Bursting in Substantia Nigra Pars Reticulata Neurons In Vitro: Possible Relevance for Parkinson Disease

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INTRODUCTION

Together with the internal globus pallidus, neurons of the substantia nigra reticulata (SNr) constitute the “output” of the basal ganglia (Albin et al. 1989; Graybiel 2004; Grillner et al. 2005a; Smith et al. 1998; Wichmann and DeLong 2003). SNR neurons exhibit a tonic firing rate of activity (Atherton and Bevan 2005; Richards et al. 1997) regulated by inhibition coming in part from the direct pathway of the basal ganglia (BG) conveyed by strionigral GABAergic afferents (Albin et al. 1989; Graybiel 2004; Smith and Bolam 1991) and also by excitation coming from the subthalamic nucleus (STN) (Irike et al. 1999; Nakanishi et al. 1987; Robledo and Feger 1990), the last relay of the indirect pathway. Balance between direct and indirect pathways selects and unselects, respectively, motor programs, by regulating the firing rate of SNR output neurons (Graybiel 2004, 2005; Grillner et al. 2005a; Obeso et al. 2002).

The rate model of Parkinson’s disease (PD) posits that the absence of dopamine induces, among other changes, an over-activation of the indirect pathway ending at glutamatergic subthalamo-nigral afferents, thus leading to overexcitation of SNR neurons. This model also proposes that PD patients have an underactivation of the GABAergic direct pathway, leading to a decrease in inhibition from strionigral afferents (e.g., Albin et al. 1989; Day et al. 2006; Mallet et al. 2006; Murer et al. 2002; Walters et al. 2007; Windels et al. 2005). The end result of these synaptic changes is proposed to be a generalized increase in firing rate of SNR neurons (Albin et al. 2002; Smith et al. 1998; Takakusaki et al. 2004), producing overinhibition of the nuclei in charge of movement. This would explain hypokinesia, a hallmark of PD.

In the present work, we tested this prediction of the rate model in SNR neurons from normal animals. The excitatory drive on SNR neurons was increased by the application of the glutamatergic agonist N-methyl-D-aspartate (NMDA), and a decrease in inhibition of SNR neurons was produced by blocking their GABA_A receptors.

The result of these manipulations was not just an increase in firing rate. Instead, SNR neurons entered into a bursting firing pattern (e.g., Hutchinson et al. 2004; Walters et al. 2007; Wichmann et al. 1999). However, the palette of ionic channels that bestow SNR neurons with the ability to generate rhythmic bursting has not been described. Therefore a preliminary search into the ionic mechanisms of bursting in SNR is presented.

The oscillatory model of PD disease posits that dopamine absence discloses pathologic central pattern generators (CPGs) that display unusual numbers of neurons engaged in rhythmic bursting in different BG nuclei (Hutchison et al. 2004). In fact, bursting patterns are exhibited by active CPGs (Bevan et al. 2002; Grillner et al. 2005a,b; Plenz and Kitai 1999). NMDA is capable of activating CPG circuits in vitro (e.g., Alford et al. 2003; Enomoto et al. 2002; Gordon and Whelan 2006; Grillner et al. 1981; Guertin and Hounsgaard 1999; Hochman et al. 1994; Hsiao et al. 1998; Kiehn 2006; Takakusaki et al. 2004). The rate and oscillatory models of PD are apparently incompatible. Nevertheless, this work shows that simulating the synaptic changes proposed by the rate model results in firing behaviors similar to those proposed by the oscillatory model. It also demonstrates that intrinsic ion channels possessed by SNR neurons can explain the bursting firing pattern. A report of...
these results has been presented in abstract form (Ibáñez-Sandoval et al. 2005).

METH ODS

Preparation of slices

All procedures were carried out in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care Committees of the UNAM. The experiments were performed on brain slices obtained from Wistar juvenile rats (PD 14–21). The rats were anesthetized and decapitated. The brain was rapidly obtained and immersed for one min in cold oxygenated saline (ca 4°C; 95% O2-5% CO2) of the following composition (in mM): 124 NaCl, 2.5 KCl, 1.3 MgCl2, 26 NaHCO3, 1.2 NaH2PO4, 2.4 CaCl2, and 10 glucose. The same saline but with choline chloride (124 mM) instead of NaCl was used during the slicing procedure. Parasagittal slices (300 μm) containing the SNr were cut on a vibratome (Pelco 101 Series 1000; Pelco, St. Louis, MO) and transferred to the saline with NaCl (preceding text). The slices were left for equilibration for 1 h in oxygenated saline at room temperature (ca 25°C). After equilibration, a single slice was transferred to a recording chamber placed on the stage of an upright microscope and was continuously superfused (2–3 ml/min) with oxygenated saline at room temperature.

Whole cell recordings

Recordings were made at room temperature (≈25°C) from neurons located inside the SNr boundaries as seen in the parasagittal slice. This region mainly contains GABAergic neurons (see following text). Neurons were visualized using infrared differential interference videomicroscopy with an ×40 water-immersion objective. Micropipettes for whole cell recordings were pulled (Sutter Instrument, Novato, CA) from borosilicate glass tubes (1.5 mm OD, WPI, Sarasota, FL) for a final resistance of 2–5 MΩ when filled with internal saline of the following composition (in mM): 120 KSO4, 10 NaCl, 10 K2EGTA, 10 HEPES, 1 CaCl2, 2 MgCl2, 2 ATP-Mg, 0.3 GTP-Na (pH 7.3, 290 mosM/l). Voltage-clamp recordings were made with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Liquid junction potentials (≈8 mV) were not corrected. Current-voltage relationships made in voltage-clamp mode with 60–80% compensated series resistance, were performed by stepping the membrane potential to hyperpolarized and depolarized values with near 1-s voltage commands. Current values in response to these commands were measured at the end of the step and plotted as a function of voltage so that I-V plots represent activity of currents underlying the spikes. Action currents were unclamped. Firing was readily obtained after switching to current-clamp mode. Coincidence between input resistance measured in voltage- and current-clamp suggested that neither bridge balance nor series resistance (<15 MΩ) represent a problem (see Ibáñez-Sandoval et al. 2006).

Immunocytochemical procedures

To identify the recorded cells, 1% biocytin was included in the pipette solution. For immunocytochemistry, the slices were incubated in streptavidin conjugated with Cy3 (1:200 dissolved in PBS, Zymed Laboratories, San Francisco CA). This allowed us to visualize the recorded neuron (1 per slice). Thereafter slices were incubated 30 min with 1% bovine albumin to block nonspecific binding sites and for 36 h with a mouse monoclonal antibody against parvalbumin (anti-PV; 1:2000, Sigma-Aldrich dissolved in PBS containing 0.25% Triton-X). The slices were then rinsed thrice with PBS and incubated with a goat versus mouse secondary antibody during 1 h. This antibody was conjugated with fluorescein (Vector Laboratories, Burlingame, CA). Next, slices were mounted on covered slides and observed with different fluorescent filters (Vetashield, Vector Laboratories) in an transillumination or in a confocal microscope (Bio-Rad Microscience, London, UK). Omission of primary anti-sera resulted in no detectable signal (data not shown).

Drugs

Drugs were stored as dry aliquots and stock solutions were prepared just prior to each experiment and added to the perfusion solution in the final concentration indicated. NMDA, biocytin, TEA-Cl, ATP-Mg, ATP-Na, picROTOXIN, and bicuculline methiodide or hydrochloride were obtained from Sigma (St. Louis, MO). ZD 7288 was obtained from Tocris (Ellisville, MO). Tetrodotoxin (TTX), calcicludine, ibotekoxin and apamin were obtained from Alomone Labs (Jerusalem, Israel).

Data analysis

Digitized data were imported for analysis and graphing into commercial software (Origin v.6, Microcal, Northampton MA). All data are given as means ± SE plus median and range, unless stated otherwise. Free-distribution statistical tests were used to assess statistical significance: Mann-Whitney’s U test or Wilcoxson’s t-test (depending on nonpaired or paired samples; Systat, Richmond CA) and Friedman’s one-way ANOVA with post hoc Dunn’s test or post hoc Student-Newman-Keuls test were also employed to compare more than two samples. Statistical differences of fitted functions were assessed by comparing the obtained parameters and their estimation errors with Student’s t-test.

RESULTS

SNr projection neurons may burst

Ninety percent (n = 63/70) of SNr neurons recorded in vitro in current-clamp mode exhibited spontaneous activity (Fig. 1A1). As previously described (Atherton and Bevan 2005; Nakashishi et al. 1987; Richards et al. 1997), spontaneous activity is characterized by regular single-spike firing at a frequency of 10 ± 2 Hz (n = 20; range: 4–29 Hz) with action potential threshold at −46 ± 0.5 mV. Action potential duration at half-amplitude is 0.87 ± 0.02 ms (n = 35). Regularity of spontaneous firing was assessed by several means. First, a phase-portrait of membrane potential versus its time derivative exhibited superimposed regular orbits with clear and fixed landmarks (Fig. 1A2). Second, distribution of interspike intervals had a single mode (Fig. 1B1) lasting 77 ± 0.3 ms with a coefficient of variation of 0.11 ± 0.01 (n = 20). Third, a diagram of interspike intervals (ISIs) against subsequent ones showed a single cluster (Fig. 1B2). Sixty-five percent of these recorded neurons (Fig. 1, C, I–3) were immunoreactive against parvalbumin (PV). PV+ neurons have been described before to be GABAergic projection neurons (Gerfen et al. 1985; González-Hernández and Rodríguez 2000; Ibáñez-Sandoval et al. 2006).

In our hands, 11% (n = 7/63) of tonically firing neurons exhibited spontaneous transitions in their firing pattern from...
single-spiking to bursting (Fig. 2A, 1) (Gongora-Alfaro et al. 1997; Yuan et al. 2004). As discussed elsewhere, bursting behavior is favored by more physiological cationic concentrations in the external and internal saline solutions (Sanchez-Vives and McCormick 2000; Vergara et al. 2003). Transitions from tonic to bursting were accompanied by spontaneous shifts in the membrane potential to more polarized voltages (Beurrier et al. 1999; Zhu et al. 2004). Percentage of neurons spontaneously entering into the bursting mode is less than in the case of subthalamic neurons (Beurrier et al. 1999; Zhu et al. 2004). These neurons could reside for several minutes in any of these two modes of firing (Fig. 2A). Burst frequency in control conditions was 0.07 ± 0.01 Hz (n = 8). Action potential frequency inside a burst is not significantly different from that in the tonic mode: 12 ± 0.9 Hz (range: 2.5 ± 0.2 to 34 ± 5 Hz).

Current-voltage relationships (I-V plots) of several SNr neurons recorded in voltage-clamp mode (Fig. 2B; n = 25/80—some neurons were only recorded in voltage-clamp mode) exhibited three crossing points in the zero current axis (Fig. 2C) (Ibáñez-Sandoval et al. 2006) when measured at the end of the voltage commands, suggestive of bistable membrane potential (Izhikevich 2007). Middle crossing point was near firing threshold (−46 ± 1 mV; n = 14) at the start of a “negative slope conductance region” (NSCR) of the I-V plot in the depolarizing direction, indicative of intrinsic and persistent inward currents—“unstable equilibrium” (e.g., Hsiao et al. 1998; Izhikevich 2007; Lee and Heckman 1998). Spontaneous voltage transitions between the tonic single-spike pattern and the bursting pattern was not observed in most cells with NSCR and three crossing points recorded in both voltage- and current-clamp modes.

Blockage of tonically activated NMDA receptors with 50 μM 2-amino-5-phosphonovaleric acid (APV, Fig. 2D) eliminated the two right crossing points, rendering a topologically different I-V plot (a “bifurcation”) (Izhikevich 2007). This occurs without losing the NSCR completely. A conclusion from these experiments is: SNr neurons possess the ionic machinery and/or connections to fire in two different firing patterns, tonic and bursting, in the control conditions (i.e., without any drug added). We then wanted to observe if NMDA administration would favor the bursting mode as the preferred firing pattern (Zhu et al. 2004).
Enhancing the excitatory drive on SNr neurons

The subthalamonic glutamatergic connection is the last step of the indirect pathway of the basal ganglia (BG) (e.g., Albin et al. 1989; Hammond and Yelnik 1983; Iribe et al. 1999; Ibañez-Sandoval et al. 2006; Nakanishi et al. 1987; Robledo and Feger 1990; Smith et al. 1998). This connection preserves some of its fibers in parasagittal in vitro slices (see Ibañez-Sandoval et al. 2006 and also: Beurrier et al. 2006; Loucif et al. 2005). This synapse has a great contribution of NMDA receptors to the postsynaptic response ("tonic drive") (see Ibañez-Sandoval et al. 2006) (see also Fig. 2D). PD models posit that a tonic overactivation coming from this connection results in some motor signs of PD (e.g., Albin et al. 1989; Bergman et al. 1994; Graybiel 2004; Kaneda et al. 2005; Smith et al. 1998; Windels et al. 2005). Therefore to observe the actions of an increase in the tonic excitatory drive on SNr neurons, we added 10 μM NMDA to the bath saline in a sample of tonically firing neurons (n = 15) (Grillner et al. 1981; Guertin and Hounsgaard 1998; Hsiao et al. 1998; Johnson et al. 1992). The result of this maneuver can be observed in Fig. 3: the spontaneous single-spike tonic firing was changed to a spontaneous burst firing (Fig. 3A). Moreover I-V plots of neurons undergoing this transition showed an increase in the NSCR (Fig. 3, A and B) or produced the three crossing points in the voltage axis if they did not have them. However, burst frequency of NMDA bathed cells did not change significantly when compared with spontaneous bursting without NMDA (cf., Fig. 2; see preceding text): 0.08 ± 0.01 Hz. Both bursting and NSCR enhancement were blocked by 50 μM APV. Frequency of action potentials inside a burst was increased during NMDA to 23 ± 2 Hz as compared with spontaneous bursts (see preceding text; range: of 8 ± 2 Hz to 52 ± 4 Hz; n = 15; P < 0.05). It was concluded that similarly to neurons belonging to CPG networks in the nervous system, an increase in the tonic excitatory drive on SNr neurons, posited to happen in PD (Graybiel 2004; Grillner 2006; Wallén and Grillner 1987), renders a rhythmic bursting output (e.g., Zhu et al. 2004). Each burst appeared to be present on top of depolarizing plateau potentials (Grillner 2006).

Blocking inhibition on SNr neurons

The strionigral connection configures the direct pathway of the BG (e.g., Albin et al. 1989; Graybiel 2004; Smith et al. 1998). This connection is inhibitory and GABAergic (Kita and Kitai 1988; Yoshida and Precht 1971). The underactivity of strionigral inhibitory afferents is posited as crucial in the pathogenesis of PD (Graybiel 1996, 2004). To test this postulate of the model in vitro, we added 10 μM bicuculline to the bath saline to a sample of tonically firing neurons. The result of this maneuver can be observed in Fig. 4: the spontaneous tonic firing pattern was changed to burst firing (Fig. 4A) (Yuan et al. 2004). Bursting frequency during bicuculline was rather low: 0.01 ± 0.002 Hz (n = 7; P < 0.001; as compared with both spontaneous bursting and NMDA-induced bursting; see preceding text).
ceding text). *I*-*V* plots of all neurons bathed with bicuculline showed an increase in the NSCR and displayed bursting (Fig. 4, A and B), indicating that tonic inhibition is capable of restraining intrinsic persistent inward currents. The same result was obtained in two experiments after the application of picrotoxin (50 μM). Frequency of action potentials inside a burst was increased: 20 ± 1 Hz (range: 3–47 Hz) with respect to spontaneous bursting (see preceding text; n = 9; P < 0.05) after GABAA receptor blockage.

Reduction of inhibition is also known to induce rhythmic patterns in CPG networks by unbalancing a tonic excitatory drive (Grillner 2006; Schmitt et al. 2004; Takakusaki et al. 2004), including the SNr (Yuan et al. 2004). Therefore the blockade of an inhibitory tone discloses and confirms the presence of a tonic excitatory tone. Previous experiments using extracellular recordings could not observe the subthreshold behavior during bursting (Gongora-Alfaro et al. 1997; Yuan et al. 2004).

Figure 4 also shows that addition of 10 μM NMDA in the continuous presence of bicuculline made the oscillations more robust and frequent, suggesting that both maneuvers have additive actions and that the system is far from saturation with any of the maneuvers. Burst duration increased from 3.5 ± 0.1 to 6.2 ± 0.2 s, and action potential frequency inside bursts became 27 ± 0.8 Hz (n = 5; range: 13–61 Hz). Because the *I*-*V* plot was clearly changed after agonists and antagonists of both transmitter receptors: glutamate and GABA, it is suggested that SNr neurons receive important excitatory and inhibitory tonic synaptic drives in such a way as to restrain the intrinsic conductances that generate bursting, thus limiting the firing pattern to tonic single-spike firing.

**Ionic mechanisms of bursting in SNr neurons**

Having demonstrated that pharmacological procedures that disclose pattern generation in other parts of the nervous system, disclose bursting firing pattern in SNr neurons, we began searching into the ionic mechanisms underlying this bursting. Ionic substitution experiments were then performed in the presence of 10 μM NMDA.

In a sample of neurons (n = 6) exhibiting oscillatory bursting induced by 10 μM NMDA (cf., Figs. 5A1 and 2), addition of 1 μM TTX, abolished the firing of action potentials (Fig. 5A3), and disclosed the slow and repetitive membrane potential plateaus, that appear to underlie burst firing. Thus the oscillation persisted after TTX in all neurons tested in this way (e.g., Beurrier et al. 1999; Guertin and Hounsgaard 1998; Hsiao et al. 1998; Zhu et al. 2004). Plateaus had longer duration than control NMDA-induced bursts but exhibited a lower frequency (Fig. 5A3). However, the NSCR in the *I*-*V* plot was decreased by TTX in all neurons of this sample by 11 ± 1% (n = 6; cf., Figs. 5B1 and 2) as though persistent Na+ currents contributed, in part, to generate the slow depolarizations underlying the bursts (Grillner 2006; Hsiao et al. 1998; Zhu et al. 2004).

**FIG. 3.** Bursting induced by NMDA. A: spontaneous tonic firing in the control (top) is changed to burst firing after addition of 10 μM NMDA (bottom). -60 mV. B: current traces in response to hyperpolarizing and depolarizing voltage commands before (top) and during NMDA administration (bottom, 1) were used to build *I*-*V* plots in both conditions (2). Note enhancement of NSCR during NMDA (●).

**FIG. 4.** Bursting induced by bicuculline. A: spontaneous tonic firing in the control (top) is changed to burst firing after addition of 10 μM bicuculline (middle). Burst frequency is further enhanced by the subsequent addition of 10 μM NMDA (bottom). -60 mV. B: current traces in response to hyperpolarizing and depolarizing voltage commands (1) in all 3 conditions are plotted in 2. *I*-*V* plots show that bicuculline is enough to augment the NSCR. NMDA has additive effects on those produced by bicuculline.
Importantly, this result also shows that firing of incoming afferent axons and terminals (blocked by TTX) are not essential to generate the oscillation, although, as mentioned before, they modulate its frequency and appearance.

Because membrane potential oscillations and NMDA-induced NSCR were not completely blocked by TTX, we next added a global blocker of all high-voltage-activated (HVA) Ca\(^{2+}\) channels (L, N, R, and P/Q types): calcicludine (50 nM—at saturating concentrations) (Kochevarov 2003; Schweitz et al. 1994), in the continuous presence of NMDA and TTX. The rationale being that if any of the known classes of HVA Ca\(^{2+}\) channel was indispensable, or contributed, to the NMDA-induced plateau depolarizations, they would then be either blocked or reduced. Figure 5A4 shows that the oscillating plateaus were reduced in amplitude, but not completely blocked, by application of calcicludine. Oscillations persisted and their frequency increased. This result, obtained also in neurons with NMDA-induced bursting without TTX (n = 8), suggests that HVA Ca\(^{2+}\) channels do contribute to the duration of plateau potentials. Accordingly, calcicludine produced a great reduction in the NSCR of the I-V plot: 51 ± 3.4% in all cells of the sample (Fig. 5B3; n = 6; P < 0.01) but did not eliminate it. Similar results were obtained by the application of 100–200 μM Cd\(^{2+}\) to the bath saline (n = 6). However, further research with specific peptidic blockers (e.g., Perez-Rosello et al. 2005) is needed to know what HVA Ca\(^{2+}\) channels are in charge of the duration and maintenance of plateau potentials. In any case, these results suggest that HVA Ca\(^{2+}\) channels are not a necessary condition for the oscillation.

Importantly, because among the HVA Ca\(^{2+}\) channels blocked by both calcicludine and Cd\(^{2+}\) are those that participate in transmitter release (Ca\(_{\alpha}2.1\) and/or 2.2), the results show again that afferent synaptic connections are not necessary to generate the oscillations. Because neither blockage of Na\(^{+}\) channels nor blockage of HVA Ca\(^{2+}\) channels, or decreases in the function of synaptic connections, interrupted membrane potential oscillations, another source of intrinsic inward current was investigated.

Figure 5A5 shows that 40 μM NiCl\(_{2}\) abolished all oscillations. Oscillations blocked by NiCl\(_{2}\) were observed in a sample of oscillating neurons during NMDA with or without TTX (n = 10). NiCl\(_{2}\) induced the complete disappearance of both the plateau potentials and the NSCR in the I-V plot (Fig. 5B4) (Lee et al. 1999). Note that NiCl\(_{2}\), but not APV, blocked the NSCR (cf., Figs. 5B4, 6B, and 2D), confirming that, at these concentrations, NiCl\(_{2}\) is very selective for Ca\(_{\alpha}3.2\) channels (Lee et al. 1999). Application of 1 μM mibebradil had the same effects (n = 4) (Martin et al. 2000).

These results suggest that the ionic conductance, essential to induce the oscillatory bursting pattern in SNr neurons, is a low-voltage-activated (LVA) Ca\(^{2+}\) channel (a T channel). This conclusion is supported by a previous report showing that SNr neurons express α1H subunits or Ca\(_{\alpha}3.2\) Ca\(^{2+}\)-channels (Talley et al. 1999). Accordingly, Fig. 6 shows that NMDA-induced oscillations in the presence of TTX, as well as the NSCR, can be blocked completely by 40 μM NiCl\(_{2}\) without first blocking HVA Ca\(^{2+}\)-channels (Fig. 6A, 1–3). It is also shown that this blockage is reversible (Fig. 6, A, 2–4, and B, I and 2), suggesting that Ca\(_{\alpha}3.2\) Ca\(^{2+}\)-channel activation is the necessary step to subsequently activate HVA Ca\(^{2+}\) channels and produce sustained plateaus. Interestingly, Ca\(_{\alpha}3.2\) Ca\(^{2+}\) channels are, precisely, the Ca\(^{2+}\) channels more susceptible to NiCl\(_{2}\) blockage (Lee et al. 1999).

During rhythmic bursting, the phase-portrait of V versus dV/dt shows two intermingled stable trajectories (Fig. 6C), one
for the action potentials (in black) that now exhibits some inactivation and another for the oscillatory plateaus (in red; dotted trajectory indicates plateau orbit without the spikes). The fixed point that unifies both orbits (blue dot in Fig. 6C) is the quiescent voltage after NiCl2 (Fig. 6A3). Finally, immunocytochemistry experiments (Fig. 6D) using antibodies against the α1H subunit indicated that it was coexpressed with parvalbumin in the same cells (n/10/11 neurons, or 91% of the tested sample, were immunoreactive for the α1H subunit).

Taken together, the experiments suggest that rhythmic bursting pattern use intrinsic ionic conductances additional to those used by the single-spike tonic firing pattern. They also suggest that the ionic machinery expressed by SNr neurons is enough to generate bursts but that synaptic drives regulate its frequency and appearance in control conditions. A main conductance in this behavior is the CaV3.2 Ca2+ channel, which is necessary for initiating the oscillatory cycle and the plateaus. However, the afterhyperpolarizations that follow bursts or plateau potentials (in TTX) may reach −90 mV and then return to potentials that activate CaV3.2 Ca2+ channel. This behavior suggested the participation of hyperpolarization-activated cation channels (HCN).

Participation of hyperpolarization-activated cation channels in the generation of bursting

Figure 7A1 shows how regular the burst firing pattern becomes after several minutes (>20 min) in 10 μM NMDA. Bursting frequency was 0.06 ± 0.004 Hz (n = 6), and the pattern exhibits regular intervals characterized by afterhyperpolarizations (AHPs) that, after each burst, slowly return to the voltages where T channels are activated. Interburst intervals (IBIs) plotted against themselves (subsequent ones) show a single cluster around 18 ± 0.5 s (Fig. 7C1, inset) with a coefficient of variation (CV) of 0.15 ± 0.002 (Fig. 7C1, inset). Action potential frequency inside the bursts was 28 ± 1 Hz (range: 6 ± 0.9 to 45 ± 0.7 Hz; Fig. 7C2, ○).

HCN channels can be reduced by 50 μM ZD 7288 (Fig. 7A2) (Robinson and Siegelbaum 2003). Accordingly, ZD 7288 significantly increased IBIs, decreasing the frequency of bursts to 0.03 ± 0.003 Hz as compared with NMDA alone (see preceding text; n = 5; P < 0.01). The amplitude of the post bursts AHPs was increased in 34 ± 2% (from 18 ± 0.2 to 24 ± 0.3 mV; P < 0.0001; Fig. 7B1) as well as burst duration from 3.7 ± 0.23 to 5.3 ± 0.3 s (P < 0.001), confirming that HCN channels contribute to NSCR and bursting (Lee and Tepper 2007). These changes were accompanied by an increase in the
CV of IBIs to 0.24 ± 0.02 (Fig. 7C1, • and inset) \( (P < 0.05) \), suggesting a loss in regularity. Action potential frequency inside the bursts increased to 35 ± 0.5 Hz (range: 6 ± 1 to 91 ± 2 Hz; \( P < 0.05 \); Fig. 7C2) as it can be appreciated in the corresponding diagram for interspike intervals (Figs. 7 and 2C).

Because ZD 7288 blocked inward current carried by HCN channels (Fig. 7D), the increase in the AHPs after the bursts, with the consequent decrease in frequency and regularity, may be interpreted as outward currents taking over on the “territory” of HCN channels during the IBI, suggesting that IBIs are made up by a combination of HCN, outward currents probably carried by K\(^+\) channels, and the inward currents mentioned in the preceding text (e.g., T channels). Likely candidates to carry a part of the outward current during AHPs are apamin-sensitive Ca\(^{2+}\)-activated K\(^+\) channels (SK channels) (Grillner 2006; Yanovsky et al. 2005).

**Participation of SK channels in bursting**

The participation of the SK channels in SNr neurons tonic firing has been documented (Atherton and Bevan 2005; Vergara et al. 1998; Yanovsky et al. 2005). As shown in Fig. 8A, 200 nM apamin increased firing frequency in a sample of control tonically firing neurons from 30 ± 0.9 Hz (range: 22–53 Hz) to 42 ± 0.7 Hz (range: 31–67 Hz; Fig. 8A, I and 2; \( n = 5; P < 0.01 \)) by reducing the ISIs (Fig. 8A, 3–5). Phase-plane analysis shows that this change in frequency is accompanied by a partial inactivation of action potentials because the spike orbit had a reduction in perimeter (Fig. 8A6). In fact, the continuous presence of apamin in the bath saline (>40 min) was followed by a gradual more pronounced depolarization and blockage of spikes (not shown), but by itself, apamin administered alone did not induce bursting. Thus apamin-sensitive K\(^+\) channels are important to pace the repetitive firing of SNr neurons (Atherton and Bevan 2005; Yanovsky et al. 2005) and thus may participate in the shortening of bursts (Zhu et al. 2004).

Figure 8B shows that apamin confirms this inference: it reduced burst frequency in a sample of neurons after NMDA-induced bursting from 0.06 ± 0.002 to 0.016 ± 0.0005 Hz (\( n = 8; P < 0.001 \); Fig. 8B, I and 2) and increased burst duration from 4 ± 0.04 to 7.4 ± 0.3 s (Fig. 8B, 3 and 4; \( P < 0.01 \)), that is, an 86 ± 8% increase. IBIs clearly formed a
different cluster after apamin (Fig. 8B6; \(P < 0.05\)). As expected, firing frequency inside the bursts was increased from 33 ± 0.5 Hz (range: 16 ± 0.5 to 52 ± 2 Hz) to 45 ± 1.1 Hz in this sample (range: 16 ± 1.2 to 98 ± 3 Hz; \(P < 0.05\)).

The diagram of ISIs was simply spread toward minor intervals in both tonic and burst firing allowing the firing of more spikes (Fig. 8, A5 and B5). In contrast, IBIs clearly separated into different clusters (Fig. 8B6), suggesting an inverse relationship between ISIs and IBIs duration. This suggests that SK-channel reduction may slow down burst repolarization with a consequent increase in burst duration, allowing more Ca\(^{2+}\) influx to generate more prolonged AHPs and IBIs. This suggests that other K\(^+\) channels, perhaps BK channels, shape the discharge during the burst.

**Participation of BK channels**

Submillimolar concentrations of tetraethylammonium (TEA) are known to be selective in blocking BK channels (Bargas et al. 1999; Pineda et al. 1992; Vergara et al. 1998). Therefore 500 \(\mu\)M TEA were used to infer the participation of these channels in tonic and burst firing (Fig. 9). Basically, TEA slowed down the repolarization of the action potential and blocked the initial part of the AHP during control tonic firing (Fig. 9A, 1–3). This prolonged the action potentials from 0.83 ± 0.02 to 1.16 ± 0.02 ms in a sample of neurons recorded in control conditions (Fig. 9A, 3 and 5; \(n = 20\); \(P < 0.0001\)). Phase portrait shows that only the repolarization phase and the peak of the AHP were affected. In contrast with apamin actions, these effects were not enough to change firing frequency: from 9.3 ± 1.7 in the control to 9.4 ± 1.3 Hz after TEA (NS). ISIs or their CVs were not changed significantly.

In the same way, TEA had minor effects in either burst duration or IBIs in a sample of neurons during bursting induced by NMDA (\(n = 6\); Fig. 9B). The most detectable actions were on spikes. Action potential frequency decreased from 24 ± 0.4 Hz (range: 10 ± 1 to 46 ± 2 Hz) to 16 ± 0.3 Hz (range: 1 ± 0.05 to 34 ± 3 Hz; \(P < 0.05\)), suggesting that at high firing frequencies, the small change on the fast AHP does have an effect in frequency. Action potentials during the bursts tended to inactivate (Fig. 9B, 3 and 4) disabling the neuron for maintaining high frequencies during the bursts. Instead of action potentials, high-frequency oscillations on top of the plateau potentials were evident (Fig. 9B, 3 and 4). The duration of bursts suffered a decrease, from 2.6 ± 0.06 to 1.6 ± 0.07 s (\(P < 0.01\)). However, neither IBIs nor ISIs distributions showed significant changes (Fig. 9B, 5 and 6). Iberiotoxin, a selective BK-channel blocker had the same effects as micromolar TEA (\(n = 5\)). To conclude, both BK and SK channels participate in the firing of actions potentials during bursts but only SK channels participate in fixing bursting frequency.
Further research is needed to disentangle the complete set of outward currents in charge of shaping the bursts and bursting firing pattern (e.g., Na-activated K channels, slowly inactivating voltage-dependent K channels, and so on) (Grillner 2006), but clearly, HCN, SK, and T channels are important in fixing the temporal structure of oscillatory bursting in SNr neurons.

DISCUSSION

NMDA bursting

Because the rate model of PD posits that pathological changes are a result of an overexcitation of the indirect pathway and an overinhibition of the direct pathway of the BG (Albin et al. 1989; Day et al. 2006; Graybiel 1996; Murer et al. 2002; Windels 2005), we decided to test this model by observing what happens to normal SNr neurons after producing an increase in their excitatory tonic drive and/or a decrease in their incoming inhibition.

As in many other studies (Grillner et al. 1981; Hochman et al. 1994; Hsiao et al. 1998; Wallén and Grillner 1987), NMDA increase the excitatory drive on SNr neurons. As in circuits embodying CPGs, the increase in the excitatory drive was transformed into patterns of bursting activity (Grillner 2006; Takakusaki et al. 2004). Coincidently, the oscillatory model of PD posits that synaptic changes induced by dopamine depletion disclose pathological or uncontrolled CPGs, manifested by bursting and abnormal rhythmic firing (Hutchinson et al. 2004; Plenz and Kitai 1999). Indeed, bursting in SNr neurons is observed during parkinsonism (Bergman et al. 1994; Hutchinson et al. 2004; Walters et al. 2007; Wichmann et al. 1999).

In a comparable way, a reduction of inhibition on SNr neurons also induced bursting (Grillner 2006; Takakusaki et al. 2004; Yuan et al. 2004). Accordingly, it was found that by simulating the synaptic changes proposed by the rate model of PD, we obtained pattern generation, a sign proposed as a main electrophysiological characteristic of PD by the oscillatory model. To our knowledge, neither bursting nor its ionic and induction mechanisms had been reported previously for SNr neurons, much less in the context of synaptic changes proposed to characterize PD.

Note that according to the rate model of PD, GABAergic inhibitory afferents connecting the globus pallidus (external) and the SNr (Graybiel 2004, 2005; Kita 2001; Parent and Hazrati 1995) would also be underactive in PD because the striatal neurons that inhibit pallidal neurons would be overactive (Day et al. 2006; Mallet et al. 2006). However, it has been proposed that pallidal neurons participate in the connections of
the CPG that emerges after dopamine depletion (Hutchinson et al. 2004; Plenz and Kitai 1999). Therefore GABA concentration may in fact be fluctuating (Bevan et al. 2002; Walters et al. 2007; Windels et al. 2005) in PD to CPG activity.

The present experiments also revealed that SNr projection neurons have an intrinsic pacemaking capability to burst similar to that found in STN neurons (Beurrier et al. 1999). Neither the blockage of all synaptic transmission with Cd2+ nor the blockage of action potential firing with TTX abolished the subthreshold oscillation underlying bursting. It is known that both NMDA and bicuculline initiate locomotion or augmented muscle tone if injected in locomotor regions controlled by the SNr (Takakusaki et al. 2004).

**Ionic conductances necessary for bursting in SNr neurons**

An initial investigation into the ionic conductances responsible for bursting in SNr neurons showed that several ionic currents participate in burst generation, such as: HCN, SK, and HVA Ca2+ channels. However, it was found that activation of CaV3.2 Ca2+ channels is the necessary step to induce the oscillatory pattern. In SNr neurons, Na+ and HVA Ca2+ conductances participate in fixing the duration and amplitude of the plateau potential that underlies the bursts, and HCN and SK conductances participate in fixing the temporal structure of bursting, but it is the CaV3.2 Ca2+ channel that links both sets of conductances to produce bursting (Grillner 2006). The experiments also show that both excitatory and inhibitory tonic drives are readily detected on SNr neurons by means of changes in the NSCR of the steady-state I-V plot after pharmacological manipulations (NMDA, APV, bicuculline). Therefore the fact that neither TTX nor HVA Ca2+-channel blockade affected the generation of membrane oscillations (pacemaking properties) does not imply that synaptic drives are unimportant. On the contrary, synaptic changes had to be disposed to disclose the bursting pattern, which means that the absence of this pattern during tonic firing implies important on-going and balanced synaptic influences, even in the in vitro slice preparation (Beurrier et al. 2006). In this respect, bursting frequencies found in the present study are rather low probably due to incomplete circuitry connections (Loucif et al. 2005), favoring the view that the cortex may be the origin of the main pacemaker (Magill et al. 2001; Yuste et al. 2005).

**Functional implications**

Bursting SNr neurons is a common finding in PD patients and parkinsonian animals (Bergman et al. 1994; Meissner et al. 2006; Tseng et al. 2001; Walters et al. 2007; Wichmann et al. 1999). The fact that bursting can appear on SNr neurons after pharmacological manipulations of their synaptic inputs and, in addition, the fact that all dopaminergic actions on SNr neurons rely on the activity of the dopamine presynaptic receptors present at these synaptic inputs (Ibáñez-Sandoval et al. 2006; Misgeld et al. 2007) (no dopamine postsynaptic receptors have been described for SNr neurons), gives a cue about why dopamine depletion may lead to impaired transmission and bursting: synaptic inputs are impeding bursting. The derangement of their control, by dopamine depletion, allows the appearance of this firing pattern. Therefore presynaptic receptors become potential targets for therapeutics.

By the same token, CaV3.2 channels turn to be the essential intrinsic component of the bursting mechanism disclosed by uncontrolled synaptic entries. Therefore these channels, too, become a potential therapeutic target.

It has been proposed that BG select among a group of CPGs to carry out innate or acquired behavioral routines and motor programs (Barnes et al. 2005; Graybiel 1995, 1996; Grillner et al. 2005a,b, 2006; Takakusaki et al. 2004). The essential properties of these circuitries are maintained throughout vertebrate evolution (Graybiel 1995; Grillner 2006). Disinhibition is the mechanism proposed for the BG to release (select) some CPGs and then elicit a motor behavior or synergy (Graybiel 1995, 1997, 2004; Grillner 2005a,b, 2006). Therefore BG circuitry can be seen as part of a set of CPGs.

Although more research is needed into the mechanisms of SNr firing in animal models of PD to further substantiate the present findings, our results indicate that the rate and oscillatory models of PD could be made compatible if one thinks that the synaptic changes proposed by the rate model will have as a consequence changes in firing pattern. In this respect, CPG theory proposes that unbalancing synaptic drives draws forth firing pattern generation.

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