z*-1-[*N*-(3-ammoniopropyl)-*N*-(*n*-propyl)amino]diazen-1-ium-1,2-diolate (PAPA/NONOate) enhanced DA release with increasing stimulus frequency. This NO-mediated enhancement of frequency sensitivity of DA release was not prevented by inhibition of soluble guanylyl cyclase (sGC), DA transporters, or large conductance Ca^{2+}-activated K^+ channels, and did not require glutamatergic or GABAergic input. However, experiments to identify whether frequency-dependent NO effects were mediated via changes in powerful acetylcholine–DA interactions revealed multiple components to NO modulation of DA release. In the presence of a nicotinic receptor antagonist (dihydro-β-erythroidine), NO donors increased DA release in a frequency-independent manner. These data suggest that NO in the NAc can modulate DA release through multiple GC-independent neuronal mechanisms whose net outcome varies depending on the activity in DA neurons and accumbal cholinergic interneurons. In the presence of accumbal acetylcholine, NO promotes the sensitivity of DA release to presynaptic activation, but with reduced acetylcholine input, NO will promote DA release in an activity-independent manner through a direct action on dopaminergic terminals.
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INTRODUCTION

The nucleus accumbens (NAc), the major part of the limbic ventral striatum, has an important role in normal goal-directed or motivated behaviors as well as in maladaptive states, including drug addiction and schizophrenia. Here, inputs from major limbic-associated brain regions like the medial prefrontal cortex, basolateral amygdala, and ventral subiculum of the hippocampus converge (Finch, 1996; French and Totterdell, 2002, 2003; Groenewegen *et al*, 1987, 1999; Mulder *et al*, 1998; O'Donnell *et al*, 1999; Sesack and Grace, 2010; Wright and Groenewegen, 1995), are integrated with thalamic inputs (Berendse and Groenewegen, 1990; Smith *et al*, 2004), and interface with motor loops of the basal ganglia (Groenewegen and Trimble, 2007; Groenewegen *et al*, 1996; Mogenson *et al*, 1980; Sesack and Grace, 2010; Zahm, 2000). These interactions are powerfully modulated by dopaminergic innervation from the ventral tegmental area (Ikemoto, 2007; Voorn *et al*, 1986). Dopaminergic neurons signal unpredicted rewards or other salient contextual stimuli and their conditioned cues by a shift in firing rates from tonic low frequencies to brief bursts at high frequency (Matsumoto and Hikosaka, 2009; Schultz, 1986, 2002), and corresponding accumbal DA release influences accumbal output and long-term plasticity (Morris *et al*, 2010; Reynolds and Wickens, 2002; Schultz, 2010).

How the release of DA reflects dynamic changes in activity in dopaminergic neurons is governed by the local regulation of release probability within the NAc (Cragg, 2003, 2006). For example, DA release is powerfully modulated by acetylcholine (ACh) arising from intrinsic cholinergic interneurons (ChIs) (Cragg, 2006; Exley *et al*, 2008; Rice and Cragg, 2004; Threlfell *et al*, 2010). Nitric oxide (NO) is another potent neuromodulator that is thought to be produced locally by neuronal nitric oxide synthase (uNOS) containing accumbal interneurons (French *et al*, 2005; Hidaka and Totterdell, 2001; Kraus and Prast, 2001), but how NO influences the dynamic signaling of activity by DA in NAc is currently unknown.

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Interactions between NO and DA have been extensively studied in the dorsal striatum, where NO modulates the excitability of striatal GABAergic projection neurons (West and Grace, 2004), corticostriatal synaptic plasticity (Calabresi *et al*, 1999a, b), and the release of various neurotransmitters including glutamate, GABA, ACh, serotonin, and DA (Prast *et al*, 1995, 1998; Prast and Philippu, 2001; Trabace and Kendrick, 2000; West and Galloway, 1996, 1997a, b, 1998). However, NO in the dorsal striatum is reported either to facilitate (Black *et al*, 1994; Buyukuysal, 1997; Iravani *et al*, 1998; Liang and Kaufman, 1998; Lonart *et al*, 1993; Stewart *et al*, 1996; Trabace and Kendrick, 2000; West and Galloway, 1996, 1997a, b, 1998; Zhu and Luo, 1992) or inhibit DA release (Guevara-Guzman *et al*, 1994; Segovia and Mora, 1998; Silva *et al*, 1995, 2003). The effector mechanisms have been suggested to include inhibition of DA transporters (DATs) (for a review see, Kiss and Vizi, 2001) as well as indirect mechanisms that involve NO activation of soluble guanylyl cyclase (sGC) in striatal projection neurons, which through their projections to the substantia nigra subsequently modify the activity of DA neurons (West and Grace, 2000) and NO-mediated increases in local glutamate levels (Bogdanov and Wurtman, 1997; Guevara-Guzman *et al*, 1994; Trabace and Kendrick, 2000; West and Galloway, 1997a, b), which might subsequently modify DA release (for a review see, David *et al*, 2005).

We used fast-scan cyclic voltammetry (FCV) at carbon-fiber microelectrodes in striatal slices to identify how exogenous NO modulates endogenous DA release in NAc core during a range of evoked activity that spans the frequencies seen for dopaminergic neurons *in vivo*. We show that the outcome of NO on DA release in NAc varies depending on the activity in DA axons as well as in other accumbal neurons.

MATERIALS AND METHODS

Brain Slice Preparation and Voltammetry

Coronal striatal slices (300 μm) containing the NAc were prepared from brains of 26–35 g CD-1 male mice (Harlan, Oxon, UK). The sections were cut on a Vibratome (Leica) in ice-cold oxygenated HEPES-buffered artificial cerebrospinal fluid (HEPES-aCSF) containing NaCl (120 mM), $NaHCO_3$ (20 mM), D-glucose (10 mM), HEPES acid (6.7 mM), HEPES salt (3.3 mM), KCl (5 mM), $CaCl_2$ (2.4 mM), KH_2PO_4 (1.25 mM), and $MgSO_4$ (2 mM) saturated with 95% O_2/5% CO_2. After maintaining slices for at least an hour in HEPES-aCSF at room temperature, they were transferred to the recording chamber and allowed to equilibrate for another hour with the superfusion medium of the recording chamber, namely bicarbonate-buffered aCSF containing NaCl (125 mM), $NaHCO_3$ (26 mM), D-glucose (10 mM), KCl (3.8 mM), $CaCl_2$ (2.4 mM), KH_2PO_4 (1.2 mM), and $MgSO_4$ (1.3 mM), and saturated with 95% O_2/5% CO_2. Recordings were carried out in aCSF at a flow rate of 1.3 ml/min and a bath temperature of 32–33°C.

Extracellular DA concentration ($[DA]_o$) was monitored and quantified using FCV as described previously (Cragg, 2003; Rice and Cragg, 2004; Threlfell *et al*, 2010). Briefly, recordings were made with 7- to 10 μm-diameter carbon-fiber microelectrodes of tip lengths ~50–100 μm that were fabricated in-house. The carbon-fiber electrode tip was inserted 100 μm into the tissue in the NAc core, ventral to the anterior commissure, and voltammetry was performed using a Millar Voltammeter (PD Systems, Surrey, UK). The applied voltage was a triangular waveform, with a voltage range of −0.7 to +1.3 V and back *vs* an Ag/AgCl reference electrode at a scan rate of 800 V/s and a sampling frequency of 8 Hz.

All evoked currents were recorded in the faradaic mode, showing currents after an electronic subtraction of background currents. These background-subtracted currents were monitored and recorded on a computer for analysis using Strathclyde Whole Cell Program (University of Strathclyde, Glasgow, Scotland). The evoked current profiles were attributed to DA by comparison of their potentials for peak oxidation and reduction currents with those of DA in calibration media (500–600 and −200 mV *vs* Ag/AgCl, respectively). Profiles of $[DA]_o$ *vs* time were obtained by sampling the current at the DA oxidation peak.

Electrode Calibrations

Electrode sensitivity to DA (nA/μM) in the presence of each added drug compound was determined from standard curves for DA oxidation current *vs* applied DA concentration for a physiological range of DA concentrations (1–3 μM) in aCSF in the presence of all experimental drugs, singly, and in combination as used experimentally. Some of the applied drugs decreased absolute electrode sensitivity to DA, for example, electrode sensitivity to DA was decreased in the presence of 3-morpholinosydnonimine hydrochloride (SIN-1) (500 μM) by ~63%, in the presence of *z*-1-[*N*-(3-ammoniopropyl)-*N*-(*n*-propyl)amino]diazen-1-ium-1,2-diolate (PAPA/NONOate) (300 μM) by ~74%, and in the presence of 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) (100 μM) by ~40%. We used appropriately modified calibration factors determined from each and every drug condition to calibrate the electrodes. Note, we also confirmed that these effects of the parent compound on DA sensitivity occurred when electrodes were situated in the tissue environment by performing additional calibrations in tissue (data not illustrated; required higher applied DA concentrations and inclusion of a DA uptake inhibitor (cocaine) to allow applied DA in the striatum to reach levels approaching low micromolar). Importantly, the relationship between $[DA]_o$ and oxidation current remained linear in all drugs used for the range of $[DA]_o$ seen *in situ* (data not illustrated).

Local Electrical Stimulation

Local stimulations used to evoke DA release were applied by a surface, bipolar concentric electrode (25 μm diameter Pt/Ir; FHC, Bowdoinham, ME). Under a binocular microscope, the stimulating electrode was positioned flush with the tissue at a distance of ~100 μm from the recording electrode. Stimulus pulses of 200 μs duration were generated out-of-phase with FCV scans to prevent interference with the voltammetric current and applied at peri-maximal currents (0.5–0.7 mA). Release evoked with stimulation used here (either a single pulse or brief 4–5 pulse trains) is

inhibited by tetrodotoxin and is calcium-dependent (Cragg, 2003), but not modulated by ionotropic glutamate or GABA receptor activation (Cragg, 2003; Exley *et al*, 2008; Threlfell *et al*, 2010). However, DA release is controlled by ACh acting at presynaptic nicotinic ACh receptors on DA axons (Exley *et al*, 2008; Rice and Cragg, 2004; Zhang and Sulzer, 2004; Zhou *et al*, 2001). This local cholinergic input results from the activity of ChIs, which have been shown to be tonically active in slices as they are *in vivo* (Aosaki *et al*, 1994; Bennett and Wilson, 1999). ACh evoked by local electrical stimulation does not seem to add to already tonic levels generated by high tonic activity of ACh interneurons. DA release evoked by remote pathway stimulations was shown to be regulated by striatal ACh similarly to release evoked by local stimulations (Exley *et al*, 2008; Rice and Cragg, 2004).

Experimental Design and Analysis

Stimulus protocols were repeated at a minimum of 2-min intervals, which ensured stable, consistent release. To test the effect of frequency on DA release, a range of stimulations consisting of either a single pulse (1p) or four pulses (4p) at a range of frequencies spanning 5–100 Hz were applied in a randomized order in triplicate at each given recording site. These stimulus frequencies include the full range of physiological DA neuron firing rates reported *in vivo*, consisting of tonic firing rates (<10 Hz) and phasic bursts (firing rates approx. 15–25 Hz or higher) that accompany salient events (Bayer and Glimcher, 2005; Hyland *et al*, 2002; Schultz, 1986; Morris *et al*, 2004), and also higher frequencies as used previously that are particularly useful for probing for changes in release probability. We have established that the peak value of $[DA]_o$ for 1 Hz is indistinguishable from 1p (data not illustrated), and for simplicity, we have used 1p data to represent 1 Hz outcome. Single-pulse stimulations were distributed regularly in time across each experiment (one 1p stimulation after three consecutive pulse train stimulations), to provide a reference value of $[DA]_o$ against which $[DA]_o$ evoked by other stimuli could be compared.

All data are means ± standard error of the mean (SEM) and the sample size, n is the number of observations. The number of animals in each data set is $\geqslant 3$. Data are expressed as $[DA]_o$ normalized to release by a single pulse in control conditions. Mean value of mean peak $[DA]_o$ for a single pulse across experiments were 0.67 ± 0.06 μM (range 0.39–1.7 μM). Comparisons for differences in means were assessed by two-way ANOVAs and *post hoc* Bonferroni multiple comparison *t*-tests using GraphPad Prism.

Drug Application

NO donors of two different classes were used: SIN-1, purchased from Tocris Bioscience (Bristol, UK), and PAPA/NONOate, purchased from Alexis Biochemical (Nottingham, UK). SIN-1 was prepared fresh immediately before use in aCSF and protected from light. SIN-1 spontaneously generates NO in aqueous solution by decomposition (Feelisch and Noack, 1987) at rates expected to be in the low micromolar range per minute for 500 μM SIN-1 as inferred by Feelisch *et al* (1989) and Hogg *et al* (1992). PAPA/NONOate was dissolved in 0.01 M NaOH to a stock concentration of 60 mM and diluted in aCSF before use to the desired final concentration. A concentration of 300 μM PAPA/NONOate as used in this study is expected to generate NO bath concentrations in the low micromolar range as inferred by Garthwaite *et al* (2002). Resulting tissue NO concentrations are likely to be several orders of magnitude lower than bath concentrations (eg, Garthwaite *et al*, 2002) owing to significant consumption of NO by tissue (Hall and Garthwaite, 2006).

D-AP5, bicuculline, dihydro-β-erythroidine (DHβE), GYKI-52466 hydrochloride, iberiotoxin (IbTx), (*S*)-MCPG, ODQ, and saclofen were purchased from Tocris Bioscience or Ascent Scientific (Bristol, UK). Trolox was purchased from Merck (Hull, UK) and nomifensine was purchased from Sigma Aldrich (Cambridge, UK). The drugs were dissolved in water, aqueous acid (GYKI-52466 hydrochloride, nomifensine), aqueous alkali ((*S*)-MCPG, saclofen), aqueous DMSO (ODQ), or ethanol (Trolox), and were either prepared fresh or stored as stock aliquots of 500–2500 × final concentrations at -20°C until required. Stock aliquots were diluted with oxygenated aCSF to final concentrations immediately before use.

Each drug condition involved drug application for approximately 60 min to include wash-on and a complete set of stimulations (40–45 min). Drug effects could be observed already 3–7 min after drug application and were maximal after 15 min. Thereafter, drug effects remained constant for the whole course of frequency testing.

RESULTS

NO Donors Increase Evoked DA Release in a Frequency-Dependent Manner

We explored how NO modulates DA release evoked by a range of different frequencies (1–100 Hz, 4p), which are in the range of firing rates that DA neurons display *in vivo*, but also include higher frequencies as used previously that are particularly useful for probing of changes in release probability. Dopaminergic neurons respond to salient stimuli by shifting from tonic (approx. 0.5–10 Hz) frequencies to short phasic bursts of high-frequency firing (approx. 15–25 Hz or higher, durations <200 ms) (Hyland *et al*, 2002; Schultz, 1986).

In control conditions, evoked $[DA]_o$ varied slightly but significantly with stimulus frequency in mouse NAc core (Figure 1) by up to $172 \pm 6\%$ of release by a single pulse, according to an inverted U relationship, as described previously (Exley *et al*, 2008). Application of the NO donor SIN-1 (500 μM) significantly increased the dependence of evoked $[DA]_o$ on stimulus frequency (Figure 1a and b; two-way ANOVA, frequency: $F_{4,133} = 72.55$, $P < 0.001$; treatment: $F_{2,133} = 34.49$ $P < 0.001$; interaction: $F_{8,133} = 10.19$, $P < 0.001$). Release by lower frequencies ($\leqslant 10$ Hz) remained unchanged, but release by higher frequencies ($\geqslant 25$ Hz) was significantly increased compared with control. Maximum $[DA]_o$ were evoked by 100 Hz (4p; ~300% of release by a single pulse). Drug effects were reversible upon washout (Supplementary Figure S1a) and were concentration-dependent (data not illustrated).

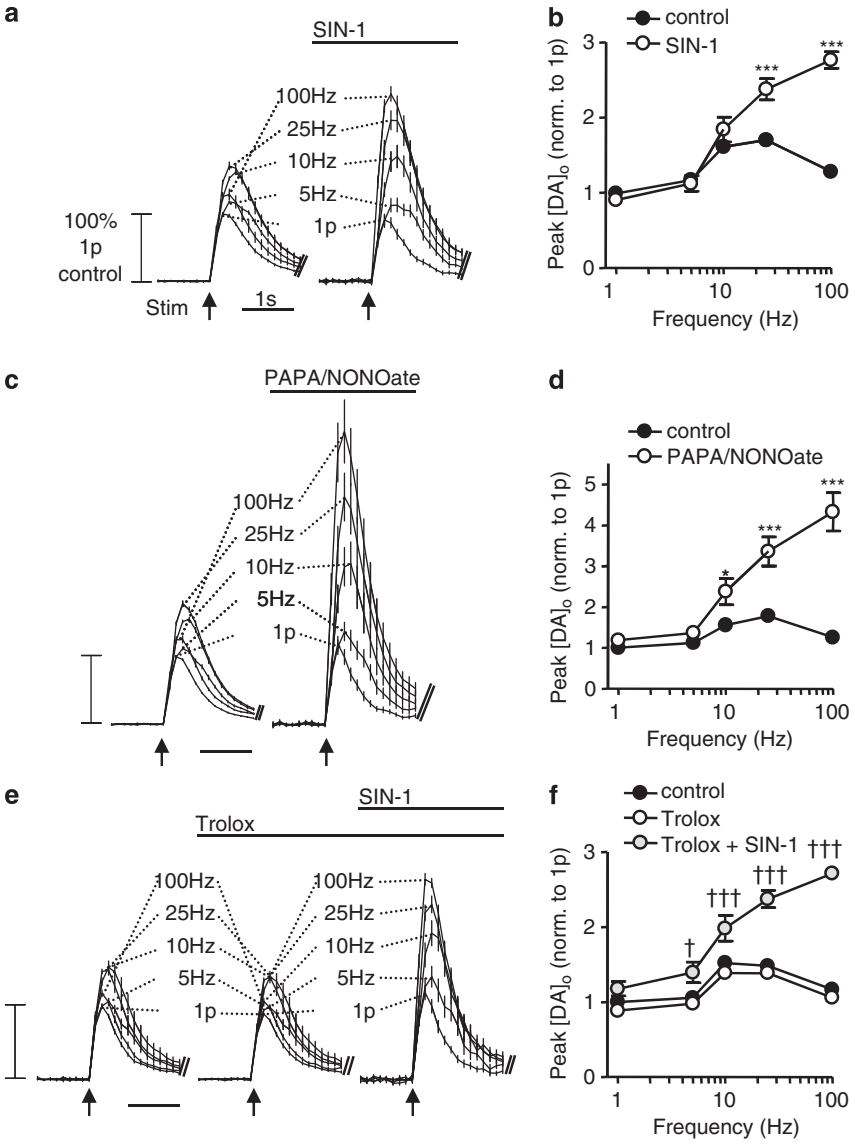

Figure 1 Nitric oxide (NO) donors increase evoked dopamine (DA) release in a frequency-dependent manner. (a, c, e) Profiles of mean extracellular DA concentration ($[DA]_o$) ± standard error of mean (SEM) *vs* time following stimuli (arrows) of either a single pulse (p) or 4p (5–100 Hz) in (a) control conditions (left) and NO donor 3-morpholinosydnonimine hydrochloride (SIN-1) (500 μM) (right), (c) control conditions (left) and NO donor *z*-1-[*N*-(3-ammoniopropyl)-*N*-(*n*-propyl)amino]diazen-1-ium-1,2-diolate (PAPA/NONOate) (300 μM) (right) or (e) control conditions (left), Trolox (200 μM) (center), and Trolox + SIN-1 (right). Data are normalized to peak $[DA]_o$ evoked by 1p in controls. (b, d, f) Mean peak $[DA]_o$ ± SEM *vs* frequency at 1p or 4p (5–100 Hz) in (b) control conditions (filled circles) and NO donor SIN-1 (unfilled) ($n = 9–15$), (d) control conditions (filled circles) and NO donor PAPA/NONOate (unfilled), ($n = 9–17$), or (f) control conditions (filled circles), Trolox (unfilled), and Trolox + SIN-1 (gray fill) ($n = 9–14$). Data are normalized to peak $[DA]_o$ evoked by 1p in controls. Asterisks indicate significance level in *post hoc* Bonferroni *t*-test for drug treatment *vs* controls, $^*P < 0.05$, $^{***}P < 0.001$. Crucifixes indicate significance level in Bonferroni *post hoc* *t*-tests for Trolox *vs* Trolox + SIN-1, $^{\dagger}P < 0.05$, $^{\dagger\dagger\dagger}P < 0.001$.

The effects of an alternative NO donor, PAPA/NONOate, was also explored. PAPA/NONOate (300 μM), like SIN-1, significantly increased $[DA]_o$ in an activity-dependent manner (Figure 1c and d; two-way ANOVA, frequency: $F_{4,92} = 29.59$, $P < 0.001$; treatment: $F_{1,92} = 89.09$, $P < 0.001$; interaction: $F_{4,92} = 18.25$, $P < 0.001$), which was reversible upon washout (Supplementary Figure S1b). Release evoked by lower frequencies ($\leqslant 5$ Hz) remained unchanged, but release by higher frequencies ($\geqslant 10$ Hz) was significantly increased compared with control. Greatest $[DA]_o$ were evoked by 100 Hz bursts (4p; 364% of release by a single pulse). The similar effects observed with two different NO donors suggest that these effects were due to NO rather than any nonspecific effects of each donor or their different breakdown products. Thus, in subsequent experiments, NO action was explored using a single example donor only, SIN-1.

To confirm that the effect of SIN-1 were not due to the concurrent release of superoxide anions, the subsequent formation of peroxynitrite and consequent modification of striatal DA release (Trabace and Kendrick, 2000), we identified whether the effects of SIN-1 were prevented by

the peroxynitrite scavenger Trolox (Edwards and Rickard, 2005; Halliwell *et al*, 1999; Regoli and Winston, 1999). Trolox alone (200 μM) did not significantly modify evoked $[DA]_o$ compared with control conditions (Figure 1e and f). Furthermore, Trolox (200 μM) did not prevent the subsequent effects of SIN-1 on increasing the activity dependence of evoked $[DA]_o$ (Figure 1e and f; two-way ANOVA, frequency: $F_{4,134} = 50.72$, $P < 0.001$; treatment: $F_{2,134} = 141.9$ $P < 0.001$; interaction: $F_{8,134} = 15.74$, $P < 0.001$). These data suggest that peroxynitrite did not contribute to the effect of SIN-1 on evoked $[DA]_o$.

NO Donors Regulate DA Transmission via Guanylyl Cyclase-independent Mechanism

NO is reported to act through a variety of effector mechanisms. One major target of NO is sGC (Bellamy *et al*, 2002; Garthwaite and Boulton, 1995). NO activation of sGC generates cyclic guanosine monophosphate (cGMP), which has a variety of downstream targets, for example, ion channels, phosphodiesterases, and protein kinases (Garthwaite and Boulton, 1995). However, the involvement of sGC in reported facilitatory effects of NO on DA levels in the striatum remains controversial. This facilitatory effect of NO on DA levels has been shown to be both sGC-dependent (Guevara-Guzman *et al*, 1994; Trabace and Kendrick, 2000) and sGC-independent (Buyukuysal, 1997; Rocchitta *et al*, 2004; Stewart *et al*, 1996; West and Galloway, 1996). We explored whether the effect of NO donors on the frequency-dependent control of DA release identified here in NAc was sGC-dependent or -independent.

The sGC inhibitor ODQ (100 μM) alone did not significantly change evoked $[DA]_o$ (Figure 2a–c), and furthermore, ODQ did not prevent the subsequent effects of SIN-1 (Figure 2a–c). SIN-1 significantly increased evoked $[DA]_o$ in a frequency-dependent manner (two-way ANOVA, frequency: $F_{4,130} = 44.09$, $P < 0.001$; treatment: $F_{2,130} = 42.98$, $P < 0.001$; interaction: $F_{8,130} = 7.71$, $P < 0.001$), which was not different from the effect of SIN-1 alone (two-way ANOVA, treatment: $F_{1,84} = 2.22$, $P > 0.05$; interaction: $F_{4,84} = 1.32$, $P > 0.05$). These data suggest that NO-mediated effects on $[DA]_o$ observed here were sGC-independent.

Another frequently reported action of NO is S-nitrosylation of proteins such as ion channels. A commonly used approach to explore whether this mechanism underlies actions of NO is to block S-nitrosylation with N-ethylmaleimide (NEM). However, in pilot studies, NEM (2 mM) induced stimulus-independent continuous release of DA that prevented subsequent evoked DA release (data not illustrated), presumably via disruption of the SNARE complex. Thus, NEM is unsuitable as a tool to explore the role of S-nitrosylation in these experiments.

Effect of NO on Evoked DA Release is not Mediated by Modulation of DA Reuptake

Previous studies have reported that NO-mediated enhancement of extracellular DA levels occurs by inhibiting DA reuptake via blockade of the DAT *in vitro* (Buyukuysal, 1997; Lonart and Johnson, 1994; Pogun *et al*, 1994) and *in vivo* (Kiss *et al*, 1999; Lin *et al*, 1995). Our data have sufficiently high temporal resolution to enable changes in re-uptake rates to be indicated by changes in the time course of disappearance of the evoked extracellular DA signal. We compared the falling phases of the DA transients evoked by 100 Hz pulse trains in control conditions *vs* those obtained during application of SIN-1. However, SIN-1 did not modify the decay of the DA signal (Figure 2d; contrast with Figure 2e, the change in decay of the DA signal seen after re-uptake blockade). Comparisons of the time required for peak evoked $[DA]_o$ to decay by 50% (t_{50}) following 100 Hz pulse trains in control *vs* during application of SIN-1 did not reveal significant differences (control: $t_{50} = 0.61 \pm 0.03$ s; SIN-1: $t_{50} = 0.62 \pm 0.03$ s, paired *t*-test, $P > 0.05$, $n = 9$). Furthermore, to ensure that modulation of the function of the DAT was not responsible for the SIN-1-induced changes in the activity dependence of evoked $[DA]_o$, we explored the effect of SIN-1 in the presence of DAT inhibition. Application of the DAT inhibitor nomifensine alone (10 μM) enhanced peak evoked $[DA]_o$ and significantly prolonged the extracellular lifetime of $[DA]_o$ (Figure 2f and g) as shown previously (Jones *et al*, 1995a, b, 1996; Schmitz *et al*, 2002). Subsequent application of SIN-1 significantly modified $[DA]_o$ in an activity-dependent manner (Figure 2f–h; two-way ANOVA, frequency: $F_{4,181} = 16.50$, $P < 0.001$; treatment: $F_{2,181} = 140.5$ $P < 0.001$; interaction: $F_{8,181} = 5.75$, $P < 0.001$) not different to the effect of SIN-1 alone (see Figure 2h), suggesting that modulation of DA re-uptake is not responsible for these NO-mediated effects on $[DA]_o$.

Major Component of Effect of NO on DA Release is BK Channel-Independent

One candidate sGC-independent mechanism through which NO has been reported to influence cellular excitability is via modulation of large conductance Ca^{2+}-activated K^+ (BK) channels. The BK current has been shown to be directly modulated by NO through S-nitrosylation of cysteine residues (and indirectly by activation of sGC depending on local NO concentrations) (Ahern *et al*, 2002). We explored whether BK channels might mediate NO effects on DA transmission. Blockade of BK channels with IbTx (100 nM) significantly increased the inverted U-dependence of the relationship between evoked $[DA]_o$ and frequency. IbTx significantly increased $[DA]_o$ evoked by 10 and 25 Hz compared with control (Figure 2i and j; two-way ANOVA, *post hoc* Bonferroni *t*-tests, 10 Hz: $P < 0.05$, $n = 9$; 25 Hz: $P < 0.01$, $n = 9$). The presence of IbTx, however, did not prevent SIN-1 effects. SIN-1 (500 μM) significantly increased evoked $[DA]_o$ in an activity-dependent manner, with greatest effect at highest frequencies (Figure 2i–k; two-way ANOVA, frequency: $F_{4,119} = 208.1$, $P < 0.001$; treatment: $F_{2,119} = 312.0$, $P < 0.001$; interaction: $F_{8,119} = 22.29$, $P < 0.001$). IbTx did, however, slightly change the effect of SIN-1. In the presence of IbTx, SIN-1 appeared to more generally increase evoked $[DA]_o$ throughout the range of stimulation frequencies applied compared with the effects of SIN-1 in the absence of IbTx (Figure 2k; compare solid *vs* dotted line). This apparent shift in the actions of SIN-1 may be due to a small component of NO action being via, or being shunted due to, a change in BK channel function. Nonetheless, a major activity-dependent component of NO action was independent of BK channels.

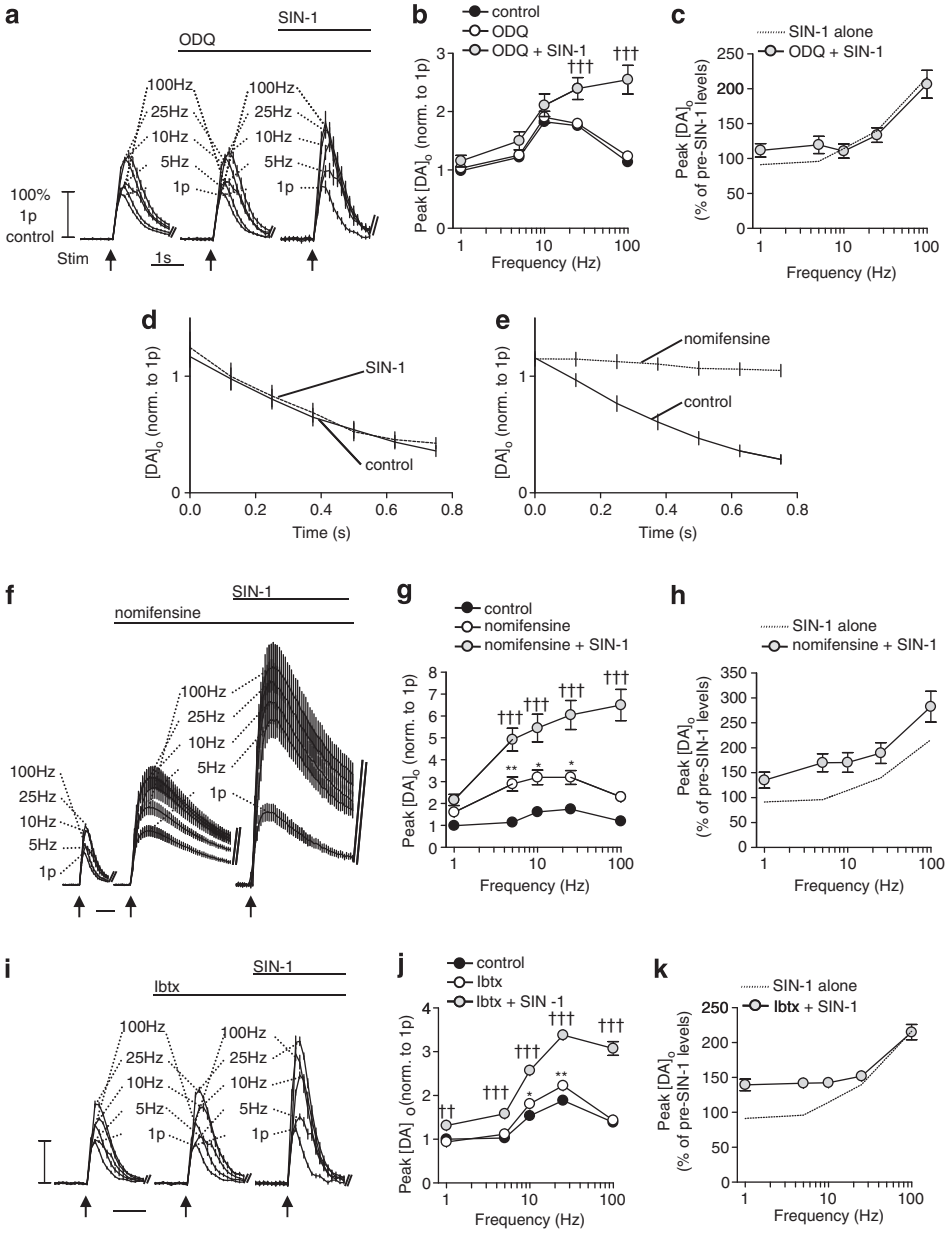

Figure 2 The effect of nitric oxide (NO) on evoked dopamine (DA) release is independent of soluble guanylyl cyclase (sGC) or DA re-uptake modulation and only a small component is dependent on Ca^{2+}-activated K^+ (BK) channels. (a, f, i) Profiles of mean extracellular DA concentration ($[DA]_o$) ± standard error of mean (SEM) *vs* time following stimuli (arrows) of either 1p or 4p (5–100 Hz) in control conditions (left), various antagonists (center) (a, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ), 100 μM; f, nomifensine, 10 μM; i, iberiotoxin (IbTx), 100 nM), and antagonist + 3-morpholinosydnonimine hydrochloride (SIN-1) (500 μM) (right) normalized to peak $[DA]_o$ evoked by 1p in controls. (b, g, j) Mean peak $[DA]_o$ ± SEM *vs* frequency during 1p or 4p (5–100 Hz) in control conditions (filled circles), antagonist (unfilled) (b, ODQ ($n = 8$–15); g, nomifensine ($n = 12$–20); j, IbTx ($n = 9$)), and antagonist + SIN-1 (gray fill) normalized to peak $[DA]_o$ evoked by 1p in controls. Asterisks indicate significance level in *post hoc* Bonferroni *t*-test *vs* controls, $^*P < 0.05$, $^{**}P < 0.01$. Crucifixes indicate significance level in Bonferroni *post hoc* *t*-tests for antagonist *vs* antagonist + SIN-1, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$. (c, h, k) Mean peak $[DA]_o$ *vs* frequency expressed as % of peak $[DA]_o$ evoked at that frequency before SIN-1 cocktail application, to compare the effect of SIN-1 on $[DA]_o$ at each applied frequency in the presence of (c) ODQ, (h) nomifensine, or (k) IbTx *vs* SIN-1 alone (dashed line; determined from data in Figure 1b). Error bars are also percentage of pre-SIN-1 levels. (k) Although IbTx does not prevent the activity-dependent increase in DA release (two-way ANOVA, $P < 0.001$, $n = 9$), which is still prominent at 100 Hz, the effects of SIN-1 may be slightly modified towards a general frequency-independent increase in DA release. (d, e) Falling phases of mean $[DA]_o$ ± SEM profiles *vs* time released by high-frequency bursts (4p/100 Hz) in control (straight line) and (d) SIN-1 or (e) nomifensine (dashed line) normalized to peak $[DA]_o$ evoked by 1p in controls.

NO Modulation of DA Release is Multifactorial

We explored whether the effects of NO on DA release reported here are mediated directly by an action on DA axons, or indirectly via regulation of an intermediary neurotransmitter(s). Accumbal glutamate and GABA do not normally have significant roles in regulating DA release probability during discrete subsecond stimuli (Cragg, 2003; Exley *et al*, 2008; Threlfell *et al*, 2010). However, as GABAergic and glutamatergic transmission can be

modulated by NO *in vivo* (Bogdanov and Wurtman, 1997; Guevara-Guzman *et al*, 1994; Trabace and Kendrick, 2000; West and Galloway, 1997a), we investigated whether a potential change of the local glutamatergic or GABAergic tone by NO contributed to the SIN-1 effect on DA release. Application of a cocktail of antagonists for glutamate (NMDA: D-AP5, 50 μM; AMPA: GYKI-52466, 10 μM; mGluR: (*S*)-MCPG, 200 μM) and GABA receptors ($GABA_A$: bicuculline, 10 μM; $GABA_B$: saclofen, 50 μM) did not significantly modulate evoked $[DA]_o$ at any frequency applied compared with control (Figure 3a–c) as shown previously (Cragg, 2003; Exley *et al*, 2008; Threlfell *et al*, 2010). Furthermore, glutamate and GABA receptor blockade did not prevent the activity-dependent effect of subsequent SIN-1 application (Figure 3a–c; two-way ANOVA, frequency: $F_{4,136} = 35.22$, $P < 0.001$; treatment: $F_{2,136} = 68.30$, $P < 0.001$; interaction: $F_{8,136} = 9.72$, $P < 0.001$), suggesting that the effect of NO on $[DA]_o$ is not via modulation of local glutamatergic or GABAergic tone.

Accumbal nicotinic ACh receptors (nAChRs) on dopaminergic terminals have a powerful control over DA release in the NAc (Exley *et al*, 2008; Rice and Cragg, 2004). Normally, endogenous accumbal ACh, released by tonically active ChIs, maintains ACh tone at accumbal nAChRs located on DA axons. This ACh tone ensures that initial DA release probability by a single stimulus pulse is high (Rice and Cragg, 2004; Zhou *et al*, 2001), that short-term depression of DA re-release at subsequent stimuli is prominent, and that sensitivity of DA release to frequency of activation is consequently limited (Cragg, 2003; Rice and Cragg, 2004). Changes in nAChR activation in turn modify the frequency sensitivity of DA transmission. We explored whether NO effects on DA transmission were mediated wholly or in part via an action involving ChIs/ACh. Application of the nicotinic receptor antagonist, DHβE (1 μM), to block cholinergic input significantly modified evoked DA release in an activity-dependent manner (Figure 3d and e; two-way ANOVA, frequency: $F_{4,134} = 463.9$, $P < 0.001$; treatment: $F_{2,134} = 602.4$, $P < 0.001$; interaction: $F_{8,134} = 127.1$, $P < 0.001$), by reducing release by lower frequencies and enhancing release by higher frequencies as shown previously (Exley *et al*, 2008; Rice and Cragg, 2004). In the presence of DHβE, subsequent application of SIN-1 (500 μM) only slightly modified further the activity dependence of evoked $[DA]_o$ (Figure 3f; two-way ANOVA, frequency: $F_{4,134} = 463.9$, $P < 0.001$; treatment: $F_{2,134} = 602.4$, $P < 0.001$; interaction: $F_{8,134} = 127.1$, $P < 0.001$), but moreover, resulted in a significant increase (63–89%) in $[DA]_o$ evoked by all frequencies compared with DHβE alone (Figure 3f; two-way ANOVA, frequency: $F_{4,134} = 463.9$, $P < 0.001$; treatment: $F_{2,134} = 602.4$, $P < 0.001$; interaction: $F_{8,134} = 127.1$, $P < 0.001$).

These data suggest a combination of both direct and indirect effects of NO on DA release. In the absence of cholinergic input to DA terminals (ie, in the presence of nAChR antagonist DHβE), NO seems to act directly at the level of DA terminals to enhance release in a manner independent of stimulation frequency. However, in the presence of cholinergic input (ie, in the absence of nAChR antagonist DHβE), these direct effects of NO at the level of the DA terminal to increase release at all frequencies appear

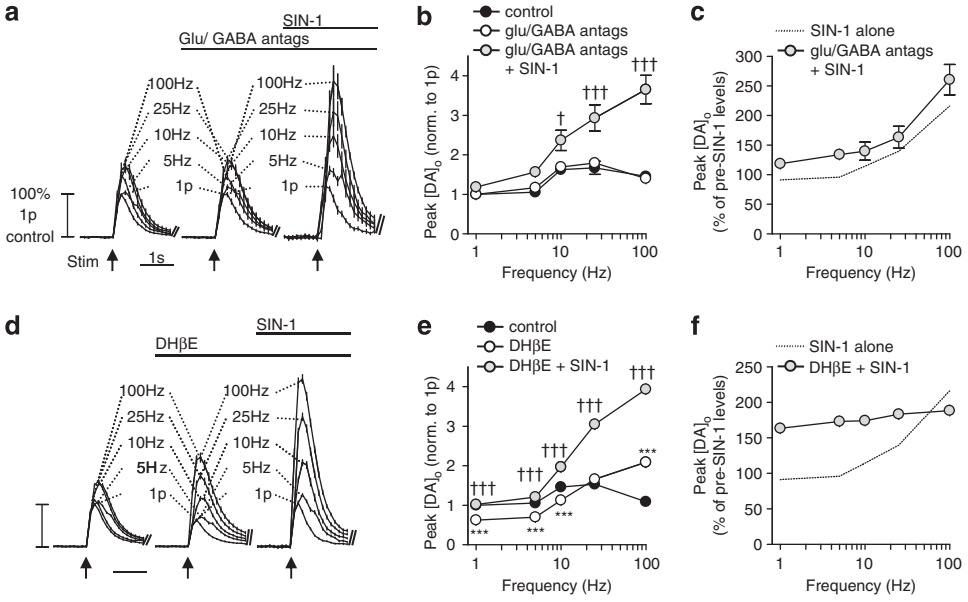

Figure 3 The effect of nitric oxide (NO) on dopamine (DA) release is independent of striatal glutamate or γ-aminobutyric acid (GABA) input, but varies with cholinergic input. (a, d) Profiles of mean extracellular DA concentration ($[DA]_o$) ± standard error of mean (SEM) *vs* time following stimuli (arrows) of either one pulse (1p) or four pulses (4p) (5–100 Hz) in control conditions (left), antagonists (center) (a, glu/GABA antagonist cocktail (10 μM bicuculline, 50 μM saclofen, 50 μM D-AP5, 10 μM GYKI-52466, 200 μM (*S*)-MCPG); d, DHβE, 1 μM), and antagonists + 3-morpholinosydnonimine hydrochloride (SIN-1) (500 μM) (right) normalized to peak $[DA]_o$ evoked by 1p in controls. (b, e) Mean peak $[DA]_o$ ± SEM *vs* frequency during 1p or 4p (5–100 Hz) in control conditions (filled circles), antagonists (unfilled) (b, glu/GABA antagonists, $n = 9–16$; e, DHβE, $n = 9–16$) and antagonists + SIN-1 (gray fill) normalized to peak $[DA]_o$ evoked by 1p in controls. Asterisks indicate significance level in *post hoc* Bonferroni *t*-test *vs* controls, $^{***}P < 0.001$. Crucifixes indicate significance level in Bonferroni *post hoc t*-tests for antagonist *vs* antagonist + SIN-1, $^{\dagger}P < 0.05$, $^{\dagger\dagger\dagger}P < 0.001$. (c, f) Mean peak $[DA]_o$ *vs* frequency expressed as % of peak $[DA]_o$ evoked at that frequency before SIN-1 cocktail application, to compare the effect of SIN-1 on $[DA]_o$ at each applied frequency in the presence of (c) Glu/GABA antagonists or (f), DHβE, *vs* SIN-1 alone (dashed line; determined from data in Figure 1b). Error bars are also the percentage of pre-SIN-1 levels.

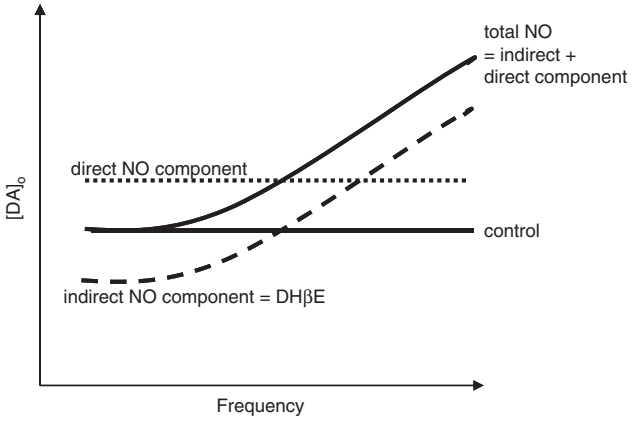

Figure 4 Direct and indirect effects of nitric oxide (NO) on dopamine (DA) release supplement each other at high-frequency DA neuron activity, but cancel out at low frequency. Cartoon to explain net outcome of the 'direct' and 'indirect' effects of NO on DA release evoked by varying frequencies of stimulation. 'Indirect' modulation of DA release by NO involving a net reduction of nicotinic ACh receptors (nAChR) control of DA would be expected to reduce DA release at low frequencies, but consequently enhance frequency sensitivity and then even enhance DA release at high frequencies (see, Cragg, 2006; Rice and Cragg, 2004). 'Direct' modulation of DA release by NO (seen in the absence of nAChR activation) increases DA concentration ($[DA]_o$) uniformly regardless of stimulus frequency. When nAChR tone is intact, these two mechanisms acting in concert will enhance $[DA]_o$ at high frequencies, but cancel out at low frequencies.

to be set against an indirect action via the cholinergic system, with a net outcome to increase the sensitivity of DA release to frequency (Figure 4).

DISCUSSION

This study reveals that exogenous NO can powerfully and variably modulate DA release in the NAc core and enhance the frequency dependence of DA release. These NO-mediated effects are independent of sGC activation, and largely independent of two other candidate NO targets, DATs and BK channels. Furthermore, this frequency-dependent modulation of DA release by NO appears to be multifactorial, involving an indirect action via (or interaction with) ACh released from ChIs, as well as a direct action on DA axons. These data reveal a variable neuromodulatory influence of local NO on DA in the NAc that depends on the activity in DA neurons as well as local accumbal circuits. Given the central role of DA neurotransmission in the NAc on motivated behaviors, interactions between NO, ACh and DA may be important for regulating these behaviors in normal as well as pathological states.

NO Increases the Contrast of DA Signals Released by Phasic *vs* Tonic Frequencies of Activity via GC-Independent Mechanism(s)

Two separate NO donors, SIN-1 and PAPA/NONOate, increased evoked $[DA]_o$ and enhanced the frequency sensitivity of DA release. Thus, NO donors enhanced the contrast between $[DA]_o$ evoked by phasic *vs* tonic frequencies of activation. Donors were used at concentrations that are those typically used to produce effects of NO that are thought to be physiologically relevant (Bon and Garthwaite, 2001; East *et al*, 1991; Garthwaite *et al*, 2002; Luchowski and Urbanska, 2007; Yang and Cox, 2008). Here, the similar effects on DA release of these two different NO donors suggest that their outcomes are due to their common property to generate NO with physiological consequences rather than any nonspecific effects or other breakdown products of each compound.

The concentrations of NO that are physiological are still debated (Hall and Garthwaite, 2009). Current estimates of NO concentrations found during normal tissue functioning are in the range of hundreds of picomolar to low nanomolar (ie, 10^{-10}–10^{-8} M) (Hall and Garthwaite, 2009; Sammut *et al*, 2006) and are a function of the rates of NO production, diffusion, and consumption. Tissue concentrations of NO that result from the NO donors applied here will depend on the NO concentrations generated in solution (100- to 1000-fold lower than the donor itself, for example, Feelisch *et al* (1989), Garthwaite *et al* (2002), and Hogg *et al* (1992)) and also on tissue penetration by NO. The high rate of consumption of NO by tissue is thought to result in a substantial concentration difference between NO applied in solution and the limited NO reaching tissue (estimated to be 1000- to 10 000-fold lower in tissue; Hall and Garthwaite, 2006, 2009). Thus, taking these different 'dilution' factors into account in this study, the NO concentrations in tissue resulting from the donor concentrations applied in solution (10^{-4} M) may be between 10^5 and 10^7 times lower, that is, in the range of 10^{-9}–10^{-11} M. These picomolar to low nanomolar estimates are very similar to estimates of NO concentrations found physiologically. Indeed, the effects here were consistent with physiological and not pathological effects because they were completely reversible within minutes of washout. Furthermore, previous studies using isolated rat optic nerve preparations found no signs of nervous tissue damage after 2-h exposure to 300 μM PAPA/NONOate or a 4-h exposure to concentrations of SIN-1 (2 mM), an order of magnitude higher than those used here (Garthwaite *et al*, 2002). In addition, NO donor effects persisted in the presence of the peroxynitrite scavenger Trolox, indicating that they did not depend on a pathological conversion to peroxynitrite.

The enzyme sGC is an effector mechanism for some actions of NO. In the dorsal striatum, the sGC dependence of facilitatory effects of NO on DA levels remains debated and has been shown to be sGC-dependent *in vivo* (Guevara-Guzman *et al*, 1994; Trabace and Kendrick, 2000), but also sGC-independent both *in vitro* (Buyukuysal, 1997; Stewart *et al*, 1996) and *in vivo* (Rocchitta *et al*, 2004; West and Galloway, 1996). In our study in NAc, the effects of NO on dynamic DA signaling were not prevented by an inhibitor of sGC, indicating that they are sGC-independent. This is in line with previous *in vitro* studies in the dorsal striatum revealing sGC-independent effects of NO on DA release (Buyukuysal, 1997; Stewart *et al*, 1996).

Many target molecules have been identified in various systems to mediate the many physiological functions of NO. It has been suggested that NO might increase extracellular striatal DA levels via inhibition of DATs in some studies (Buyukuysal, 1997; Lonart and Johnson, 1994; Pogun *et al*, 1994). However, NO donors modified DA transmission in

this study via a mechanism that did not involve any modulation of, or dependence on, DA uptake via DAT.

The conductance of BK channels (among other K^+ channels) has also been reported to be modulated by NO, via both sGC-dependent and -independent mechanisms (Ahern *et al*, 1999; Klyachko *et al*, 2001). In posterior pituitary nerve terminals, NO has been reported to increase BK channel conductance, therefore promoting spike after-hyperpolarization and Na^+-channel recovery from inactivation, and thus reducing action potential failures during spike trains (Klyachko *et al*, 2001). Such a mechanism would be expected to give rise to a short-term, frequency-dependent enhancement of transmitter release, and was thus an attractive mechanism to explain NO effects on $[DA]_o$ described here. Although BK channel expression/function has to date not been reported in DA neurons or axons, BK channels regulate neurotransmitter release from some other central neurons (eg, Xu *et al*, 2005) and are also present in the striatum, for example, in dorsal striatal ChIs where they contribute to action potential repolarization (Bennett *et al*, 2000). As striatal ACh potently regulates DA transmission in a manner that varies with presynaptic activity (Cragg, 2006; Rice and Cragg, 2004; Zhang and Sulzer, 2004), these channels on ChIs might in turn modulate DA release. However, although IbTx, a blocker of BK channels, slightly modified evoked $[DA]_o$, it did not prevent significant frequency-dependent effects of SIN-1 on DA release in NAc. Taken together, these data suggest that the sGC-independent effector mechanisms involved in NO-mediated modulation of DA release do not require either the DAT or BK channels, and must involve an alternate target(s), of which there are numerous candidates, for example, Na^+ channels (Hammarstrom and Gage, 1999), the ryanodine receptor (Sun *et al*, 2001; Xu *et al*, 1998), L-type Ca^{2+} channel (Campbell *et al*, 1996; Summers *et al*, 1999), and cyclic nucleotide-gated channels (Broillet, 2000; Broillet and Firestein, 1996).

NO Modulates DA Release via an Indirect ACh-Dependent Mechanism and via Direct Actions on Dopaminergic Terminals

To identify which accumbal neuron type(s) mediate NO regulation of DA transmission, we explored whether these effects required local accumbal glutamatergic, GABAergic, or cholinergic inputs (eg, Bogdanov and Wurtman, 1997; Guevara-Guzman *et al*, 1994; Trabace and Kendrick, 2000; West and Galloway, 1997a). NO-mediated modulation of evoked DA release was independent of glutamate and GABA inputs, consistent with previous studies showing that neither glutamate nor GABA modulate DA release evoked by single pulses and brief 4–5 pulse trains (Cragg, 2003; Exley *et al*, 2008; Threlfell *et al*, 2010).

NO has been shown to powerfully modulate the activity of ChIs in the dorsal striatum (Centonze *et al*, 2001) as well as the release of ACh in the dorsal and ventral striatum (Guevara-Guzman *et al*, 1994; Prast *et al*, 1995, 1998; Prast and Philippu, 2001; Trabace and Kendrick, 2000). Notably, ACh at nAChRs on DA axons has a major role in governing the frequency sensitivity of DA release (Exley *et al*, 2008; Rice and Cragg, 2004; Zhang and Sulzer, 2004). In this study, we reveal that when ACh action at nAChRs in NAc is prevented, the frequency-dependent effects of NO on DA transmission are also prevented. Without nAChR activity, NO then increases DA release independently of the frequency of activation. The simplest explanation for these data is that NO operates two partly opposing mechanisms that control DA release. One mechanism is indirect, involving regulation of ACh input to nAChRs akin to switching nAChRs off. Switching nAChRs off is expected to decrease DA release at low frequencies, but enhance frequency sensitivity of DA release, ultimately enabling enhanced DA release at high frequencies, and can result from either a decrease in ACh release or an increase sufficiently large to cause nAChR desensitization as seen with nicotine (Rice and Cragg, 2004; Zhang and Sulzer, 2004). The second mechanism, revealed in the absence of nAChR activation, appears to be directly located to DA axons, and increases evoked $[DA]_o$ uniformly regardless of stimulus frequency. When nAChR tone is intact, these two mechanisms acting in concert would be expected at low frequencies to oppose each other, resulting in no net effect. By contrast, at high frequencies, they would be expected to boost DA signals. These outcomes are indeed those observed here, and are summarized in a cartoon representation of individual and net effects (Figure 4).

Mechanistically, the increase in DA release by both direct and indirect actions of NO could be explained by an increase in vesicle fusion events as suggested for hippocampal synaptosomes by Meffert *et al* (1996, 1994). NO may increase the docking and fusion of dopaminergic vesicles at dopaminergic terminals, leading to an increase in release independent of frequency. In addition, NO may also increase the docking and fusion of cholinergic vesicles at cholinergic terminals, leading to an increase in ACh release that, as described above, could desensitize nAChRs with the effect of increasing DA release at high frequencies.

An action of NO at multiple neuronal sites with variable outcome would certainly be in keeping with the body of literature to date, indicating that NO that has diverse target molecules and proposed effector mechanisms and various reported outcomes on DA release (in the dorsal striatum). However, these data do not preclude an alternative explanation that NO regulation of DA transmission is via action at a single site, through a single mechanism that is in some way shunted at low frequencies in the presence of nAChR tone.

Summary and Concluding Remarks

Whether there are single or multiple effector mechanisms, these effects of NO donors, suggest that the action of endogenous NO on accumbal DA signaling may be highly dynamic, depending on DA axon activity and also on the state of the local ventral striatal network, especially ChIs. Our data suggest that during ChI and nAChR activation, accumbal NO might enhance how DA release conveys high frequencies of activation. This postulated action for NO would be in opposition to those of ACh, which limits the frequency dependence of DA signaling (Cragg, 2006). However, in the absence of nAChR activation by ACh, when the frequency dependence of DA signaling is great, NO might boost this outcome by promoting all DA signals uniformly. We speculate that NO might co-operate in

outcome with the pauses in ChIs that signal motivationally significant stimuli (Aosaki *et al*, 1994; Apicella, 2002; Morris *et al*, 2004; Ravel *et al*, 2001; Shimo and Hikosaka, 2001), when nAChR activation will be minimal.

The neurons that are the most likely source of endogenous accumbal NO are nNOS-expressing, GABAergic interneurons that also contain somatostatin and neuropeptide Y (Beal *et al*, 1986; French *et al*, 2005; Smith and Parent, 1986) and are highly interconnected to form a local nNOS-containing interneuron network (French *et al*, 2005). Knowledge of their functions within the accumbal network is limited, but our data suggest that they might have a role in promoting transmission by DA of phasic *vs* tonic activity in DA neurons. The outcome of endogenous NO on accumbal DA function might vary dynamically with activity within the accumbal neuron network and might also impact significantly on the behavioral outcome of activation of limbic basal ganglia loops.

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DISCLOSURE

The author(s) declare that, except for income received from my primary employer, no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional service and that there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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