Nitric Oxide Modulates Striatal Neuronal Activity via Soluble Guanylyl Cyclase: An In Vivo Microiontophoretic Study in Rats

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KEY WORDS basal ganglia; in vivo; electrophysiology; extracellular recording; nitrergic neurons

ABSTRACT It is now well established that nitric oxide (NO) acts as a neuromodulator in the central nervous system. To assess the role of NO in modulating striatal activity, single-unit recording was combined with iontophoresis to study presumed spiny projection neurons in urethane-anesthetized male rats. Striatal neurons recorded were essentially quiescent and were therefore activated to fire by the iontophoretic administration of glutamate, pulsed in cycles of 30 sec on and 40 sec off. In this study, iontophoresis of 3-morpholinosydnonimine hydrochloride (SIN 1), a nitric oxide donor, produced reproducible, current-dependent inhibition of glutamate-induced excitation in 12 of 15 striatal neurons, reaching its maximal inhibitory effect (76.2 ± 5.6% below baseline) during the application of a 100 nA current. Conversely, microiontophoretic application of N-ω-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide synthase, produced clear and reproducible excitation of glutamate evoked firing in 7 of 10 cells (51.4 ± 2.3%, at 100 nA). To evaluate the involvement of cyclic guanosine monophosphate (cGMP) in the electrophysiological effects produced by the NO donor, the effects of methylene blue, an inhibitor of guanylyl cyclase, on the responses of nine neurons to SIN 1 were tested. In six of nine neurons the effect of SIN 1 was significantly reduced during continuous iontophoretic administration (50 nA) of methylene blue. Taken together, these data show that NO modulates the striatal network and that inhibitory control of the output neurons is involved in this effect. These results also suggest that the effects of nitric oxide on striatal neurons are partially mediated via cGMP. Synapse 48:100–107, 2003. © 2003 Wiley-Liss, Inc.

INTRODUCTION

Nitric oxide (NO), a free-radical gas, has been characterized as a novel neuronal messenger involved in a variety of neurotransmitter functions (Garthwaite, 1991; Bredt and Snyder, 1992). The synthesis of NO in the central nervous system (CNS) is mainly linked to the activation of N-methyl-D-aspartate (NMDA) receptors by glutamate (GLU) (Garthwaite, 1991). The production of NO from the amino acid L-arginine is catalyzed by the Ca2+ - and calmodulin-dependent enzyme, NO synthase (NOS). A major biochemical function of NO is to activate the soluble form of guanylyl cyclase (sGC), inducing the accumulation of cyclic guanosine monophosphate (cGMP) in target cells (Arnold et al., 1977; Feelisch and Noack, 1987; Bredt and Snyder, 1989). cGMP subsequently acts via protein kinases, phosphodiesterases, and perhaps directly on ion channels (Lincoln and Cornwell, 1993; Garthwaite and Boulton, 1995).

Within the brain, high levels of NOS activity occur in several areas, including the hippocampus, cerebellum, cerebral cortex, striatum, and brainstem (Vincent and Kimura, 1992; East et al., 1996). In the striatum NO is produced from a small population of interneurons containing two neuropeptides, somatostatin and neu...
CROZIER, 1996). Moreover, nitrergic neurons do synapse on the cholinergic striatal interneurons (Vuillet et al., 1992) that receive glutamatergic fibers and, in turn, innervate projection neurons themselves (Izzo and Bolam, 1988).

Therefore, it has been suggested that NO, released from the nitrergic neurons, plays a role in coordinating the complex neurotransmitter interactions within the striatum. Furthermore, endogenous NO may modulate the striatal release of dopamine (DA), GLU, GABA, taurine, ACh, and serotonin (5-HT) (Guevara-Guzman et al., 1994; Hanbauer et al., 1992; Stewart et al., 1996; Trabace and Kendrick, 2000; Serra et al., 2000). There have been numerous neurochemical studies, but few electrophysiological ones, investigating a regulatory role for NO on the discharge activity of striatal neurons. Only the results of in vitro electrophysiological studies are available at the moment. Among these, the recent report of Centonze et al. (2001) showed that NO regulates the striatal microcircuitry by increasing the activity of the striatal cholinergic interneurons. An NO signal is also involved in the long-term regulation of the synaptic activity within the striatum (Calabresi et al., 1999), which supports the idea that NO plays a critical role in the synaptic plasticity of the brain (Garthwaite, 1991; Bredt and Snyder, 1992).

The current study was designed to determine the effects of various pharmacological manipulations of the NO system on striatal neuronal activity in vivo using electrophysiological methods coupled with microiontophoresis. In this study, we used 3-morpholinosydnonimine hydrochloride (SIN 1), a useful tool for delivering NO in a controlled and reproducible manner into close proximity to neurons in the CNS during in vitro and in vivo electrophysiological experiments (Lovick and Key, 1996; Shaw and Salt, 1997; Xu et al., 1998; Lin et al., 1999). We examined the effects of the iontophoretic application of Nω-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO synthesis, on the activity of striatal cells. As has recently been shown (Kara and Friedlander, 1999), the effects of these NO-modulating compounds, at the concentrations used in this current study, are likely to be attributed to a direct action on a neuronal component rather than through a modification of cerebral blood flow. Lastly, to further study the possible involvement of cGMP in the electrophysiological effects produced by the NO donor, we investigated whether the pharmacological inhibition of sGC by microiontophoretic application of methylene blue (Gruetter et al., 1981) prevents SIN 1-induced changes in the firing rate of striatal neurons.

**MATERIALS AND METHODS**

**Animals**

Male Wistar rats (Morini, Italy) weighing 250–300 g were kept at constant room temperature (21 ± 2°C) and relative humidity (60%), with a 12-h light–dark cycle and free access to water and food. All experiments were performed in strict accordance with the Italian laws on animal experimentation (D.L. 116/1992) and with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Single-cell recording procedures**

Rats were anesthetized with urethane (1.2 g/kg, i.p.) (Sigma Chemical Co., St. Louis, MO, USA) and mounted on a stereotaxic instrument (David Kopf Instruments, Tujunga, CA, USA). Body temperature was maintained at 37–38°C with a heating pad placed beneath the animal. Heart rate and pupil diameter were monitored during all experimental sessions. A midline scalp incision was made and the skull was exposed. To allow electrophysiological sampling throughout the striatum, a 3-mm burr hole was drilled in the skull, centered at 8.2 mm anterior to the interaural line and 3.5 mm lateral to the midline. Extracellular recordings were performed using five-barrel glass micropipettes. These were automatically pulled and broken back to approximately 5–8 μm at the tip under a light microscope. The protruding center barrel of each pipette, filled with a 2 M NaCl solution saturated with 2% pontamine sky blue dye, was used for recording. The in vitro impedance of the recording barrel was 1–2 MΩ, measured at 135 Hz. One side barrel of the micropipette was filled with a 2 M NaCl solution for automatic balancing, whereas a second side barrel was always filled with l-glutamic acid monosodium salt (GLU, 100 mM in 100 mM NaCl, pH 8; Sigma) to activate the quiescent striatal neurons. The two remaining side barrels contained combinations of the following drugs: SIN 1 (40 mM, pH 4.5; Sigma), L-NAME (50 mM, pH 6.5; Sigma), and methylene blue (40 mM, pH unadjusted; Sigma). Retaining currents (positive for GLU and negative for the other solutions) of 8–10 nA were applied to the drug barrels between ejection periods. The impedance of the side barrels was typically between 20 and 70 MΩ. Micropipettes were lowered via a hydraulic microdrive into the lateral striatum. The coordinates for recording were 7.6–8.7 mm anterior to the interaural line, 3–3.8 mm lateral to the midline suture, and 3.5–5.5 below the cortical surface (Paxinos and Watson, 1996). Recordings were made from each animal for no more than 6 h. Frequently, more than one cell per rat was studied. Most striatal neurons were essentially quiescent and were therefore activated by the iontophoretic administration of GLU. The ejection current for GLU was set to activate the quiescent cells to fire at about 5–10 spikes/sec for most neurons, which required a current of 20–35 nA. At the beginning of
TABLE I. Descriptive statistics of studied parameters of single unit extracellular potentials and of interspike interval histograms (values are means ± SEM) (n = 34)

<table>
<thead>
<tr>
<th>Waveform</th>
<th>Interspike interval histograms</th>
</tr>
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<tbody>
<tr>
<td>Total duration (ms)</td>
<td>Mean 24.93 ± 1.8</td>
</tr>
<tr>
<td>Negative phase duration (%)</td>
<td>Mode 7.80 ± 1.27</td>
</tr>
<tr>
<td>Time to negative peak (ms)</td>
<td>CV 0.85 ± 0.04</td>
</tr>
<tr>
<td>Time to positive peak (ms)</td>
<td>Skewness 1.45 ± 0.14</td>
</tr>
<tr>
<td>Peak to peak amplitude (mV)</td>
<td>2.09 ± 0.13</td>
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Determination of drug effects on striatal neurons

The responses of striatal neurons to the microiontophoretic administration of drugs were determined by comparing the total number of spikes occurring during the administration of the test compound with the basal firing rate. To activate silent neurons, GLU was administered using a pulsing paradigm in which the current through the GLU barrel was automatically timed to occur for 10 min. Brains were removed and placed in 10% buffered formalin for 2 days before histological examination. Frozen sections were cut at 40-μm intervals and stained with neutral red. Microscopic examination of the sections was carried out to verify that the electrode tip was in the striatum.

Statistical analysis was performed with the SYSTAT 9.0 statistical software package (SPSS, Chicago, IL, USA). Comparisons between the current–response curves generated with the administration of the iontophoretic drug were calculated with an analysis of variance (ANOVA) for repeated measures (current). When significant effects were found, post-hoc comparisons were made with Tukey’s test. The interaction between methylene blue and SIN 1 was analyzed by ANOVA, two-factor mixed design, followed by Tukey’s test. In all cases, P < 0.05 was considered statistically significant.

RESULTS

A total of 34 striatal neurons, histologically verified to be in the striatum, were recorded and evaluated in this study. Under these experimental conditions we recorded from essentially quiescent neurons that were activated to fire by the iontophoretic administration of GLU. Nearly all tested neurons displayed smooth, sharp, biphasic action potentials (+/−), with amplitudes of about 2 mV (Table I). These features resemble those of sporadically discharging neurons previously identified in the neostriatum of anesthetized animals (Alexander and DeLong, 1985; Sardo et al., 2002), which were defined as spiny projection neurons (Kimura et al., 1990; Gerfen and Wilson, 1996; West, 1998). An example of such a recorded neuron is shown in Figure 1 (inset waveform). According to the ISIHs and the autocorrelograms, the neurons that we have examined presented a burst firing pattern, characterized by the appearance of bursts separated by pauses or by low-frequency tonic activity. These cells have autocorrelograms with no peak or with a single initial peak and an ISIH with a very asymmetric distribution of interspike intervals (Fig. 1). The mode of the ISIH is usually smaller than 10 ms. Descriptive statistics of ISIHs are reported in Table I. No changes in spike morphology were evident during microiontophoretic application of all drugs used in this current study (data not shown).
Effects of 1,3-morpholinosydnonimine hydrochloride

Within the striatum, local application of SIN 1 by microiontophoresis caused a clear decrease in the GLU-induced excitation of most striatal neurons (n = 12/15) (Fig. 2A). The effective ejection currents required to produce responses in cells recorded late in an experiment did not differ from those used for the earliest recorded cells. In responsive cells, firing began to slow down within 2.5 min of the onset of the drug ejection period. Maximum inhibition was attained within 15–22.5 min (mean ± SEM = 20 ± 0.8 min). In the majority of neurons the depression of firing rate was maintained until the end of the ejection period (Fig. 2A). At the end of drug ejection, the firing rate began to return to the control level. However, in four cells (33% of the responsive population) the neuronal activity did not return to the baseline firing frequency. As can be seen in Figure 2B, the inhibitory effect of SIN 1 was related to the amount of current applied, which ranged from 20–100 nA. The current–response curve shows that 100 nA caused a marked inhibition (76.2 ± 5.6%, P < 0.01) of striatal neurons (Fig. 2B). Ejecting currents required to produce a 50% reduction in firing rate ranged from 20–80 nA (mean ± SEM = 45.8 ± 4.8 nA). Although the inhibitory response varied among the different neurons sampled, it did not depend on the magnitude of evoked basal firing rate of the neurons. In unresponsive cells (n = 3/15), the microiontophoretic administration of SIN 1 caused a slight excitatory response but the overall effect was not statistically significant (17.8 ± 2.5 at a current of 100 nA; data not shown).

Effects of N-ω-nitro-L-arginine methyl ester

Microiontophoretic application of L-NAME, an NOS inhibitor, on striatal neurons induced rate changes in GLU-evoked firing. The most frequently encountered response (7/10) was a current-dependent increase in firing rate. A representative rate histogram is presented in Figure 3A, which shows the typical excitatory effect of L-NAME. The effect was already evident at a current of 50 nA, which significantly enhanced the basal firing rate of striatal cells by 25.7 ± 3.5%. The excitatory response did not depend on the magnitude of GLU-evoked basal firing rate of the neurons. The maximum excitatory effect (51.4 ± 2.3%) was observed during the application of a 100-nA current. Administration of higher currents did not cause further excitation of cell activity (data not shown). The current–response curves, showing the effect of L-NAME on the responsiveness of striatal neurons, are presented in Figure 3B.

Effects of methylene blue on neuronal responses to SIN 1

The effect of methylene blue, an inhibitor of guanylyl cyclase, on the responses of nine neurons to SIN 1 was tested. Methylene blue was ejected using a 50-nA current applied for a period of 17.5–20 min. At 2.5–5 min after the beginning of methylene blue application, responses to the ejection of SIN 1 (at 50–100 nA) were tested (Fig. 4A). Responses to SIN 1 were quantified by comparing the number of spikes fired during its coadministration with methylene blue and alone. In three
cells the response to SIN 1 was unchanged. However, in six cells the inhibitory response to SIN 1 was significantly reduced from 76.2 ± 5.6% to 20 ± 2.2% (ANOVA, two-factor mixed design, F\(_{1,17}\) = 40.7; \(P < 0.01\)) at a current of 100 nA (Fig. 4B). Typically, the effect of methylene blue took several minutes to develop (2.5–5 min). Furthermore, recovery of the response to SIN 1 was observed 8–15 min after the end of the ejection of methylene blue (data not shown).

**DISCUSSION**

NO mediates numerous neuronal processes and acts as a second messenger in the CNS, including the striatum (Bredt et al., 1990; Garthwaite and Boulton, 1995). Previous work in this laboratory using striatal depth electroencephalogram (EEG) recording demonstrated that the intraperitoneal administration of 7-nitroindazole (7-NI), an inhibitor of neuronal NOS (nNOS), induced an augmentation of low-frequency band power and a simultaneous attenuation of higher-frequency band power (Ferraro et al., 2002). Yurek and Randall (1991) have proposed that striatal depth EEG measures can be considered an electrophysiological parameter of postsynaptic activity. In view of this hypothesis, the synchronization of striatal bioelectrical activity induced by 7-NI suggests that NO plays a crucial role in the integration of input signals modulating the output of the basal ganglia. The current study extended these findings by examining whether pharmacological manipulation of the NO system induces a modification in the discharge of striatal projecting neurons.

Several observations provide evidence for the existence of two distinct neuronal types in the context of the striatum, based on pharmacological and electrophysiological criteria (Alexander and DeLong, 1985; Nisenbaum and Berger, 1992; Nisenbaum et al., 1988;
Sardo et al., 2002). Under our experimental conditions, all neurons examined showed biphasic negative/positive extracellular waveforms, which are characteristic discharge features (generally silent or with a very low spontaneous firing rate), and these units appear to correspond to medium spiny projection neurons (Kimura et al., 1990; Gerfen and Wilson, 1996; West, 1998).

In the present study, local microiontophoretic application of SIN 1 caused a decrease in GLU-induced excitation of most striatal neurons, recorded extracellularly in vivo. The inhibitory effect of SIN 1 was current-dependent and reproducible responses of similar magnitude could be evoked in individual cells using the same ejecting current. However, the NO donor caused a slight increase in firing rate in some striatal neurons, indicating that the neurons do not constitute a homogeneous population with regard to their responses to NO.

As a further demonstration of the critical role of NO in the regulation of the discharge activity of striatal neurons, we examined the effects of L-NAME, a nonselective NOS inhibitor. Iontophoretic application of this compound produced clear and reproducible excitation in most of the cells studied. The increase in GLU-induced excitation of striatal neurons observed in the present study following microiontophoretic application of L-NAME is in full agreement with previous observations made in our laboratory showing that systemic administration of 7-NI, a specific inhibitor of nNOS, induced an increase in the firing rate of slowly discharging striatal neurons (Sardo et al., 2002). However, it is interesting to note that some cells were completely unaffected by the inhibition of NOS and unresponsive to the application of NO via the donor, SIN 1. It has previously been suggested that locally produced NO may affect cells within a volume of tissue with a diameter of approximately 300–400 μm (Gally et al., 1990; Wood and Garthwaite, 1994; Garthwaite and Boulton, 1995). Therefore, cells with no apparent response to SIN 1 or L-NAME may lack the biochemical machinery to respond to NO-manipulating agents, rather than simply being beyond the reach of the applied compounds. Indeed, in the same electrode penetrations we have found both responsive and unresponsive to these compounds. Thus, it may be argued that there is a different distribution of NO-responsive neurons, probably linked to theirs different target structures. Obviously, further investigations regarding the effects of NO on different striatal projection neurons are necessary to test this exciting possibility.

It has been shown that NO can increase cerebral blood flow (Prado et al., 1992) by releasing vascular smooth muscle cells (Moncada et al., 1991). Therefore, microiontophoretic applications of SIN 1 and L-NAME could influence the microvascular tone by interfering with NO formation. However, it seems unlikely that the effects of these compounds on striatal neuronal activity might be secondary to modifications in local microcirculation, inasmuch as recent data show that microiontophoretic application of different NO-modulating compounds in the visual cortex do not induce any changes of cortical blood flow measured with laser Doppler flow meter (Kara and Friedlander, 1999). Moreover, our hypothesis that the effect of NO-modulating compounds on striatal neuronal activity is primarily attributable to a direct effect on neuronal targets is corroborated by the finding that topical application of L-arginine, which is known to be the endogenous substrate of the synthesis of NO, induces only a slight dilatation (6%) of pial arterioles in normotensive rats (Riedel et al., 1995).

In the light of our in vivo results with SIN 1 and L-NAME, it is likely that there is a physiologically relevant inhibitory nitricergic control of striatal neuronal activity. Endogenous NO within the striatum is released by a small population of interneurons (1–2% of the total striatal cells) coexisting with neuropeptide Y, somatostatin, and probably GABA (Kubota et al., 1993; Vincent and Kimura, 1992). These aspiny neurons have a crucial anatomical role within the striatal circuitry. In fact, nitricergic neurons receive inputs arising in the cortex (Vuillet et al., 1989; Rudkin and Sadikot, 1999), from the nigral dopaminergic neurons (Fujiyama and Masuko, 1996), and make synaptic contacts with spiny GABAergic projecting neurons (Kharazia et al., 1994) and ACh interneurons (Vuillet et al., 1992). Therefore, it has been suggested that this type of striatal interneuron mainly mediates the feedforward processing of the inputs to the projection cells modulating the local circuit and the afferent inputs to the striatum. NO release by nitricergic neurons occurs only during the prolonged depolarizations (Kawaguchi et al., 1995) induced by the activation of corticostriatal and/or thalamostriairal fibers (Calabresi et al., 1999; Consolo et al., 1999) and appears to be linked to the stimulation of NMDA receptors (East et al., 1996). It seems that many of the NO effects are mediated by cGMP, through the activation of sGC that induces the accumulation of this cyclic nucleotide in target cells (Arnold et al., 1977; Feehish and Noack, 1987; Bredt and Snyder, 1989; Southam and Garthwaite, 1991). Because immunohistochemical studies have revealed the presence of high levels of sGC in striatal spiny neurons (Ariano et al., 1982; Ariano, 1983), it is conceivable that the NO donor, SIN 1, acts through the NO/cGMP pathway to decrease the firing rate of striatal projection neurons. To investigate this possibility, we used a very common guanylyl cyclase inhibitor, methylene blue (Gruetter et al., 1981), to block the effects of the NO donor. The effects of SIN 1 were reduced in a reversible manner by the iontophoretic application of methylene blue. Interestingly, the blockade was slow to develop and often did not become apparent until several minutes after the
initial ejection of methylene blue. However, methylene blue does not pass freely through the cell membrane (Kontos and Wei, 1993). Therefore, the slow onset of its blocking effect may reflect the time required for the drug to penetrate the cell membrane and reach the intracellular sites.

The inhibitory effect of the NO donor on striatal neurons could be explained by the following mechanism. According to the widely accepted version of NO action on the CNS, this gaseous neurotransmitter, produced by striatal nitricergic neurons, increases cGMP levels in neighboring cells via the activation of sGC present in those cells. It is probable that the cyclic nucleotide can activate cGMP-dependent protein kinase, cGMP-dependent cation channels, and cGMP-dependent phosphodiesterases to induce its effects (Uhler, 1993; Garthwaite and Boulton, 1995; Juilfs et al., 1999). On the other hand, NO increases striatal release in cGMP-dependent mechanism of ACh, 5-HT, GABA, and DA (Trabace and Kendrick, 2000). Because methylene blue does not block the effect of SIN 1 in some neurons, it is possible that NO also acts via other mechanisms. In fact, NO inhibits NMDA receptor function (Manzoni et al., 1992), either by promoting the oxidation of a redox modulatory site (Lei et al., 1992), or by another as yet unidentified mechanism (Hoyt et al., 1992; Fagni et al., 1995). Moreover, it has also been demonstrated that the formation of NO from SIN 1 is accompanied by a liberation of superoxide anions (Feelisch et al., 1989) that react rapidly, in the brain, with NO to form peroxynitrite (OONO−) (Blough and Zafiriou, 1985). Recent studies have demonstrated the capacity of OONO− to induce the release of neurotransmitters in vitro and in vivo (Okhuma et al., 1995a,b; Moro et al., 1998) and these, at least in part, modulate the concentrations of striatal neurotransmitters (Trabace and Kendrick, 2000). Furthermore, by increasing the activity of the striatal cholinergic interneurons (Centonze et al., 2001), nitricergic neurons regulate indirectly the excitability of spiny projection neurons. There is a strict cooperation between nitricergic and ACh interneurons on modulation of the striatal plasticity. In fact, NOS-positive and cholinergic interneurons actively participate in the induction of long-term depression and long-term potentiation, respectively (Centonze et al., 1999).

In addition to these influences, striatal NO signaling also regulates the responsiveness of DA nigral neurons to the striatal input that enhances the firing rate of DA cells (West and Grace, 2000) and directly modulating striatal gap junction permeability (O’Donnell and Grace, 1997).

In conclusion, our findings extend previous electrophysiological studies demonstrating that NO modulates striatal excitability (Calabresi et al., 1999; Centonze et al., 2001). This is the first in vivo study to show that, besides affecting the transmission efficacy at synapses, NO also modifies the firing rate of striatal projecting neurons. Furthermore, our results provide evidence that NO acts mainly by stimulating the formation of cGMP in the striatum. It is very difficult to predict the mechanism involved in the action of NO on the striatal microcircuitry. Indeed, there is increasing evidence of further possible cellular targets and pathways involved in NO-mediated modulation. Obviously, our results do not provide a definitive answer for the questions raised, but offer new insights into the mechanisms underlying NO-induced modulation of striatal function that may contribute to an understanding of the pathogenesis of movement disorders, such as Parkinson’s disease and Huntington’s disease.

REFERENCES


