

Nitrgic Tone Influences Activity of Both Ventral Striatum Projection Neurons and Interneurons

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Abstract Nitric oxide (NO) is a well-established striatal neuromodulator, effecting both the activity and electrical coupling of striatal projection neurons. The NO-producing interneurons within the striatum are altered in schizophrenia brain tissue, and they may be key to the pathophysiology and future treatment of schizophrenia. We investigated in vivo the effect of locally applied NO-active drugs on the firing rate of electrophysiologically and anatomically identified, medium-sized densely spiny neurons and interneurons in the ventral striatum.

Juxtacellular recording and labelling experiments were performed on ventral striatal neurons during prefrontal cortex electrical stimulation. A NO donor, precursor or scavenger were applied microiontophoretically and single unit responses were recorded; after labelling, neurons were examined morphologically to determine neuronal type.

Correlation of electrophysiological and anatomical findings revealed four drug response profiles and four types of neurons. The nitrgic modulation of ventral striatal neurons is neuronal-type specific and may be effector-mechanism dependent, and it is involved in the gating of cortically driven ventral striatal output and the temporal and spatial synchrony of the striatal networks.

1 Introduction

The ventral striatum (VST), which includes the nucleus accumbens, plays an essential role in incentive reward responding/motivational behaviour and is implicated in pathologies such as schizophrenia and addiction. A striatal neuromodulator that may be altered in schizophrenia is nitric oxide (NO); the NO-generating neurons show abnormal morphology and reduced number (Lauer et al. 2005), and neuronal

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NO synthase (nNOS) promoter polymorphisms are linked with the disease (Reif et al. 2006).

NO is key to the normal function of the striatum; it is critical to the synaptic modulation of gap junctions following the activation of the glutamatergic corticostriatal fibres (O'Donnell and Grace 1997), as well as exerting a powerful tonic modulatory influence over the membrane activity of the striatal medium-sized densely spiny neurons (MSNs) via the activation of guanylate cyclase and stimulation of cGMP production (West and Grace 2004). NO modulation of striatal interneurons may also act to regulate the functioning of individual MSNs and may co-ordinate the MSNs to form neuronal ensembles (Pennartz et al. 1994). There is a well-described network of nNOS-immunopositive interneurons (French et al. 2005) in the VST. The influence of nitric tone from and on these and other interneurons, as well as on the MSNs, is of critical importance to our understanding of the modulation that occurs at both the single cell and microcircuit levels in the striatum and what may therefore occur in the diseased state.

2 Materials and Methods

2.1 *Animal Preparation*

Experimental procedures were performed on male Wistar rats, 210–280 g (Harlan), and were conducted in accordance with the Animals (Scientific Procedures) Act of 1986 (UK). Anesthesia was induced with and maintained with urethane (1.3 g kg⁻¹, i.p.; ethyl carbamate; Sigma, UK) and supplemental doses of ketamine (30 mg kg⁻¹, i.p.; Ketaset; Willows Francis) and xylazine (3 mg kg⁻¹, i.p.; Rompun; Bayer). The animals were then placed in a stereotaxic frame (Kopf). Anesthesia levels were assessed by examination of the electrocorticogram (ECoG) and supplemental doses of the ketamine/xylazine mixture were given when necessary. The ECoG was recorded via two 1-mm diameter steel screws juxtaposed to the dura mater; raw ECoG was amplified (2,000×; NL104 AC preamplifier; Digitimer) before acquisition. Two discrete craniotomies (3–4 mm²) were performed above the right prefrontal cortex (PFC) and VST and the dura mater was removed for insertion of stimulating and recording electrodes, respectively.

2.2 *Microelectrodes and PFC Activation*

The VST neurons act to integrate a large number of cortical inputs, thus continuous stimulation of the PFC was applied during the entire duration of all experiments using a platinum bipolar twisted electrode (outer diameter 0.075 mm, Plastics One). Current pulses were timed using a Master 8 stimulator (Intracel) and generated with

a constant-current stimulus isolation unit (A360, WPI). A 500–700 μ A square-wave pulse was delivered at 1 Hz.

The multibarrel microiontophoretic pipette and extracellular recording/labelling microelectrode assembly was cemented together in parallel with the microelectrode tip protruding 15 μ m beyond the tip of the multibarrel pipette. The extracellular recording microelectrode was filled with saline solution (0.5 M NaCl) and neurobiotin (1.5% w/v; Vector Labs). One barrel of the multibarrel pipette was used for automatic balancing and was filled with 2 M NaCl. The other barrel of the microiontophoretic pipette was filled with a solution of one of the following drugs (all purchased from Sigma): the NO donor 3-morpholinosydnonimine hydrochloride (SIN-1), 40 mM in water, pH 4.5; the NO precursor hydroxy-L-arginine (H-Arg), 50 mM in water, pH 4.5; the NO scavenger 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazol-1-yl-oxy-3-oxide (C-PTIO), 50 mM in water, pH 4.5. The resistance of the recording microelectrode was 15–25 M Ω .

2.3 *Electrophysiological Recordings and Microiontophoresis*

Extracellular recordings of action potentials of VST neurons were made from 110 cells in 35 rats. Electrode signals were amplified (10 \times) through the active bridge circuitry of a Neurodata amplifier (Cygnus Technologies), AC-coupled and amplified a further 100 \times (NL106 AC-DC Amp; Digitimer), before being filtered between 0.3 and 5 kHz (NL125; Digitimer). Spikes were approximately 1 mV in amplitude and always exhibited an initial positive deflection. As verified in ECoG recordings, neuronal activity was recorded during slow-wave activity.

To determine the role of NO in VST cell firing, microiontophoretic techniques were used on 99 cells. SIN-1 and H-Arg were retained in the microiontophoretic pipette using a negative current of 10 nA and expelled using a positive current of 10–100 or 5–50 nA, respectively, with a Neurophore microiontophoresis BH-2 system (Medical Sciences Corp); C-PTIO was held with positive current and expelled with negative current (5–50 nA).

All biopotentials were digitized online using a Micro1401 Analog–Digital converter (CED) and a PC running Spike2 acquisition and analysis software (version 5; CED). Unit activity and the ECoG were sampled at 20 kHz. Baseline mean firing rates were calculated from 5 min immediately prior to drug ejection. Over the same period the average waveform was plotted, and the average action potential (AP) duration was determined. All data were expressed as spike events in 6-s bins. Frequency distribution histograms were generated automatically with a custom CED Spike 2 script; the script calculates the mean baseline firing rate of the 6-s bins and the mean \pm [2 \times standard deviation (SD)]. These levels were superimposed upon the histogram and the cell was considered to be significantly activated/inhibited if the firing frequency in three consecutive bins was beyond mean \pm 2SD of the baseline (illustrated in Fig. 1a–c).

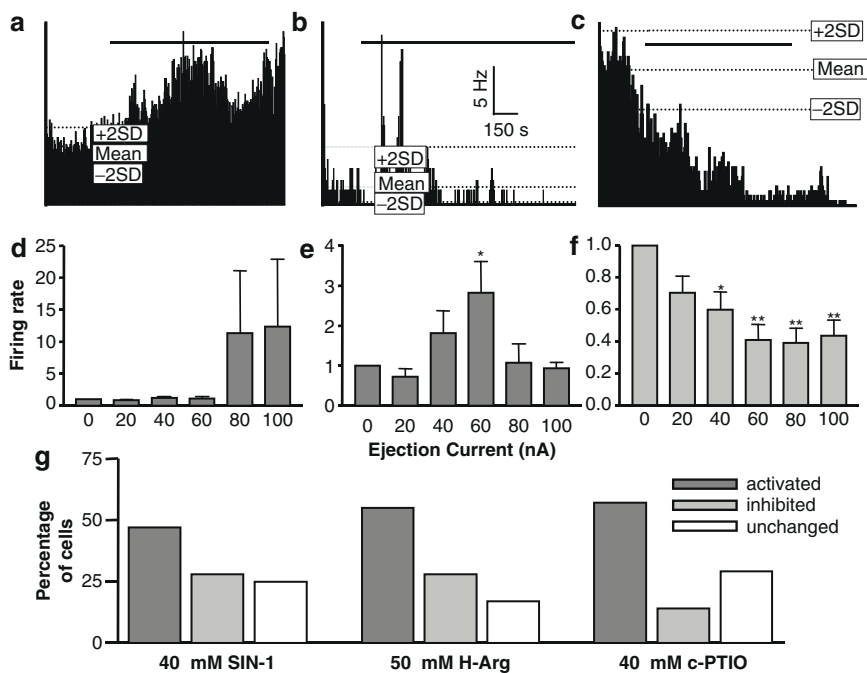


Fig. 1 A comparison of the neuronal responses to the NO donor SIN-1. Representative individual neurons were significantly activated in a more-linear current-dependant manner (**a**), or in a bell-shaped manner (**b**) or inhibited (**c**). Normalised current response graphs for all neurons activated by SIN-1 activation [$n = 27 + 4$; (**d**, **e**)] and all neurons inhibited by SIN-1 [$n = 19$; (**f**): The application of SIN-1 at 60 nA significantly increased the firing rates of neurons that responded with a bell-shaped curve ($F = 3.1$, $P < 0.05$, ANOVA; 20 vs. 60 nA, $P < 0.05$, Tukey's post hoc test). The application of SIN-1 at currents ranging from 40 to 100 nA significantly reduced the firing rates of inhibited neurons ($F = 6.5$, $P < 0.0001$, ANOVA; 0 vs. 40 nA, $P < 0.05$, 0 vs. 60/80/100 nA, $P < 0.001$, Tukey's post hoc test). (**g**) A comparison of the neuronal responses to the NO donor SIN-1, precursor H-Arg and scavenger c-PTIO

2.4 Juxtacellular Labelling and Histochemistry of Single Neurons

To identify the locations and morphological properties of recorded units, neurons were then labelled with neurobiotin by the juxtacellular method (Pinault 1996). After the recording and labelling sessions, the animals were given a lethal dose of ketamine (150 mg kg^{-1}) and perfused as described previously (French and Totterdell 2002). The fixed brains were parasagittally sectioned at $70 \mu\text{m}$ on a vibrating Microtome (VT1000M; Leica). The biotin was revealed and the tissue was processed for microscopy by methods described previously (French et al. 2002). After examination under a light microscope, reconstructions of neural structures were performed with the aid of a *camera lucida*. In addition, to create light micrograph

montages with enhanced depth of field, several micrographs were taken at sequential focal depths and then processed in Adobe Photoshop software.

3 Results

3.1 *Effects of the NO Manipulation VST Neurons*

The responses of 67 neurons to application of the NO donor SIN-1 were recorded (Fig. 1). Of these neurons, 31 were activated (46%; Fig. 1a, d). Nineteen of the 67 recorded cells (28%) were significantly inhibited by the microiontophoresis of SIN-1; these cells decreased their firing rate to either no activity (six cells) or to 55–4% of their baseline activity (Fig. 1c, f). A further 17 cells did not respond significantly to the application of SIN-1. In responsive cells, firing rates significantly altered within 5.3 ± 4 min of the onset of the drug ejection period and were maintained for 6.4 ± 5.5 min. As can be seen in Fig. 1d–f, the excitatory or inhibitory effect of SIN-1 was related to the amount of current applied. Four of the 31 activated cells responded in a bell-shaped fashion (Fig. 1b, e); they were activated at intermediate currents only and inhibited at higher currents.

To confirm the activation of MSNs by NO and to demonstrate that stimulating the natural release pathway of NO that involves NOS could also lead to a similar effect to that seen with SIN-1 we undertook a second series of experiments with local application of the NO precursor H-arginine (H-Arg). H-Arg is a substrate for the NOS enzyme in NOS-containing interneurons. The responses of 18 cells to H-Arg were examined in the VST. Ten of the 18 neurons (55%) were significantly activated by the drug. Seven of these ten neurons were activated at the highest currents applied, in a similar manner to that seen with SIN-1. The other three activated cells displayed a bell-shaped response to the drug. Five of the 18 examined neurons (27%) were significantly inhibited by the drug. Three cells (17%) did not display a significant response to the drug (data summarised in Fig. 1g). The timescale of the responses to H-Arg were approximately twice as long as that displayed by neurons responding to SIN-1.

As there is likely to be an endogenous nitric tone within the striatum (West and Galloway 1997), we also tested for nitric tone with an NO scavenger (c-PTIO) and looked for the effects on the VST neurons (Fig. 1g). Eight of the 14 recorded cells (57%) were activated by the drug. Of these, five cells were activated at the highest currents applied. The increase in firing rate under application of the NO scavenger was in the range between 192 and 423% of the baseline activity. Three of the eight activated neurons exhibited a bell-shaped response to the drug. Two neurons were significantly inhibited by the drug; they decreased their firing rate to 15.98% and 22.29% of the initial baseline firing rate. Four neurons did not show a significant response to the NO scavenger. The time scale was similar to that observed for H-Arg with a significant effect seen by 10.7 ± 10.3 min, which on an

average lasted for 11.5 ± 8.3 min, with the majority of neurons also not returning towards baseline activity rates after the ejection current was stopped.

3.2 Anatomical Identification of Neurons

The 20 VST neurons successfully labelled and reconstructed fell into four anatomical classes: MSNs, giant aspiny/sparingly spiny interneurons, medium-sized sparsely spiny interneurons and medium-sized aspiny interneurons.

The labelled MSNs showed the morphology typical of this class of striatal neurons (Fig. 2a–c). Of the 12 labelled MSNs, 6 received the NO donor, 3 responded with a significant increase in their firing rate and 3 significantly decreased their firing rate.

The two giant sparsely spiny labelled neurons had very similar morphology; one was within the most ventral aspect of the VST and the other within the ventral core of the nucleus accumbens. The giant sparsely spiny neuron (cell body approx. 36 μm length) from the ventral core is illustrated in Fig. 2d. The density of spines was much lower than those of the MSNs (Fig. 2g). This neuron responded to SIN-1 by increasing its firing rate at the highest currents applied.

The three medium-sized sparsely spiny interneurons were of varied morphologies, with soma sizes between 18 and 33 μm diameter (Fig. 2e). The response of these three medium-sized sparsely spiny interneurons to increased VST nitroergic tone was as follows: one significantly increasing its firing rate to H-Arg, one significantly decreasing its firing rate to SIN-1 and one did not respond to SIN-1 application.

All the medium-sized aspiny interneurons that we recorded and labelled had the shorter AP durations ≤ 1.4 ms. These neurons had medium- to large-sized soma (15–18 μm diameter) and varicose non-spiny dendrites. The application of SIN-1 to all the labelled medium-sized aspiny interneurons resulted in an increase of firing rate in a bell-shaped manner.

In the current study 20 VST neurons had short AP durations, ranging between 1.0 and 1.4 ms, and 90 neurons had an AP duration greater than 1.5 ms (Fig. 3); these AP durations had a Gaussian distribution. Other than the medium-sized aspiny interneurons, all the interneurons had AP durations between 1.9 and 2.1 ms (when using minimum AP amplitude of 0.9 mV). These latter values fall within the distribution shown for the MSNs.

4 Discussion

4.1 Modulation of Striatal Activity by Alterations of Nitroergic Tone

This study investigated the impact of alterations in nitroergic tone on the activity of different populations of VST neurons with ongoing PFC stimulation. A major finding of our study is that, by either increasing or decreasing the nitroergic tone, cells are

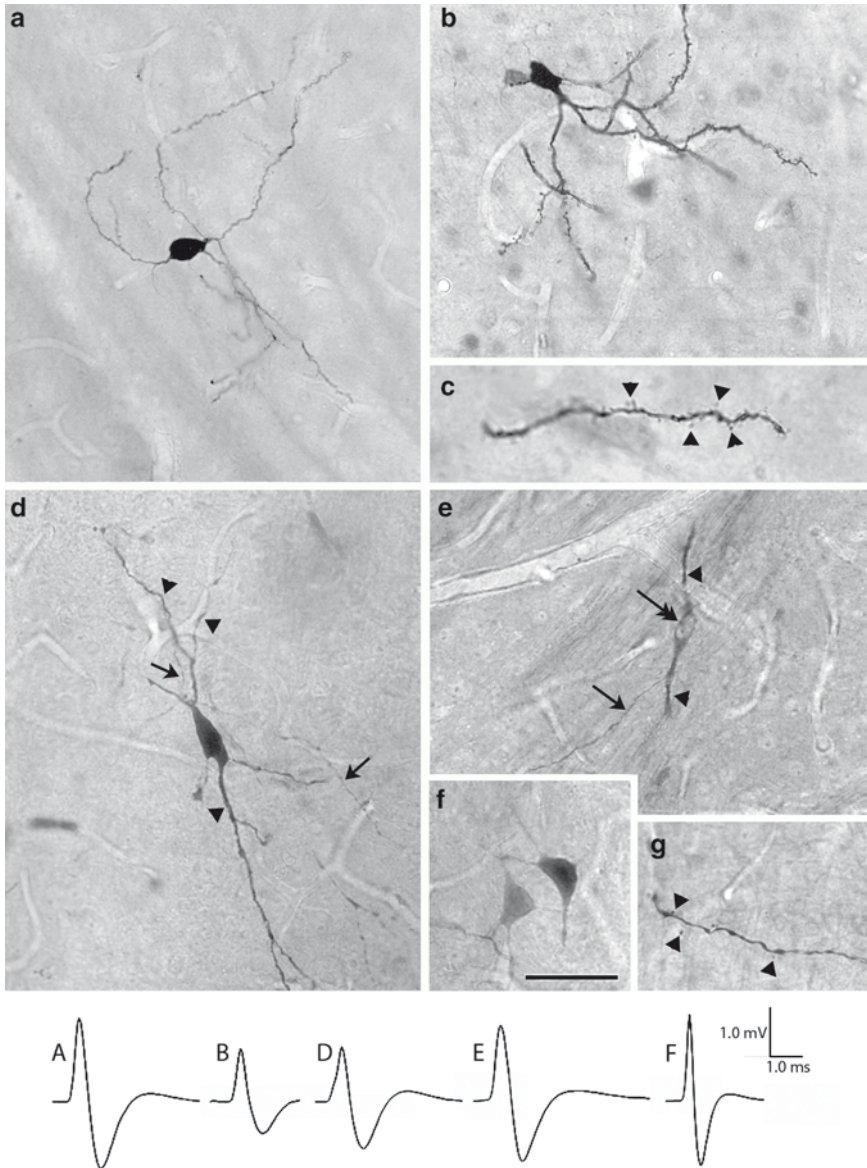


Fig. 2 Light level photomicrographs of juxtacellularly labelled VST neurons and their action potential waveforms. (a, b) MSNs with torturous densely spiny dendrites: the piece of dendrite is shown at higher magnification in (c), and spines are indicated by *arrowheads*. (d) Giant sparsely spiny neuron, clearly demonstrating dendritic (*arrowheads*) and axonal (*arrows*) labelling. (e) Labeled sparsely spiny medium-sized interneuron: the neuron is lightly labelled but the dendrites (*arrowheads*) and axon (*arrow*) can be clearly seen; the lighter labelling also allows the indented nucleus to be visualised (*double arrow*). (f) Medium-aspy neurons with varicose dendrites: note the short duration of this neurons waveform. (g) A high-power photomicrograph of a distal sparsely spiny dendrite of the cell in (d) – spines indicated by *arrowheads*. Scale bar = 50 μ m [apart from (c) and (g) = 20 μ m]

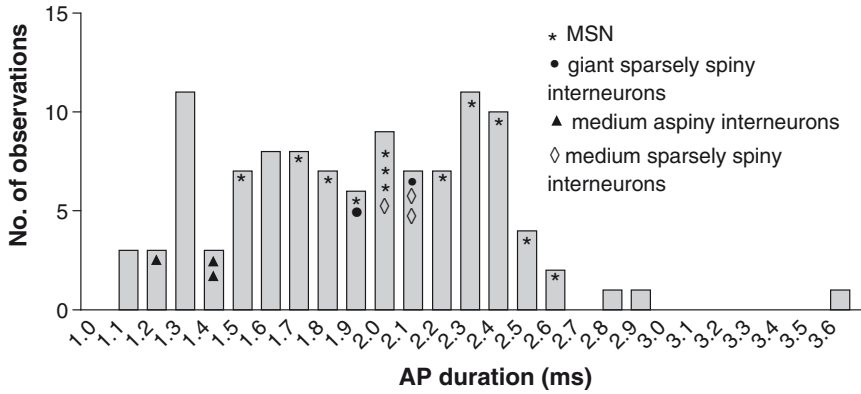


Fig. 3 A frequency distribution histogram of the AP durations of all recorded neurons illustrating the Gaussian distribution. Superimposed upon the distribution are the AP duration positions of the anatomically identified interneurons and MSNs. Note the clustering of the medium-sized aspiny interneurons at shorter AP durations and also the distribution of the other interneuron classes within that of the MSNs

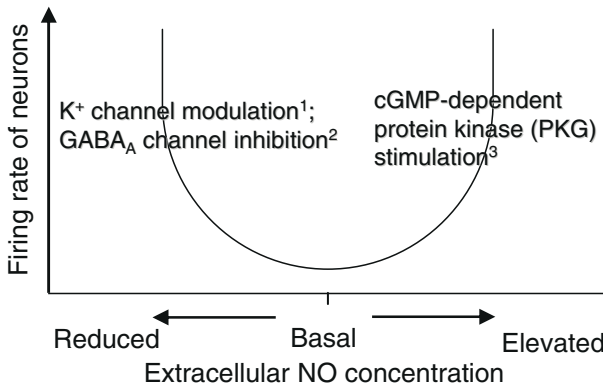


Fig. 4 A hypothetical schematic of the biphasic response of striatal neurons to alterations in the basal concentration of nitric oxide, with suggested effector mechanisms (Fagni and Bockaert 1996; Robello et al. 1996; Garthwaite and Boulton 1995)

predominantly excited. Moreover, within the cells that responded by increasing their firing rate, two patterns of response were seen; the majority of cells respond at only the highest microiontophoresis currents, whilst a small proportion respond only at intermediate currents.

Our findings demonstrate that enhancing striatal NO tone significantly increased the firing activity of 41, and inhibited 24, of the 85 neurons investigated. Reducing the nitrenergic tone also significantly excited 8 of the 14 neurons tested. Given that most neurons tested were excited by increasing nitrenergic tone, it is perhaps surprising that scavenging NO also excited most neurons tested. Thus, we hypothesise that the VST

neurons respond to an alteration of nitroergic tone in a U-shaped manner (Fig. 4). NO may employ other effector mechanisms e.g. the inhibition of chloride influx through GABA_A receptors (Robello et al. 1996) or the modulation of potassium channels (Fagni and Bockaert 1996), both of which if reduced would increase neuronal excitability.

Previous studies have investigated the action of nitroergic drugs on the activity of striatal neurons (Di Giovanni et al. 2003; Sardo et al. 2003; West and Grace 2004). One aspect of our study that is entirely novel is the correlation of single-cell anatomical properties with drug effect. In addition, no study has specifically examined the effect on striatal interneurons.

4.2 Nitroergic Modulation of VST Interneurons

We morphologically identified two giant sparsely spiny interneurons characteristic of the cholinergic interneurons (Bolam et al. 1984) that form 1–2% of the total neuronal population. In agreement with *in vitro* intracellular studies that reported that striatal cholinergic interneurons were excited by hydroxylamine (NO donor) and SIN-1 (Centonze et al. 2001), the current study provides evidence that these giant sparsely/spiny putative cholinergic neurons are excited by a NO donor.

We labelled three medium-sized sparsely spiny interneurons with soma sizes and dendritic and axonal arbours consistent with those of NADPH-D-positive neurons (Kawaguchi 1993) and somatostatin-positive neurons (Kawaguchi and Kubota 1996), which are known to be those that co-localise NOS (Vincent et al. 1983). A striking feature of these neurons is that the axonal arborisation is not dense within the region of the dendritic field, but is extensive and the dendrites are relatively unbranched for long distances. The presence of sparse spines on these labelled neurons argues against them being calretinin interneurons, which are devoid of spines (Bennett and Bolam 1993). The altered firing rate of these putative NOS-containing interneurons in response to nitroergic drugs is not unexpected, as anatomical studies have already demonstrated a NOS–NOS network of neurons (French et al. 2005). This network is proposed to widely propagate a hippocampally derived spatial signal received by individual NOS-containing neurons potentially synchronising and recruiting striatal MSNs into a functional ensemble.

The modulation of PV interneurons is of critical importance to our understanding of the feedforward inhibitory role this class of interneurons may play in striatal output synchronisation (Mallet et al. 2005) and striatal synaptic plasticity control (Pennartz et al. 1993). We labelled three neurons that are morphologically similar to those described to contain PV immunoreactivity (Kita et al. 1990). Of particular note is their aspiny varicose dendrites and medium- to large-sized soma. The soma size of our medium aspiny neurons are too large to be those belonging to the calretinin class. The PV neurons are also known to be of the FSI class (Kawaguchi 1993). The nitroergic effect on these FSIs was of particular interest as they increased their firing only at intermediate currents, thus they are recruited to fire when the NO tone is initially increased, but stop firing when it is higher. These cells have dense but

spatially limited axonal arbour; the increased NO would thus lead to a subsequent but limited-duration inhibitory influence on the MSNs within their strict vicinity.

The influence of nitric signalling in the striatum is functionally relevant to the flow of information through the MSNs output neurons, as well as the specific gating of signal transduction across corticostriatal synapses. This modulation may have fundamentally different influences on the spatial information processing and co-ordinated network synchrony depending on the neuron subtype involved. This ability to either increase or decrease the cell activity may represent the ability of NO to balance output activity such that neurons within a restricted region of the VST can act as a group or ensemble.

These findings also have implications for our understanding of the processes that may be dysfunctional in the VST of patients with schizophrenia. These neurons may normally generate a balanced gating of VST excitation by cortical glutamatergic afferents, contributing to the temporal and spatial regulation of information processing, and so their dysfunction may explain why the cognitive and motivational functions and the reward processes attributed to VST are disrupted in this disorder.

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