A Dynamic Role for GABA Receptors on the Firing Pattern of Midbrain Dopaminergic Neurons

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Abbreviated Title: A Dynamic Role for GABA Receptors on Dopaminergic Neuron Firing Patterns
Abstract

Dopaminergic neurons are subject to a significant background GABAergic input in vivo. The presence of this GABAergic background might be expected to inhibit dopaminergic neuron firing. However, dopaminergic neurons are not all silent but instead fire in single-spiking and burst firing modes. Here we present evidence that phasic changes in the tonic activity of GABAergic afferents are a potential extrinsic mechanism that triggers bursts and pauses in dopaminergic neurons. We find that spontaneous single-spiking is more sensitive to activation of GABA receptors than phasic NMDA-mediated burst firing in rat slices (P15-P31). Since tonic activation of GABA\(_A\) receptors has previously been shown to suppress burst firing in vivo, our results suggest that the activity patterns seen in vivo are the result of a balance between excitatory and inhibitory conductances that interact with the intrinsic pacemaking currents observed in slices. Using the dynamic clamp technique, we applied balanced, constant NMDA and GABA\(_A\) receptor conductances into dopaminergic neurons in slices. Bursts could be produced by disinhibition (phasic removal of the GABA\(_A\)R conductance) and these bursts had a higher frequency than bursts produced by the same NMDAR conductance alone. Phasic increases in the GABA\(_A\)R conductance evoked pauses in firing. In contrast to NMDAR, application of constant AMPAR and GABA\(_A\)R conductances caused the cell to go into depolarization block. These results support a bidirectional mechanism by which GABAergic inputs, in balance with NMDAR-mediated excitatory inputs, control the firing pattern of dopaminergic neurons.

Keywords: dopamine, burst, GABA\(_A\) receptors, GABA\(_B\) receptors, disinhibition
Introduction

Dopamine released by the activity of midbrain dopaminergic neurons plays an important role in Parkinson’s disease (Blandini et al., 2000), reinforcement learning (Schultz 1998), and schizophrenia (Grace 1991). At least 70% of all inputs onto dopaminergic neurons are GABAergic, the majority of which arise from the striatum, globus pallidus (GP) and substantia nigra pars reticulata (SNr; Bolam and Smith 1990; reviewed in Tepper and Lee 2007). Since spontaneously active neurons in the GP and SNr fire at rates as high as 50 and 60 Hz, respectively (Guyenet and Aghajanian 1978; Deniau et al., 1978; Kita and Kitai 1991; Celada et al., 1999), the activity of dopaminergic neurons is subject to a large GABAergic input. Dopaminergic neurons under the influence of this GABAergic inhibition would be expected to be mostly silent. Although some dopaminergic neurons are silent (Grace et al., 2007; but see Dai and Tepper, 1998), many of them are spontaneously active in anesthetized or in awake, behaving animals (Freeman et al., 1985; Schultz et al., 1997; Kiyatkin and Rebec 1998; Hyland et al., 2002). They typically fire single spikes with varying degrees of regularity and can generate high-frequency bursts by a mechanism activating NMDA receptors (Overton and Clark 1992, 1997; Chergui et al., 1993; Deister et al., 2009; Zweifel et al., 2009). Dopamine is then released either tonically or phasically depending whether the neuron is in a single-spiking mode or burst-firing mode (Wilson et al., 1977; Grace and Bunney 1984a,b; Goto et al., 2007). Does tonic GABAergic input simply hinder these firing modes or could it play a more integral role in their generation?

Dopaminergic neurons receive a combination of tonic inhibitory and excitatory inputs in vivo. Dopaminergic neurons are bombarded by chloride-mediated IPSPs in vivo.
Local application of GABA\textsubscript{A} receptor (GABA\textsubscript{A}R) antagonists shifts the firing pattern of dopaminergic neurons from a single-spike mode into a burst-firing mode, while application of GABA\textsubscript{B} receptor antagonists regularizes the firing pattern (Engberg 1993; Paladini and Tepper 1999; Brazhnik et al., 2008). These results were interpreted to mean that tonic activation of GABA\textsubscript{A} receptors inhibits burst firing and that the action of GABA\textsubscript{B} receptors is mostly presynaptic. The shift into the burst-firing mode suggests that dopaminergic neurons are also subject to tonic excitation. Local application of NMDA receptor (NMDAR) antagonists, but not an AMPA receptor (AMPAR) antagonist, significantly reduced burst firing (Overton and Clark 1992; Chergui et al., 2003), suggesting that the majority of the tonic excitation driving bursts is NMDAR-mediated.

Here we show that spontaneous, single-spike firing is more sensitive to activation of both GABA\textsubscript{A} and GABA\textsubscript{B} receptors than phasic, NMDAR-mediated burst firing evoked by either iontophoresis or dynamic clamp. This suggests that the activity seen in dopaminergic neurons \textit{in vivo} is the result of a balance between excitatory and inhibitory conductances. Single spiking continued after application of balanced, constant NMDAR/GABA\textsubscript{A}R, but not AMPAR/GABA\textsubscript{A}R, conductances by dynamic clamp. Applying tonic NMDAR and GABA\textsubscript{A}R conductances, we show that phasic removal of the GABA\textsubscript{A}R conductance causes burst firing and phasic increases in the GABA\textsubscript{A}R conductance causes a pause in firing. Bursts generated by disinhibition have a higher frequency than bursts generated by identical excitation alone. Our results provide evidence for an extrinsic mechanism by which phasic changes in GABAergic drive can generate bursts and pauses in firing in midbrain dopaminergic neurons.
Materials and Methods

Slice preparation and recordings. Electrophysiological experiments were performed on slices obtained from Sprague-Dawley rats (Charles River Laboratories) aged 15 to 31 days. While dopaminergic neurons recorded in vivo do show changes in firing pattern with age (Tepper et al., 1990), we found no difference among neurons from 15 or 31 day old animals during in vitro recordings. All experimental procedures were approved by the University of Texas at San Antonio Institutional Animal Care and Use Committee. Rats were anesthetized with ketamine/xyzaline, decapitated and the brains rapidly removed and cooled. 240µm horizontal slices were cut using a vibrating microtome (Microm HM 650V) in oxygenated, cold ACSF containing (in mM): 110 CholineCl, 2.5 KCl, 1.25 NaH2PO4, 7 MgCl2, 0.5 CaCl2, 10 dextrose, 25 NaHCO3, 1.3 ascorbic acid and 2.4 sodium pyruvate. Slices were then transferred to an incubation chamber containing (in mM): 126 NaCl, 2.5 KCl, 1.25 NaH2PO4, 4 MgCl2, 2 CaCl2, 10 dextrose, 25 NaHCO3, 1.3 ascorbic acid, 2.4 sodium pyruvate and 0.05 glutathione. Slices were incubated at 32°C for at least an hour before recording and then kept at room temperature thereafter. Prior to recording, a slice was held in a submerged chamber filled with ACSF similar to the incubating solution except that 2 mM MgCl2 was used and glutathione was not added. The slice was superfused at a rate of 2 ml/min by a gravity feed system and heated to 32-34°C with an inline heater. SNc neurons were visualized with a gradient contrast imaging system. Perforated-patch or whole-cell recordings were then made from presumed SNc dopaminergic neurons. Perforated patch recordings were made with the whole-cell internal described
below or an internal solution containing (in mM): 140 KMeSO₄, 0.2 EGTA, 7 NaCl, 10 HEPES. The antibiotic gramicidin A or D (dissolved in DMSO, 100µg/ml of internal solution) was used to maintain natural intracellular Cl⁻ levels (Kyrozis and Reichling 1995) and to reduce run-down. Accidental break-in was determined by a large, instantaneous jump in spike height. Cells in which accidental break-in occurred were rejected. Whole cell recordings were made with an internal solution containing (in mM): 138 K-gluconate, 10 HEPES, 2 MgCl₂, 0.2 EGTA, 0.0001 CaCl₂, 4 Na-ATP, 0.4 Na-GTP. All internal solutions were adjusted to a pH of 7.3 using 1M KOH and an osmolarity of 270-275 mOsm. Recordings were acquired with a Multiclamp 700B and digitized (Instrutech) under command of the AxographX software program.

Dopaminergic neurons were identified by a slow, spontaneous firing rate (usually 1-4Hz), a prominent spike AHP, and a large Iₜ current upon passage of a hyperpolarizing voltage step. The presence of a large mGluR1–mediated hyperpolarizing response to iontophoresis of glutamate following a burst (Morikawa et al., 2003) was also used to identify dopaminergic neurons (Marino et al., 2001).

**Dynamic clamp.** Dynamic clamp experiments were conducted in whole cell mode as previously described (Deister et al., 2009). The equations used to calculate the applied current are:

\[ I_{\text{NMDA}} = -g_{\text{NMDA}} \cdot \left( \frac{1}{1 + ([\text{Mg}] / 3.57)} \cdot e^{(V_m + 0.062)} \right) \cdot (V_m - E_{\text{NMDA}}) \]

\[ I_{\text{GABA}_A} = -g_{\text{GABA}_A} \cdot (V_m - E_{\text{GABA}_A}) \]

\[ I_{\text{GABA}_B} = -g_{\text{GABA}_B} \cdot (V_m - E_{\text{GABA}_B}) \]

where [Mg] = 1.5 mM, E_{NMDA} = 0 mV, E_{GABA}_A = -60 mV (-63mV was the mean reversal potential for GABAₐ receptors in perforated patch; Gulasci et al., 2003), E_{GABA}_B = -100
mV unless otherwise stated. An AMPAR-mediated current was created from the $I_{\text{NMDA}}$ equation by setting [Mg] to 0. All recordings were done with a balanced bridge in continuous current clamp (Bridge Mode). A junction potential of -6 mV was corrected online in these experiments.

**Evoked responses.** Iontophoresis was chosen over electrical stimulation of the slice to evoke phasic bursts that did not contain a GABA-mediated component, which would be concomitantly activated upon electrical stimulation. Iontophoresis was also chosen over bath application of NMDA (Paladini et al., 1999a, Komendantov et al., 2004) to isolate the effects of GABA receptor activation on single-spiking and burst firing. Bursts were generated by iontophoresis of glutamate (typically onto dendrites 50 µm from the recording electrode; 50-250 ms pulses, holding +1 to 10 nA, ejection -50 to -300 nA; Dagan ION-100) as previously described (Deister et al., 2009). The iontophoretic pipette contained 1M glutamate at pH 7-9. All experiments involving iontophoresis were performed in the presence of the AMPA receptor blockers, NBQX or GYKI-52466.

**Drugs.** All drugs were applied to the slice via superfusion. Isoguvacine hydrochloride (1-100 µM), (R)-baclofen (0.01-10 µM), picrotoxin (100 µM), NBQX (25 µM), GYKI-52466 (20-50 µM), CGP-55845 (1-2 µM) were purchased from Tocris.

**Data Analysis.** Action potentials were detected using a derivative threshold in AxographX (1-5 V/s) and changes in spiking frequency measured. Analysis was done with Mathematica 7 (Wolfram Research Inc.). A burst was defined as a series of the action potentials that occurred within 1 second after the onset of the iontophoretic pulse. Bursts evoked by dynamic clamp were analyzed for the entire time window that the
NMDA conductance was on. In the case of bursts evoked by disinhibition, the window was defined as the period in which the GABA\textsubscript{A}R conductance was phasically turned off. Maximum burst frequency was determined as the reciprocal of the minimum interspike interval (ISI) for all spikes in the burst. Mean burst frequency was determined as the reciprocal of the mean intraburst ISI. For spontaneous single-spiking, mean frequency was determined as the reciprocal of the mean ISI measured over a time window of 20 seconds in which no current was injected to the cell. Average membrane potential was calculated as the mean of all sampled voltages, including spikes.

**Statistics.** In several experiments, inhibition of single-spike or burst firing frequency by GABA receptor activation was measured. Since one of the factors (GABA\textsubscript{A}R activation) was quantitative rather than categorical, analysis of variance (ANOVA) was not appropriate. Instead, regression analysis was used to fit each curve. The data was fit with a quadratic model. This was implemented in SAS (SAS Institute Inc.) using the equation:

\[ y_i = \tau_i + \beta_1 x + (\tau\beta_1)_i + \beta_2 x^2 + (\tau\beta_2)_i x^2 + \varepsilon \]

where

\[ y_i = \text{dependent variable representing inhibited firing frequency in terms of percent of control,} \]

\[ \tau_i = \text{effect of firing frequency type (Figures 1 and 2B,E: max and mean burst frequency and mean single spiking frequency) or receptor type (Figure 2D: GABA}_A \text{ and GABA}_B),} \]

\[ \beta_1, \beta_2, (\tau\beta_2)_i, (\tau\beta_1)_i = \text{regression coefficients,} \]

\[ x = \text{continuous independent variable for GABA}_A \text{R activation in terms of} \]
concentration of agonist (µM) or conductance applied (nS),

\[ \varepsilon = \text{residuals, } \varepsilon \sim N(0, \sigma^2). \]

For dynamic clamp experiments in which a range of \( \text{GABA}_A \)R conductances were applied to the same cell, an additional random effect \( c_k \) was added to \( y_i \) to account for within cell effects.

After each curve was fit, multiple comparisons of firing or receptor type (\( \tau \)) given \( \text{GABA}_A \)R conductance were then made using the Scheffe adjustment (\( p < 0.05 \)). These comparisons permit us to compare, for example, if the inhibition of single-spike firing was statistically different than the inhibition of burst firing at a specific \( \text{GABA}_A \)R conductance.

For all other statistical tests, Prism (Graphpad Software Inc.) was used. All effects are given in terms of mean ± SEM unless stated otherwise. In all experiments presented here, statistical significance is considered at \( p < 0.05 \).

Results

**GABA\(_A\) receptor activation**

We first investigated the inhibition of spontaneous single-spiking and NMDAR-mediated burst firing by activation of \( \text{GABA}_A \) receptors. Identified dopaminergic cells fired spontaneously (typically 1-4 Hz) in a regular, single-spike firing pattern during perforated patch recordings. Bursts of action potentials (mean 22 Hz max burst frequency; similar to *in vivo*, e.g. Grace and Bunney 1984b) were evoked every 30 seconds by iontophoretic application of glutamate onto the recorded neuron in the presence of an AMPA receptor antagonist, NBQX (25 µM) or GYKI-52466 (20-50 µM).
We then applied the GABA<sub>A</sub>R agonist, isoguvacine (1, 10, 40, or 100 µM), to the bath and measured its effect on single-spike and burst firing frequency (Figure 1). Regression analysis of the concentration-response data showed that isoguvacine inhibited single-spike firing at significantly lower concentrations than burst firing (p < 0.05, 15-75 µM isoguvacine, regression analysis). Application of 100µM isoguvacine abolished both single-spike and burst firing (Figure 1B). The addition of the GABA<sub>A</sub>R antagonist, picrotoxin (100 µM), reversed the effects of isoguvacine (Figure 1A; p < 0.05, repeated measures ANOVA with Tukey’s multiple comparison test; control single-spike firing frequency: 2±0.57 Hz, 40 µM isoguvacine: 0±0 Hz, picrotoxin: 1.9±0.68 Hz, n=5). Spontaneous and burst firing frequencies in the presence of picrotoxin were not significantly different from control (p > 0.05; repeated measures ANOVA with Tukey’s multiple comparison test). These results suggest that spontaneous, single-spiking is more sensitive to GABA<sub>A</sub>R activation than burst firing.

We obtained similar results by applying a range of GABA<sub>A</sub>R conductances (0-30 nS) into cells using dynamic clamp (Figure 1C-D; Robinson and Kawai 1993, Sharp et al., 1993). Bursts (20.6 Hz maximum burst frequency on average) were evoked by application of a moderate NMDAR conductance (35nS, range 20-40nS). These conductances were high enough to cause sustained high-frequency firing but were generally not high enough to cause the cell to go into a state of depolarization block by the end of the conductance pulse (which typically occurred at conductances greater than 60 nS; data not shown). Again, single-spiking was more sensitive to GABA<sub>A</sub>R activation than burst firing (p < 0.05, 0-24 nS, regression analysis). Both single-spike and burst firing were abolished in all cells at a GABA<sub>A</sub>R conductance of 30 nS. The average
GABA$_A$R conductance at which single-spike firing was suppressed was 25% of the conductance needed to suppress burst firing (Figure 1D; single-spike 4.8±2.1 nS, burst 19.5±4.5 nS, n=4). Together, these results show that in the presence of a tonic GABA$_A$R conductance, single-spike firing is more sensitive to GABA$_A$R activation than burst firing.

$GABA_B$ receptor activation

Tonic activity in GABAergic afferents may also activate postsynaptic GABA$_B$ receptors on midbrain dopaminergic neurons. Cells were recorded in perforated patch and NMDAR-mediated bursting was evoked by iontophoresis at regular intervals as above. The GABA$_B$R receptor agonist, baclofen (0.01, 0.1, 1, or 10 µM), suppressed single-spike and burst firing in a dose-dependent manner (Figure 2). Single-spike firing was inhibited at lower concentrations than burst firing (p < 0.05, 0.6-2 µM baclofen, regression analysis).

Inhibition of single-spike and burst firing by application of a GABA$_B$R conductance was also investigated in dynamic clamp (Figure 2C,D; $E_{GABA_B} = -100$ mV). Bursts were evoked by application of a NMDAR conductance as before (Figure 1C-D). There was a significant difference in the regressions of the single-spike inhibition curves and both of the burst firing inhibition curves (p < 0.05, 0-8 nS, regression analysis).

We also tested whether GABA$_B$R activation was more effective at suppressing firing than GABA$_A$R activation. The use of dynamic clamp allowed for direct comparison of these two receptor types in the same sample (from Figure 1D). We found that there was a significant difference between GABA$_A$R and GABA$_B$R conductances for inhibition
of burst firing (p < 0.05, 0-24 nS, regression analysis) and single-spike firing (p < 0.05, 0-
7.2 nS, regression analysis).

Together these results extend our findings with GABA_A to show that with
activation of either GABA_A or GABA_B receptors single-spike firing will be abolished
prior to the suppression of burst firing mediated by the activation of an NMDAR
conductance. These results further show that GABA_B receptors are more effective at
suppressing both single-spike and burst firing than GABA_A. Since the conductances
used in Figure 2D were equal, this indicates that GABA_B are more effective due to the
more hyperpolarized reversal potential for potassium.

**Bursting by Disinhibition**

Previous studies have suggested that bursts are suppressed by tonic activation of
GABA_A in vivo (Paladini and Tepper 1999; Brazhnik et al., 2008). However, here we
show that single-spike firing is more sensitive than burst firing to activation of either
GABA_A or GABA_B receptors (Figures 1 and 2). Therefore, tonic activation of GABA
receptors in vivo would be expected to suppress single spiking, causing dopaminergic
neurons to be silent. However, single spiking is seen in vivo (e.g. Wilson et al., 1977;
Grace and Bunney 1984a; Paladini and Tepper, 1999). This suggests that the firing
pattern recorded in vivo is due to a combination of excitatory and inhibitory conductances
in addition to its pacemaking currents. These results also suggest that phasic removal of
this inhibitory conductance can evoke a burst and that a phasic increase in this inhibitory
conductance can evoke a pause in firing.

A control burst was evoked by application of a constant NMDAR conductance in
whole cell recordings, as shown above (Figures 1C, 2C). The same NMDAR conductance was then applied in the presence of a constant GABAAR conductance (\(E_{\text{GABAAR}}=-60\ \text{mV} ;-63\ \text{mV measured in perforated patch recordings in Gulácsi et al., 2003}) sufficient to counteract the NMDAR conductance-mediated burst and return single-spike firing back to control levels (Figure 3A, B; \(p > 0.05\); control mean ISI 0.55±0.11 s, balanced ISI 0.67±0.18 s, paired t-test, \(n=10\)). Overall, the ratio of NMDAR/GABAAR conductance used was 3.2±0.4 (\(n=7\)). In similar experiments using an AMPAR conductance instead of NMDAR, background firing could not be restored. Instead, the cell quickly went into depolarization block (Figure 3D, 4/4 cells). Block in the AMPA/GABA\(_A\) configuration occurred at much smaller conductances than those used in the balanced NMDAR/GABA\(_A\) configuration (typical NMDAR/GABA\(_A\) conductance: 25/8nS; AMPA/GABA\(_A\) conductances were less than 4nS each).

In the NMDAR/GABA\(_A\) balanced configuration, a burst of action potentials could be evoked by briefly turning off the GABA\(_A\)R conductance (Figure 3A). A pause in firing was evoked by transiently turning off the NMDAR conductance (Figure 3A) or by increasing the GABA\(_A\)R conductance (Figure 3C). Bursts produced by disinhibition had a greater frequency than control bursts with an identical NMDAR conductance (Figure 4A, B; \(p < 0.05\), control maximum burst frequency 22.2±4.9 Hz, disinhibition 27.8±5.4 Hz; paired t-test, \(n=10\); first 5 spikes mean burst frequency, control 13.8±1.6 Hz, disinhibition 20.0±3.4 Hz; paired t-test, \(n=10\)). Both disinhibition and excitation-only bursts displayed prominent spike frequency adaptation (e.g. Figure 4A). The increase in burst firing frequency was sustained over the first eight spikes of the burst (Figure 4C).
This suggests that the mechanism by which disinhibition bursts are faster occurs on the order of hundreds of milliseconds.

The mechanism by which disinhibition bursts are faster than control bursts may lie in differences in the availability of spiking currents just prior to the removal of the GABA conductance (Figure 5). To test this, we constructed phase plots of both spontaneous single-spiking (Figure 5A, black) and balanced conductance single-spiking modes (Figure 5A, red). There was a significant decrease in maximum $dV_m/dt$ (Figure 5BI; $p < 0.05$; control $67.8 \pm 8.3$, balanced $44.4 \pm 2.9$, paired t-test, $n=7$). There was also a significant increase in minimum $dV_m/dt$ (Figure 5BI; $p < 0.05$; control $-41.0 \pm 4.9$, balanced $-29.7 \pm 3.6$, paired t-test, $n=7$). Balanced spiking had a more depolarized threshold than control spiking (Figure 5BIII, $p < 0.05$; control $-34.4 \pm 1.3$ mV, balanced $-32.0 \pm 1.5$ mV, paired t-test, $n=7$). This suggests that an increase in sodium channel availability is not the cause of the increased burst frequency of disinhibition bursts.

Histograms of control and balanced spiking revealed that the average membrane potential was significantly more depolarized for balanced than control spiking (Figure 5C-D, also 5A; example histogram taken from cell shown in Figure 4A; $p < 0.05$; control $-46 \pm 1.0$ mV, balanced $-39 \pm 1.5$ ms, paired t-test, $n=10$). There was also a significant decrease in the latency from the time of conductance change to the peak of the first action potential in the burst (Figure 5E, $p < 0.05$; control $58 \pm 16$ ms, disinhibition $27 \pm 9$ ms, paired t-test, $n=10$). This suggests that a current that inactivates with depolarization (e.g. an A-type potassium current) may be the cause of the increased burst frequency of disinhibition bursts.
Discussion

The High Conductance State of the Dopaminergic Neuron

Dopaminergic neurons recorded in vivo display a variety of firing patterns: silent, regular single-spiking, irregular single-spiking, and bursty (reviewed in Tepper and Lee 2007). Dopaminergic neurons recorded in slices, however, fire only in the regular, single-spiking mode. This difference in firing patterns of dopaminergic neurons recorded in slices and in vivo is often ascribed to the loss of afferent input in slices (e.g. Overton and Clark 1997). This suggests that single-spiking in vivo is generated by an in vitro-like pacemaking mechanism in which spikes can be advanced or delayed due to afferent input (hereafter referred to as the ‘pacemaker’ mechanism). Under such a scheme, bursts are caused by phasic excitatory inputs and pauses in firing are caused by phasic inhibitory inputs. Such a mechanism may underlie the reward-related responses of dopaminergic neurons described by Schultz (1998). However, additional mechanisms for generating phasic bursts and pauses are likely for dopaminergic neurons in the ‘high conductance state’ (reviewed in Destexhe et al 2003).

There is much evidence that suggests the presence of a tonic inhibitory drive onto dopaminergic neurons. Dopaminergic neurons are bombarded by chloride–mediated IPSPs in vivo (Grace and Bunney 1985). These IPSPs are most likely due to spontaneously active afferent cells in the globus pallidus (GP) or substantia nigra pars reticulata (SNr), which fire at ~50Hz (Guyenet and Aghajanian 1978; Deniau et al., 1978; Kita and Kitai 1991; Celada et al., 1999). The presence of this tonic inhibitory drive can also be demonstrated by the local application of GABA antagonists in vivo. Paladini and Tepper (1999), and Brazhnik et al. (2008) found that local application of a variety of...
GABA_A antagonists by pressure ejection onto the recorded neuron shifted the firing pattern of the dopaminergic neuron from a single-spiking mode to a bursting one, indicating that tonic GABA_A receptor activation suppresses burst firing.

Our results, showing that NMDA-mediated burst firing is suppressed at greater levels of both GABA_A and GABA_B receptor activation than single-spiking, also suggest that dopaminergic neurons are tonically inhibited in vivo. However, if single-spiking in vivo is generated by a pacemaker mechanism then our results suggest that both single-spiking and bursting would be suppressed and there would be no tonic level of dopamine in efferent structures. However, many dopaminergic neurons recorded in vivo are not silent (e.g., Wilson et al., 1977; Grace and Bunney 1984a,b; Paladini and Tepper 1999; Paladini et al., 1999b) and tonic levels of dopamine are observed at target loci (Gonon 1988; Floresco et al., 2003). Therefore, our results suggest that dopaminergic neurons receive tonic excitatory input.

Local application of NMDA receptor (NMDAR) antagonists but not an AMPA receptor (AMPAR) antagonist significantly reduce spontaneous burst firing (Overton and Clark 1992; Chergui et al., 1993; see also Charlety et al., 1991), suggesting that tonic excitation is present and is NMDAR-mediated. Similar results were obtained with genetic NMDAR inactivation (Zweifel et al., 2009), and lesion of the subthalamic nucleus, a spontaneously active glutamatergic afferent, regularized the firing pattern of dopaminergic cells (Smith and Grace 1992). Incomplete antagonism of NMDAR activation may explain why the strongly bursting cells of Chergui et al. (1993) do not become silent as would be expected for a dopaminergic neuron in the high conductance state.
Are the GABA\textsubscript{A}R and NMDAR conductances applied here with dynamic clamp comparable to the conductances activated by tonic GABAergic synaptic input \textit{in vivo}? Using a GABA\textsubscript{A} receptor time constant of 6ms (\(\tau\); Brancucci et al., 2004), a single channel conductance of 5-30pS (\(\Delta g\); MacDonald and Olsen 1994; Guyon et al., 1999), and a 50 Hz input rate (F), we calculate that 887-5330 GABA\textsubscript{A} receptors must be activated by globus pallidus and substantia nigra pars reticulata inputs to achieve the 8 nS steady-state conductance (\(g_{ss}\)) used in the balanced configuration in Figure 1D (\(g_{ss} = \Delta g/(1-e^{(-1/(\tau Fn))})\); Wilson et al., 2004). Assuming 12 receptors per synapse (~5 immunogold particles per synapse (Fujiyama et al., 2002) times 2.5 GABA\textsubscript{A} receptors per gold particle (Nusser et al., 1997)), this corresponds to a minimum ~1-8% of the total GABAergic synapses onto an SNc neuron (~5600 GABAergic synapses total; Henny et al., 2009). Similarly, we calculate that 2300 NMDA receptors from 348 synapses must be activated for the NMDAR conductance used in the balanced configuration (\(g_{ss} = 25\text{nS}; \tau=43\text{ ms}, \text{Schilstrom et al. 2006}; \Delta g=50\text{pS}, \text{Edmonds et al., 1995}; F=5\text{ Hz for subthalamic nucleus, Chergui et al. 1994}; 2.9\text{ immunogold particles per synapse, Chatha et al., 2000}; \text{assuming 2.3 channels per synapse, similar to AMPA, Nusser 1999}). This corresponds to approximately 15% of the available glutamatergic synapses (~30% of synapses are VGluT2+, Henny et al., 2009). Thus, the NMDAR/GABA\textsubscript{A}R conductances used in this study are easily approachable in the intact network. These results also suggest that mechanisms that affect the properties of NMDAR and GABA\textsubscript{A}R (\textit{e.g.,} conductance, desensitization or kinetics) may also have significant effects upon the balance achieved by the dopaminergic neuron and hence influence firing pattern.
We propose that many dopaminergic neurons \textit{in vivo} may be in the high conductance state generated by tonic GABA\textsubscript{AR} and NMDAR conductances. Activation of both AMPA and GABA\textsubscript{A} receptors decrease the input resistance of the cell and shunt the pacemaker and burst generating currents (Figure 6A). Thus, the negative slope region of the IV curve, created by voltage-gated sodium and calcium channels, disappears. Similar results are expected for all other excitatory conductances such as nicotinic acetylcholine receptors. However, activation of NMDAR is unique because its negative slope conductance produces an increase in input resistance (Koch 1999). This negative slope conductance is caused by the magnesium sensitivity of the NMDA receptor. The increase in input resistance with activation of NMDA receptors counteracts the decrease in input resistance caused by activation of GABA\textsubscript{A} receptors, and the negative slope region of the IV curve persists (Figure 6B). This explains why spikes can still be produced in the balanced NMDA/GABA\textsubscript{A} configuration. Differences in input resistance measurements \textit{in vivo} (31M\textOmega; Grace and Bunney 1983) and in slices (approximately 200M\textOmega; 70-250 M\textOmega, Kita et al., 1986; 135 M\textOmega, Grace and Onn 1989; 384 M\textOmega, Paladini et al., 1999a) suggest that 27.3 nS of the total input conductance \textit{in vivo} (32.3nS measured \textit{in vivo} - 5nS measured in slices = 27.3nS) are due to tonic NMDA and GABA\textsubscript{A} conductances. We found that cells went into block at AMPA/GABA\textsubscript{A} conductances around 4nS and thus cannot account for 27.3 nS difference in input conductance. The 27.3 nS input conductance difference in the NMDA/GABA\textsubscript{A} configuration may be underestimated due the increase in input resistance with NMDAR activation. The drop in input resistance due to impalement with sharp electrode recordings will overestimate this difference in input conductance.
An imbalance in tonic inputs may drive the cell into hyperpolarization or depolarization block in some proportion of dopaminergic neurons (Dai and Tepper 1998; Grace et al., 2007). For example, silent cells are cells whose membrane potential (-65 to -70 mV; Grace and Bunney 1984a) is near the chloride reversal potential (-68 mV; Grace and Bunney 1985)). They represent the case where there is a tonic GABA<sub>A</sub> conductance sufficient to shunt intrinsic pacemaker currents (Grace et al., 2007) but lacks a strong enough NMDA conductance to return the cell to the spiking regime. Silent cells, like other shunting configurations, may still be able to produce spikes in response to input fluctuations.

A dopaminergic neuron firing mainly under the influence of a balance between excitatory and inhibitory inputs would be more sensitive to dynamic fluctuations in rate and gain of inputs within the basal ganglia network (Destexche et al 2003; Carvalho and Buonomano, 2009; Vogels and Abbott, 2009) and adopt a more irregular single-spike firing pattern, as normally seen in vivo (e.g. Paladini et al., 1999b).

_Tipping the Scales: Generating Pauses and Bursts_

NMDA receptors play an important role in the generation of bursts (Chergui et al., 1993,1994; Overton and Clark 1992; Tong et al., 1996; Zweifel et al., 2009; Deister et al., 2009). The simplest mechanism by which bursts are generated in vivo is phasic activation of NMDA receptors. However, in the presence of tonic synaptic conductances, this requires that the NMDA receptor currents overcome a substantially reduced input resistance (up to 90% from in vitro estimates) and an increased Mg<sup>2+</sup> block on the NMDA receptor (Paladini and Tepper 1999). These limitations can be overcome by an
alternative mechanism where bursting is triggered by disinhibition (e.g. Matsumoto and Hikosaka, 2007; Hong and Hikosaka 2008; Jhou et al., 2009).

Here we show that not only can bursts be produced by disinhibition but that disinhibition bursts have a greater firing frequency than bursts evoked by NMDA receptors alone. The mechanism by which disinhibition bursts are faster than excitation-only bursts is probably due to a more depolarized voltage in the balanced state as compared to spontaneous, single-spiking (Figure 5C). This depolarization is a result of the total current produced from the application of NMDA and $\text{GABA}_A$ conductances with an effective synaptic reversal potential that is a conductance-weighted average of $E_{\text{NMDA}}$ (0 mV) and $E_{\text{GABA}_A}$ (-60 mV). A depolarized membrane potential may inactivate A-type potassium channels (Liss et al., 2001; Khaliq and Bean 2008; Gentet and Williams 2007). The time to first spike measurement of Figure 5EII is influenced by both spike-generating sodium channels and A-type potassium channels, which play an important role in the determination of the interspike interval. However in control conditions, more sodium channels are available, which would overestimate the contribution of $I_A$. This suggests that A-type potassium channels are inactivated in the balanced configuration and may be responsible for the increase in burst frequency. Inactivation of A-type potassium channels would also increase single-spiking frequency (e.g. Liss et al., 2001). However, an increase is not seen here (e.g. Figure 3B) because the procedure by which we obtain the balanced configuration was designed to keep the firing rate similar to the control, spontaneous firing rate. These results also suggest that disinhibition bursts may be more precisely timed to rewarding stimuli than bursts by NMDA receptor activation alone.
Recent studies demonstrate that synaptic plasticity may enhance synaptically triggered burst firing and therefore underlie the acquisition of the burst response to a reward-predicting stimulus (Harnett et al., 2009). While changes in plasticity can be effected within minutes, changes in the balance between excitatory and inhibitory inputs are capable of modulating bursts quicker. Thus, scaling of intraburst firing rate can occur according to the probability of reward associated with a stimulus within a single trial (e.g. Morris et al., 2004) or on successive trials of a learning task (e.g. Pan et al., 2005).

Phasic increases in GABAergic drive activate postsynaptic GABA\textsubscript{A} and/or GABA\textsubscript{B} receptors to induce a pause in firing. The length of the pause would be proportional to the increase in drive. GABA\textsubscript{B} receptors would be expected to produce a longer pause than GABA\textsubscript{A} receptors and may be recruited by synaptic spillover (Galvan et al., 2006). This suggests that the role of GABA\textsubscript{B} receptors may not be only presynaptic. Pauses in firing may also be initiated or augmented by removal of excitation (Figure 4A, e.g. the STN).

The firing pattern of dopaminergic neurons is a behaviorally important signal necessary for normal reward learning (Schultz, 1998; Zweifel et al., 2009). Bursts and pauses in firing encode reward prediction error. Our results provide an extrinsic mechanism by which both reward-related responses could be evoked by phasic changes in GABAergic drive. These results suggest that the role of tonic GABAergic inhibition is not simply to suppress single-spiking and burst firing. On the contrary, the presence of tonic background inhibition allows for bidirectional changes in the firing rate and pattern
in dopaminergic neurons. Thus, GABAergic inhibition plays a fundamental role in the generation of the reward prediction error signal.
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Figure 1: Single-spiking is more sensitive to suppression by GABA<sub>A</sub>R activation than NMDAR-mediated burst firing.  A. A phasic burst (15Hz maximum burst frequency) was evoked in a perforated patch recording of an identified dopaminergic neuron by iontophoresis of glutamate (Glu; black bars beneath trace, top) in 25 μM NBQX. The GABA<sub>A</sub>R agonist isoguvacine (40 μM) was then applied to the bath (middle). Single-spike firing was completely suppressed while the burst frequency was decreased by 62%. The effect of isoguvacine was then reversed by subsequent application of the chloride channel blocker, picrotoxin (100 μM, bottom). B Summarized data show the percent inhibition of maximum burst frequency (dark circles), mean burst frequency (grey circles), and mean spontaneous firing frequency (white circles) from control frequencies after bath application of isoguvacine (1, 10, 40, and 100 μM). Numbers in parenthesis indicate n for each concentration. C. Application of a 40 nS NMDAR conductance using dynamic clamp elicits a burst (20 Hz max) in a whole cell somatic recording of an identified dopaminergic neuron (top). Application of a 2.8 nS GABA<sub>A</sub>R conductance (E<sub>GABA<sub>A</sub></sub>= -60 mV) suppresses single-spike activity while the burst remains (18 Hz max; bottom). Above each voltage trace is the truncated current applied from the dynamic clamp (downward represents an outward current). D. Summarized data (n=4) show the percent of inhibition of maximum burst frequency (dark circles), mean burst frequency (grey circles), and mean spontaneous firing frequency (white circles) to application of a series of GABA<sub>A</sub>R conductances in dynamic clamp (as in C).
Figure 2: Single-spike is more sensitive to GABA_BR activation than NMDAR-mediated burst firing. A. A phasic burst was evoked in a perforated patch recording of an identified dopaminergic neuron by iontophoresis of glutamate (Glu; black bars beneath trace, top) in 25 μM NBQX. Bath application of 1 μM baclofen abolished single-spike activity and reduced the burst firing frequency. This effect was reversible upon removal of baclofen (Return to ACSF). Spike heights are truncated. B. Summarized data for the percent of inhibition of maximum burst frequency (dark circles), mean burst frequency (grey circles), and mean spontaneous firing frequency (white circles) from control after bath application of baclofen (0.01, 0.1, 1, and 10 μM) Numbers in parenthesis represent number of cells. C. A representative recording showing inhibition of single-spike and burst firing by application of a 1.2 nS GABA_BR conductance (E_{GABA_B} = -100 mV). The total current (truncated) applied by the dynamic clamp is shown above each voltage trace. D. Summarized data (n=4) show the effectiveness of GABA_AR- and GABA_BR-mediated inhibition in suppressing maximum burst frequency (dark squares and circles, respectively) and mean spontaneous firing frequency (white squares and circles, respectively). Conductances were applied into the same group of cells.

Figure 3: Bursts and pauses can be generated by phasic changes in input. A. In a whole cell recording, balanced GABA_AR and NMDAR conductances were applied into a dopaminergic neuron. A disinhibition burst could be evoked by phasic removal of the GABA_AR conductance. A pause in firing could be evoked by phasic removal of the NMDAR conductance (A) or by a phasic increase in the GABA_AR conductance (C, increase of 2 nS). The total current applied by the dynamic clamp is shown above each voltage trace. The total current in C has been truncated. B. Single-spiking frequency in
the presence of tonic NMDAR and GABA_A conductances was not significantly different from spontaneous, single-spiking (Control; p > 0.05, paired t-test). Horizontal lines indicate the means for the sample. D. Single-spiking is not maintained with application of balanced GABA_A and AMPAR conductances.

Figure 4: Disinhibition bursts have a greater frequency than phasic NMDA bursts of the same conductance. A. Representative example of a burst produced by phasic activation of NMDA receptors (Control, top trace; g_GABA_A = 0; 18Hz max) and by disinhibition (bottom trace; removal of g_GABA_A =5.6 nS; 29 Hz max). The total current applied by the dynamic clamp is shown above each voltage trace. In both cases (control and disinhibition), an NMDA conductance of 20 nS was used. B. Summary data show a significant increase in maximum (I) and mean (II) burst frequencies of disinhibition bursts as compared to control bursts. Horizontal lines indicate the means for the sample. C. Summary data show that the interspike interval for disinhibition bursts was significantly shorter than control bursts for the first seven ISIs (*, p < 0.05).

Figure 5: Disinhibition bursts may be faster due to depolarization. A. Phase plots of spontaneous single-spiking (black) and balanced single-spiking (red). Threshold was defined as the voltage in which there was a significant break (4 times standard deviation) of dV_m/dt from baseline (illustrated in inset for control) and is shown on the control and balanced phase plots by black and red arrows, respectively. B. There was a significant decrease in maximum dV_m/dt (I), during balanced spiking. There was also a significant increase in minimum dV_m/dt (II) and spiking threshold (III) during balanced spiking. C. Overlay of all-points histograms for sampled membrane potentials during spontaneous firing (Control, black bars) and balance between excitation and inhibition (Balanced,
white bars). Black and white arrows represent the average membrane potential for Control and Balanced, respectively. The histograms were generated from 9 seconds of single spike firing in each configuration. 10 superimposed single spikes (aligned at the peak) are shown as an inset for Control (Black) and Balanced configurations (red). Scale bars for inset are 10 mV (ordinate) and 100 ms (abscissa). Dashed line in inset shows -40 mV. D. Average membrane potential for balanced spiking was more depolarized than control spiking. E. Overlay of control (black) and disinhibition (red) bursts (I; same bursts as shown in Figure 4A). The total current applied by the dynamic clamp is shown above each voltage trace. Summary data shows a significant decrease in the time to the peak of the first action potential (II) from application of an NMDAR conductance (Control, black) or removal of a GABA<sub>A</sub>R conductance (Disinhibition, red). Arrow indicates the time of conductance change. Horizontal lines indicate means for the sample in each panel.

**Figure 6: The high conductance state of the dopaminergic neuron.** The effect of tonic AMPA/GABA<sub>A</sub> (A) and NMDA/GABA<sub>A</sub> (B) conductances on the steady-state IV curve of a fictive dopaminergic neuron. Note that the negative slope region between -65 mV and -50 mV persists at much higher NMDA/GABA<sub>A</sub> conductances than AMPA/GABA<sub>A</sub> conductances. The thick black line represents the control condition where g<sub>AMPA</sub>, g<sub>NMDA</sub> and g<sub>GABAA</sub> are set to zero. The thin lines represent a 5nS increase in g<sub>AMPA</sub> (A) or g<sub>NMDA</sub> (B) and a 1.6 nS increase in g<sub>GABAA</sub>. Dashed arrows represent displacements of the IV curve as these conductances are increased.
A  AMPA/GABA_A

B  NMDA/GABA_A

Voltage (mV) vs Current