Tonic Firing Rate Controls Dendritic Ca^{2+} Signaling and Synaptic Gain in Substantia Nigra Dopamine Neurons

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Substantia nigra dopamine neurons fire tonically resulting in action potential backpropagation and dendritic Ca^{2+} influx. Using Ca^{2+} imaging in acute mouse brain slices, we find a surprisingly steep relationship between tonic firing rate and dendritic Ca^{2+} . Increasing the tonic rate from 1 to 6 Hz generated Ca^{2+} signals up to fivefold greater than predicted by linear summation of single spike-evoked Ca^{2+} -transients. This “ Ca^{2+} supralinearity” was produced largely by depolarization of the interspike voltage leading to activation of subthreshold Ca^{2+} channels and was present throughout the proximal and distal dendrites. Two-photon glutamate uncaging experiments show somatic depolarization enhances NMDA receptor-mediated Ca^{2+} signals $>400\ \mu\text{m}$ distal to the soma, due to unusually tight electrotonic coupling of the soma to distal dendrites. Consequently, we find that fast tonic firing intensifies synaptically driven burst firing output in dopamine neurons. These results show that modulation of background firing rate precisely tunes dendritic Ca^{2+} signaling and provides a simple yet powerful mechanism to dynamically regulate the gain of synaptic input.

Key words: action potential; backpropagation; calcium imaging; dendrite; dopamine; substantia nigra

Introduction

Dopaminergic neurons of the substantia nigra pars compacta (SNc) play a key role in movement and reward-based learning (Wise, 2004; Schultz, 2007). Critical to their function *in vivo*, dopamine neurons exhibit steady, tonic firing within a narrow range of 1–6 Hz (Grace and Bunney, 1984b; Hyland et al., 2002). During goal-directed behaviors, tonic firing is interrupted by brief, high-frequency bursts of action potentials (APs; Schultz et al., 1997; Schultz, 2006; Cohen et al., 2012) that at the cellular level are driven largely by excitatory synaptic inputs with a key role for NMDA receptors (NMDARs; Overton and Clark, 1992; Kuznetsov et al., 2006; Deister et al., 2009; Zweifel et al., 2009). More sustained increases in tonic firing occur in response to neuromodulators such as nicotine (Zhang et al., 2009; Li et al., 2011) or cannabinoids (French et al., 1997). It is believed that the tonic firing of dopamine neurons controls the resting concentration of dopamine in target areas (e.g., striatum) within a narrow range relative to the large, phasic dopamine signals associated with burst firing (Gonon, 1988). However, it is unknown whether the rate of tonic firing influences synaptically driven burst firing and how synaptic and intrinsic conductances respond to sustained increases in background tonic firing.

Action potential backpropagation provides a mechanism for ongoing somatic activity to influence dendritically located conductances (Stuart et al., 1997; Waters et al., 2005). Direct dendritic recordings in SNc neurons have shown that backpropagating APs (bAPs) are supported by a high-density of Na^+ channels and reliably invade the dendrites with little attenuation in amplitude (Häusser et al., 1995; Gentet and Williams, 2007). Successful backpropagation leads to dendritic Ca^{2+} influx which may trigger plasticity (Harnett et al., 2009) and the dendritic release of dopamine (Beckstead et al., 2004; Ford et al., 2010). Interestingly, carbon fiber experiments show that the somato-dendritic release of dopamine occurs in a steeply frequency-dependent manner when evoked by trains of tonic stimuli between 1 and 10 Hz (Rice et al., 1997). Therefore, it is possible that dendritic Ca^{2+} influx may exhibit a similar sensitivity to small changes in the tonic firing rate, however, this issue has not been examined.

Here we test the influence of tonic firing rate on dendritic Ca^{2+} signaling and synaptically driven burst firing in dopamine neurons using two-photon Ca^{2+} imaging. Increasing the tonic firing rate resulted in an unexpected supralinear increase in the dendritic Ca^{2+} signal. In addition, we find that burst firing in response to local stimulation of excitatory synapses is enhanced at elevated rates of tonic firing in an NMDAR-dependent manner. Therefore, these results reveal a previously unrecognized role of background firing rate in tuning the excitability of dendrites, dendritic Ca^{2+} signaling, and synaptic gain in dopamine neurons.

Materials and Methods

Slide preparation. Sagittal brain slices containing SNc were prepared from postnatal day 14–21 Swiss Webster mice of either sex. Animals were anesthetized with isoflurane and swiftly decapitated. Brains were quickly

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removed and placed in ice-cold slicing solution containing the following (in mM): 250 glycerol, 2.5 KCl, 2 MgCl_2 , 2 CaCl_2 , 1.2 NaH_2PO_4 , 10 HEPES, 21 NaHCO_3 , 5 glucose, bubbled with 95/5% O_2/CO_2 . Slices were cut (300 μm thick) and incubated for 30 min at 34°C in artificial CSF (ACSF) containing the following (in mM): 125 NaCl, 25 NaHCO_3 , 1.25 NaH_2PO_4 , 3.5 KCl, 1 MgCl_2 , 2 CaCl_2 , and 10 glucose, bubbled with 95/5% O_2/CO_2 . Slices were then stored at room temperature until time of use.

Electrophysiological recording. Slices were placed into a recording chamber and perfused continuously with warm ACSF ($32\text{--}34^\circ\text{C}$). Dopamine neurons were targeted primarily by their location within the SNc. Other criteria included the presence of slow pacemaking (<5 Hz) during cell-attached or whole-cell recordings, broad APs (halfwidth >1.35 ms) and prominent voltage sag in response to negative current injection, associated with hyperpolarization-activated cation current (I_H). Current-clamp and voltage-clamp recordings were made with a Multiclamp 700B amplifier and digitized with a Digidata 1440A (Molecular Devices). Low-resistance patch electrodes (2–4 $\text{M}\Omega$) were pulled from filamented borosilicate glass. In voltage-clamp recordings, pipette series resistance was compensated by 70–80% and was monitored throughout the experiment. Recordings were terminated if series resistance changed by $>20\%$. Current-clamp recordings were bridge balanced and monitored frequently throughout the experiment. Accurate bridge balance was achieved by adjusting the resistance of the Multiclamp 700B bridge circuit to minimize the instantaneous voltage change in response to a negative current injection. Internal recording solution contained the following (in mM): 122 K methanesulfonate, 9 NaCl, 1.8 MgCl_2 , 4 Mg-ATP, 0.3 Na-GTP, 14 phosphocreatine, 10 HEPES, 0.3 Fluo-5F ($K_D \sim 2.3$ μM) and 0.05 AlexaFluor 594 hydrazide adjusted to a pH value of 7.35 with NaOH.

Two-photon laser scanning microscopy and glutamate uncaging. Imaging experiments were performed using a custom two-photon microscope from Prairie Technologies along with a Mai Tai ultrafast Ti:Sapphire laser (Spectra-Physics) tuned to 810 nm. Cells were imaged using a $40\times$, 0.8 NA objective (Olympus). Fluorescence was split into red and green channels using a 575 nm dichroic longpass mirror and passed through 607/45 nm and 525/70 nm barrier filters before being detected by multi-alkali photomultiplier tubes (Hamamatsu). Ca^{2+} imaging was initiated 15–20 min after whole-cell break in. Line-scan imaging of dendritic Ca^{2+} was performed at 30 s intervals. Ca^{2+} imaging data are presented as G/G_s and were quantified as changes in green fluorescence divided by red fluorescence (G/R), normalized to $G_s/R \times 100\%$. G_s/R was measured by imaging a pipette filled with internal recording solution plus saturating Ca^{2+} (2 mM CaCl_2) placed directly above the slice at the end of experiment (Yasuda et al., 2004).

Simultaneous glutamate uncaging experiments were performed using a second Mai Tai laser tuned to 725 nm with an uncaging pulse width of 500 μs . External solutions for glutamate uncaging experiments contained 3 mM 4-methoxy-7-nitroindolyl-caged-L-glutamate (MNI-glutamate; Tocris Bioscience), 500 nM TTX, 10 μM nifedipine and 10 μM D-serine to prevent NMDAR rundown. Solutions were recirculated to conserve MNI-glutamate (volume = 10 ml).

Local extracellular stimulation. Local stimulation of dendritically located synapses was performed using bipolar electrodes placed in theta glass pipettes filled with ACSF (tip diameter ~ 5 μm). Electrodes were placed within 10 μm of an AlexaFluor 594-labeled dendrite. To increase the tonic firing rate, stimulation was performed at 20 Hz for 6 s and dendritic Ca^{2+} was imaged during the final 3 s of stimulation. To evoke bursts of action potentials, stimulation was performed at 100 Hz for 200 ms. Stimulus intensity was set to 15–45 V with 0.5 ms pulse duration using an Iso-Flex stimulus isolator (AMPI). Picrotoxin (50 μM), CGP 55845 hydrochloride (1 μM), sulpiride (1 μM), SCH 39166 hydrobromide (1 μM), and LY 341495 (1 μM) were added to ACSF to block activity of GABA_A, GABA_B, D₂ dopamine, D₁ dopamine, and group II metabotropic glutamate receptors.

Data analysis. Ca^{2+} imaging data were quantified using ImageJ to measure fluorescence intensities and further analyzed using custom routines written in Igor Pro (Wavemetrics). The maximal amplitude of AP-evoked Ca^{2+} transients was measured as the average value in a 5 ms

window around the peak Ca^{2+} signal. AP-evoked Ca^{2+} transients were fit by the equation:

$$\text{AP-evoked } \text{Ca}^{2+} = A_1(1 - e^{-(t-t_0)/\tau_{on}})(e^{-(t-t_0)/\tau_1}) + A_2(1 - e^{-(t-t_0)/\tau_{on2}})(e^{-(t-t_0)/\tau_2})$$

The amplitude of Ca^{2+} signals evoked by a burst of APs was measured as the average signal intensity during the last 100 ms of a 300 ms current injection. Z-stacks of the cell were acquired at the end of each experiment. A maximum intensity projection was used to measure the distances of dendritic Ca^{2+} imaging from the soma.

Nonspike voltage was taken as an average of the middle 60% of the interspike interval. All statistics are given as mean $\pm$ SEM. The liquid junction potential between the internal solution and bath solution measured -8 mV. The reported voltages have not been junction potential corrected.

Results

Reliable backpropagation into dendrites during pacemaking in dopaminergic neurons

SNc neurons recorded in brain slices fire spontaneously at low rates of 1–4 Hz, called “pacemaking,” resulting in rhythmic backpropagation of APs throughout the dendrites. We tested the effect of bAPs during pacemaking on dendritic Ca^{2+} signaling in SNc neurons. Previous work in CA1 pyramidal neurons indicates that dendritic Ca^{2+} transients mostly reflect the height of bAPs rather than changes in spike width (Magee and Johnston, 1997; Gasparini et al., 2007). Using two-photon laser scanning microscopy, we imaged dendritic Ca^{2+} influx by filling SNc neurons with a Ca^{2+} -sensitive dye, Fluo 5F, loaded into the patch pipette. In proximal dendrites (<100 μm), a clearly definable peak in the dendritic Ca^{2+} signal accompanied each somatic AP (Fig. 1A,B). The one-to-one correlation between somatic APs and dendritic Ca^{2+} transients was observed throughout every recording during pacemaking. A similar observation was made in more distal dendritic regions (Fig. 1A,C). Measuring dendritic Ca^{2+} transients in a population of cells (31 dendrites, 20 cells), the average AP-evoked Ca^{2+} signal attenuated little with distance, even beyond 400 μm away from the soma (Fig. 1D). Therefore, together with previous work using direct dendritic recordings (Häusser et al., 1995; Gentet and Williams, 2007), our results show that APs backpropagate reliably throughout the distal dendrites during low-frequency tonic firing in SNc neurons.

Supralinear dendritic Ca^{2+} -influx during synaptically driven increases in firing rate

Backpropagating action potentials increase dendritic Ca^{2+} in a rate-dependent manner that may be critical for Ca^{2+} -dependent processes, such as the somatodendritic release of dopamine (Rice et al., 1997; Beckstead et al., 2004). We measured backpropagation-evoked dendritic Ca^{2+} signals over a range of firing rates (0.6–6.2 Hz), using local stimulation of excitatory synaptic inputs to increase the rate of tonic firing. A potential confounding factor in this approach is that stimulation of excitatory synaptic inputs will result in NMDA receptor-mediated Ca^{2+} influx which may complicate the interpretation of dendritically recorded Ca^{2+} signals. Therefore, to examine the influence of firing rate on intrinsic conductances alone, we recorded Ca^{2+} signals in a dendrite contralateral to the site of stimulation where the contribution from synaptic Ca^{2+} sources is likely to be small (Fig. 2A). Excitatory synaptic inputs were stimulated for 6 s at 20 Hz and the contralateral dendrite was imaged during the final 3 s of the stimulus train when firing had settled to a relatively constant rate. We then tested firing at higher rates in interleaved trials

Figure 1. Backpropagating action potentials evoke Ca^{2+} signals throughout the dendrites of SNc dopamine neurons during pacemaking. **A**, Maximum intensity projection of a typical SNc dopamine neuron visualized using AlexaFluor 594. Sites of linescans are indicated by colored bars across the dendrite. **B**, Example linescan and dendritic Ca^{2+} signal measured at location of the red bar in **A** and simultaneously recorded somatic voltage. **C**, AP-evoked dendritic Ca^{2+} transients measured at multiple distances from the soma. Single Ca^{2+} transients are shown in gray. The average AP-evoked Ca^{2+} signals are colored according to bars shown in **A**. **D**, Plot of amplitudes of AP-evoked Ca^{2+} transients against distances from the soma. Data for individual dendrites are plotted in gray. Binned averages and SEM are plotted in red.

by adjusting the voltage of pulses delivered through our local stimulation electrode.

Figure 2D shows examples of dendritic Ca^{2+} signals recorded both during spontaneous firing, and after low and high-frequency stimulation. In this cell, we found that a relatively small increase of the firing rate from 2 to 4.1 Hz led to an unexpectedly large increase in the average the dendritic Ca^{2+} signal. A moderate increase in the recorded Ca^{2+} signal is to be expected due to

temporal summation of Ca^{2+} at higher firing rates. To estimate the amount of cytoplasmic calcium that is expected to accumulate during high-frequency tonic firing (Helmchen et al., 1996; Scheuss et al., 2006), we constructed linear predictions of the Ca^{2+} signals at low and high tonic frequencies.

To generate the predicted Ca^{2+} signals, we fit AP-evoked Ca^{2+} signals imaged during spontaneous firing (0.7–2.4 Hz), both rising and decay phases, with bi-exponential functions (Fig. 2C, inset). Next, we constructed predicted Ca^{2+} signals based on linear summation of the fit, where the placement of fits matched the timing of somatic APs (Fig. 2C, black traces). Using this method, the predicted Ca^{2+} signal represents the signal expected in the absence of backpropagation failures (Koester and Sakmann, 2000; Brenowitz and Regehr, 2007).

Surprisingly, at elevated tonic firing rates, dendritic Ca^{2+} signals were substantially larger than predicted signals. In the example cell shown in Figure 2, raising the firing rate just slightly from 2.0 to 4.1 Hz, the measured Ca^{2+} signal increased to values >2.5 times larger than the predicted Ca^{2+} signal (Fig. 2C,D). Across a population of cells, dendritic Ca^{2+} signals during tonic firing >3 Hz (3–6.2 Hz) were 3.90 ± 0.51 -fold greater than predicted based on linear predictions (Fig. 2E,F; paired *t* test, measured vs predicted 2.5–3.5 Hz, $p < 1e-5$; 3.5–4.5 Hz, $p < 1e-5$; 4.5–6.2 Hz, $p < 1e-5$; $n = 10$ –18 measurements, 17 dendrites). Therefore, Ca^{2+} influx during tonic firing at elevated rates exceeded linear predictions, and thus increases “supralinearly.”

To test our assumption that synaptic Ca^{2+} influx contributed minimally to signals recorded in the contralateral dendrites, we measured Ca^{2+} influx in response to synaptic stimulation in cells voltage-clamped at -50 mV. Synaptic stimulation evoked substantial Ca^{2+} influx into the region of the ipsilateral dendrite nearest the stimulating electrode (Fig. 2G, green trace), whereas there was no detectable Ca^{2+} influx into the contralateral dendrite (Fig. 2G, purple trace). Summary data (Fig. 2H) show that the magnitude synaptic Ca^{2+} influx falls off rapidly with distance from the stimulating electrode. Therefore, these data suggest that Ca^{2+} supralinearity we observe is likely due to intrinsic rather than synaptic conductances.

Supralinear dendritic Ca^{2+} -influx arises from intrinsic conductances throughout the dendrite

As an alternative approach to test whether fast tonic firing results in supralinear Ca^{2+} influx originating from intrinsic conductances, we altered the tonic firing rate by adjustment of holding current injected via the somatic patch pipette (Fig. 3A). We fit AP-evoked Ca^{2+} signals imaged during slow tonic firing (<2.0 Hz) as before (Fig. 3B), and constructed predicted Ca^{2+} signals by linear summation of the fit according the timing of APs (Fig. 3C). During low-frequency tonic firing (<2.0 Hz), there was excellent agreement between the values from measured and predicted signals averaged over 3 s (paired *t* test, $p = 0.11$, $n = 32$), indicating that the predicted values based on fits were accurate.

As with synaptic stimulation, dendritic Ca^{2+} signals were substantially larger than predicted signals at elevated tonic firing rates. In the example cell shown in Figure 3, the measured Ca^{2+} signal during 1.6 Hz firing was almost identical to the predicted signal. However, raising the firing rate just slightly to 3.4 Hz, the measured Ca^{2+} signal increased to a value >2.5 times larger than the predicted Ca^{2+} signal (Fig. 3C,D). Across a population of cells, dendritic Ca^{2+} signals during tonic firing >3 Hz (3–6.0 Hz) were 2.86 ± 0.19 -fold greater than linear predictions (Fig. 2E,F; paired *t* test, measured vs predicted, 2.5–3.5 Hz, $p < 1e-5$; 3.5–4.5 Hz, $p < 1e-5$; 4.5–6 Hz, $p = 0.00014$; $n = 10$ –24

measurements, 28 dendrites). Therefore, supralinear Ca^{2+} influx during tonic firing at elevated rates does not require synaptic stimulation and results from the intrinsic conductances of SNc dopamine neurons.

To ensure that this result was not biased by the injection of somatic current, we tested for the presence of the Ca^{2+} supralinearity in cells that fired spontaneously at high rates. We found a moderate positive correlation between the ratio of measured/predicted Ca^{2+} and spontaneous firing rate (Pearson's $r = 0.53418$; $p = 0.0034$; $n = 28$). We also found a significant difference between measured and predicted values for cells that fire spontaneously >2.5 Hz (G/G_S , measured vs predicted, $10.6 \pm 2.7\%$ vs $3.8 \pm 0.6\%$, $n = 6$, $p = 0.041$ paired t test).

Last, we tested for the presence of the Ca^{2+} supralinearity at distances throughout the dendrites (42–275 μm from the soma). Interestingly, we found robust Ca^{2+} supralinearity during fast tonic firing throughout the entire dendritic tree (Fig. 3*G,H*). The ratio of measured/predicted Ca^{2+} was 2.75 ± 0.25 ($n = 21$) in the proximal dendrites (<100 μm one sample t test vs 1, $p < 1e-5$), 2.71 ± 0.46 ($n = 9$) in the intermediate (100–200 μm ; $p < 1e-5$) and 2.37 ± 0.19 ($n = 4$) in the distal dendrites (200–275 μm ; $p = 0.0054$). Therefore, these data demonstrate that Ca^{2+} influx increases supralinearly with changes in firing rate, and that the Ca^{2+} supralinearity is present throughout the dendrites of SNc dopamine neurons.

Supralinear Ca^{2+} signaling requires subthreshold depolarization

Two major sources of Ca^{2+} entering SNc neurons are AP-evoked Ca^{2+} and Ca^{2+} entering at subthreshold voltages. Studies in dissociated neurons show that $\sim 80\%$ of the Ca^{2+} influx during pacemaking occurs during APs (Puopolo et al., 2007). Therefore, we tested the possibility that AP-evoked Ca^{2+} contributes to the frequency-dependent Ca^{2+} supralinearity. We held neurons steadily at -65 mV with negative current to prevent activation of low-threshold Ca^{2+} channels (Fig. 4). We then delivered short current steps to evoke APs over a range of frequencies (2–10 Hz) while recording dendritic Ca^{2+} .

Interestingly, we found little difference between measured and predicted signals when APs were evoked from fixed potentials (Fig. 4*A*). Across cells, we averaged Ca^{2+} signals near steady-state (last 0.3 s of train) then plotted these values against the

Figure 2. Fast tonic firing evoked by synaptic stimulation results in a supralinear increase in dendritic Ca^{2+} signals. **A**, Example neuron imaged by AlexaFluor 594 (red) overlaid with Dodt contrast image to visualize placement of stimulation electrode. **B**, Somatically recorded firing during pacemaking, and after low and high intensity stimulation at 20 Hz for 6 s. Stimulus artifacts have been digitally removed for clarity. **C**, Predicted linear Ca^{2+} signals for firing patterns shown in **B**. Inset, Average spontaneous AP-evoked Ca^{2+} transient and fit (black trace) used to generate linear predictions. Blue lines indicate the average values for predicted waveforms. **D**, “Measured” dendritic Ca^{2+} signals imaged during firing shown in **B**. Red lines indicate the average value of measured Ca^{2+} signal. **E**, Summary plot of measured (red) and predicted (blue) Ca^{2+} signals against the frequency of tonic firing. Light symbols are individual points; dark symbols are averages and SEM binned according to frequency. The slope of the line of best fit for measured Ca^{2+} versus frequency (red line) was 6.2% G/G_S per Hz. The slope of the line for predicted Ca^{2+} versus frequency (blue line) was 1.8% G/G_S per Hz. **F**, Plot of the ratio of measured Ca^{2+} to predicted Ca^{2+} against firing rate. Gray lines represent data from individual dendrites. Black symbols represent averaged data for all dendrites binned according to frequency. **G**, Synaptic Ca^{2+} influx recorded near the site of stimulation (green) and on a contralateral dendrite (purple) in response to synaptic stimulation of a voltage-clamped cell (-50 mV). Average synaptic currents are shown as black traces. **H**, Plot of amplitude of synaptic Ca^{2+} influx versus distance from the stimulation electrode for both ipsilateral (green) and contralateral (purple) dendrites. Gray lines connect points from same cell. Black line is exponential fit for all points; length constant = 20.1 μm .

Figure 3. Fast tonic firing evoked by somatic current injection results in a supralinear increase in dendritic Ca^{2+} signals. **A**, Example traces of tonic firing. Rates were adjusted with holding current. **B**, AP-evoked Ca^{2+} signals (93 μm from soma) during 1.6 Hz tonic firing were averaged and fit with 2 rising and 2 falling exponentials (individual and averaged traces shown in gray and red, fit shown in black). Somatic APs are shown below. **C**, Predicted Ca^{2+} signals (black) were generated for the firing rates displayed in **A**. Blue lines represent the average values of the predicted Ca^{2+} signals. **D**, Dendritic Ca^{2+} signals (black) recorded at the firing rates displayed in **A**. Red lines indicate the average Ca^{2+} signal over the course of the scan. **E**, Summary plot of measured (red) and predicted (blue) Ca^{2+} signals against the frequency of tonic firing. Light symbols are individual points; dark symbols are averages and SEM binned according to frequency. Lines are lines of best fit to each dataset. The slope of the line of best fit for measured Ca^{2+} versus frequency was 3.7% G/G_s per Hz. The slope of the line for predicted Ca^{2+} versus frequency was 1.5% G/G_s per Hz. Predicted data varies from the line of best fit due to variability in the size and kinetics of the AP-evoked Ca^{2+} transients between cells. **F**, Plot of the ratio of measured Ca^{2+} to predicted Ca^{2+} against firing rate. Gray lines represent data from individual dendrites. Black line and symbols represent averages and SEM for all dendrites binned according to frequency. **G**, Plot of the average ratio of measured Ca^{2+} to predicted Ca^{2+} for rates > 3 Hz against the distance from the soma at which dendritic Ca^{2+} was measured. Gray points represent individual recordings; black symbols represent averages binned according to distance. **H**, Example of a cell in which supralinear dendritic Ca^{2+} signaling was measured at multiple distances from the soma. Colored bars represent sites of line scans and correspond to points plotted in **G**.

firing rate (Fig. 4B). There was close agreement between measured and predicted Ca^{2+} signals when APs were evoked from fixed voltages (student's paired t test: 4 Hz, $p = 0.39$; 5 Hz, $p = 0.48$; 6 Hz, $p = 0.88$; 8 Hz, $p = 0.55$; 10 Hz, $p = 0.88$, $n = 3$ –10 for each frequency, 11 dendrites, 8 cells). The ratio of these values remained near 1 across all frequencies tested (one sample t test, ratio of measured/predicted Ca^{2+} , 4 Hz, $p = 0.45$; 5 Hz, $p = 0.48$; 6 Hz, $p = 0.67$; 8 Hz, $p = 0.61$; 10 Hz, $p = 0.82$, $n = 3$ –10, 11 dendrites, 8 cells; Fig. 4C). Ca^{2+} signaling was linear during evoked spiking in both proximal and distal dendrites (Fig. 4D). Therefore, the frequency-dependent Ca^{2+} supralinearity was completely abolished when APs were evoked from fixed membrane potentials and was unlikely due to AP-evoked Ca^{2+} .

Response of dendritic Ca^{2+} to subthreshold depolarization of the soma

Because the Ca^{2+} supralinearity was abolished when APs were evoked from a fixed membrane voltage, we predicted that activation of low-threshold Ca^{2+} channels is critical to the Ca^{2+} supralinearity. We hypothesized that increases in firing rate are accompanied by membrane depolarization and activation of subthreshold Ca^{2+} channels. Consistent with this idea, increasing the firing rate from 1 to 5 Hz led to depolarization of the nonspike membrane voltage by 8.0 ± 1.7 mV (average voltage at 0.5–1.5 Hz, -59.4 ± 0.8 mV; 4.5–5.5 Hz, -51.4 ± 0.9 mV; $p = 8.3 \times 10^{-8}$, $n = 14$ neurons, unpaired t test (Fig. 5A,B). We observe a similar depolarization when comparing the average nonspike voltage at the lowest and highest frequencies measured for each cell (-60.4 ± 1.0 mV at 0.97 ± 0.1 Hz vs -52.1 ± 0.7 mV at 4.6 ± 0.2 Hz, $p = 4.5 \times 10^{-9}$, $n = 14$, paired t test).

The depolarization observed at high rates occurs in the subthreshold voltage range, where Ca^{2+} conductances are small in amplitude (Durante et al., 2004; Puopolo et al., 2007). Therefore, we asked whether these relatively modest subthreshold depolarizations produce substantial Ca^{2+} influx. We blocked spiking with application of 500 nM TTX and delivered a family of somatic voltage steps covering the subthreshold voltage range (from -70 mV). Comparing voltages that roughly correspond to the depolarization observed between low- and high-frequency firing, we observed significantly more Ca^{2+} influx during a step to -50 mV than to -60 mV ($\Delta\text{G}/\text{G}_s$, $14.2 \pm 1.44\%$ at -50

mV vs $3.81 \pm 0.721\%$ at -60 mV, $n = 4$, $p = 0.002$, paired t test; Fig. 5C,D). This demonstrates that substantial Ca^{2+} influx results from subthreshold depolarization, even in the absence of APs.

This observation is in accord with the known expression of $\text{Ca}_v1.3$ L-type Ca^{2+} channels, which display an atypically hyperpolarized voltage dependence of activation and are steeply voltage-dependent within the subthreshold voltage range (Xu and Lipscombe, 2001). Accordingly, the L-type Ca^{2+} channel antagonist nifedipine ($10 \mu\text{M}$) dramatically decreased the amplitude of dendritic Ca^{2+} signals, resulting in a $72.1 \pm 4.3\%$ block of signals evoked by voltage steps from -60 to -50 mV ($n = 4$; Fig. 5E,F). These results show that in SNc dopamine neurons, relatively modest depolarizations of the soma lead to substantial dendritic Ca^{2+} influx through activation of L-type Ca^{2+} channels.

Ca^{2+} supralinearity is due to L-type Ca^{2+} channels

Based on our results, it is likely that L-type Ca^{2+} channels contribute to the Ca^{2+} supralinearity observed during fast tonic firing in SNc dopamine neurons. To test this, we pretreated slices with $10 \mu\text{M}$ nifedipine for 10–20 min ($n = 5$) or acutely applied $1 \mu\text{M}$ nifedipine ($n = 5$) and recorded dendritic Ca^{2+} signals during tonic firing across a range of frequencies (Fig. 6). Focusing first on the somatic membrane potential, depolarization of the nonspike voltage was similar in magnitude to control cells (-57.0 ± 0.75 mV at 0.5 – 1.5 Hz vs 49.7 ± 1.0 mV at 4.5 – 5.5 Hz; $p = 8.0 \times 10^{-5}$, $n = 5$; Fig. 6A). Across all cells, we found a modest reduction (29.7% decrease) in AP-evoked Ca^{2+} signals in the presence of nifedipine ($5.8 \pm 0.5\%$ vs $4.1 \pm 0.7\%$, control vs nifedipine, $p = 0.044$, unpaired t test). By contrast, in the presence of nifedipine total Ca^{2+} signals at elevated firing rates (3.5 – 4.5 Hz) were less than half the size of corresponding control Ca^{2+} signals ($15.8 \pm 1.5\%$ vs $7.7 \pm 1.7\%$, $p = 0.00015$, unpaired t test; Fig. 6E). Furthermore, Ca^{2+} signals measured in the presence of nifedipine more closely resembled linear predictions (Fig. 6C–F). The ratio of the measured/predicted Ca^{2+} signals was significantly reduced in the presence of nifedipine (control = 2.86 ± 0.19 , $n = 34$; nifedipine = 1.88 ± 0.18 , $n = 10$; $p = 0.011$, unpaired t test). For acute application experiments, the ratio of measured/predicted Ca^{2+} was reduced by $44.6 \pm 3.3\%$ (measured/predicted ratio at >3 Hz, control, 4.1 ± 0.5 vs nifedipine, 2.2 ± 0.2 , $n = 5$; $p = 0.0069$; Fig. 6C–G). Altogether, these results suggest that L-type channels are a primary source of the Ca^{2+} supralinearity.

It is possible that AP broadening contributes to additional dendritic Ca^{2+} influx as was shown in cortical pyramidal neu-

Figure 4. Linear dendritic Ca^{2+} signaling when APs are evoked from a fixed membrane potential. **A**, Example of measured (red) and predicted (black) dendritic Ca^{2+} signals (top), somatic voltages (middle), and current injections (bottom) to elicit spiking at 2, 4, and 6 Hz from a fixed membrane potential (-65 mV). Dashed line in Ca^{2+} recordings indicates baseline Ca^{2+} level. Dashed line in somatic voltage recordings indicates -60 mV. **B**, Plot of measured and predicted Ca^{2+} signals against firing rate. Points represent average and SEM, and lines are of best fit to all individual data points (individual data points not shown for clarity). Black symbols represent average Ca^{2+} signals measured during tonic firing (replotted from Fig. 3E). Red and blue symbols represent measured and predicted Ca^{2+} signals when APs were evoked from a fixed membrane potential predictions. The slopes of lines of best fit are as follows: control, $3.7\% \text{ G}/\text{G}_s$ per Hz; fixed V_m measured, $1.4\% \text{ G}/\text{G}_s$ per Hz; fixed V_m predicted, $1.6\% \text{ G}/\text{G}_s$ per Hz. **C**, Plot of the ratio of measured Ca^{2+} signals to predicted Ca^{2+} signals against firing rate. Black points represent averages and SEM for Ca^{2+} signals measured during tonic firing (replotted from Fig. 3F). Pink lines represent data for individual dendrites when firing was evoked from a fixed membrane voltage. Red points represent averages and SEM at each frequency. **D**, Plot of the average ratio of measured and predicted Ca^{2+} signals at firing rates >3 Hz measured in proximal and distal dendrites during tonic firing (gray) or when APs were evoked from fixed voltages (pink). Error bars are SEM.

rons (Kampa and Stuart, 2006). Indeed, we observe a small increase in AP width at elevated tonic firing rates. However, the extent of AP broadening was comparable in control (AP width at half-max, 1.7 ± 0.05 ms at <1.5 Hz vs 2.2 ± 0.1 ms at >4 Hz, $n = 14$; $p = 0.001$) and nifedipine-treated cells (1.8 ± 0.07 ms at <1.5 Hz vs 2.4 ± 0.2 ms at >4 Hz, $n = 5$; $p = 0.012$). Because nifedipine treatment did not completely abolish the Ca^{2+} supralinearity, it is possible that spike broadening may partially contribute. However, these results are consistent with the idea that AP broadening is not the primary mechanism of supralinear dendritic Ca^{2+} signaling in SNc dopamine neurons.

Dopamine neurons of the ventral tegmental area (VTA) display pacemaking similar to dopamine neurons in the SNc. However, in VTA dopamine neurons, the subthreshold currents that

Figure 5. Subthreshold depolarization of somatic V_m increases dendritic Ca^{2+} . **A**, Example of somatic voltage recordings at a low (1.4 Hz) and high (5.4 Hz) rates of firing. Dashed lines represent average threshold of AP generation and average trough. Solid lines indicate average nonspike membrane voltage. **B**, Plot of the average nonspike membrane voltage against tonic firing rate for individual cells (gray) and average values ± SEM for all cells binned according to frequency (black). **C**, Example of dendritic Ca^{2+} signals (top) recorded in response to somatic voltage steps from -70 mV (bottom). **D**, Plot of dendritic Ca^{2+} influx versus voltage for four dendrites (gray) and average signals ± SEM (black). Gray box represents subthreshold voltage range. **E**, Example of Ca^{2+} signals recorded in response to a voltage step from -60 to -50 mV before and after application of nifedipine. **F**, Amplitudes of sustained dendritic Ca^{2+} signals evoked by a voltage step from -60 to -50 mV before and after application of nifedipine.

drive spontaneous activity are mediated primarily by Na^+ conductances rather than Ca^{2+} conductances (Khaliq and Bean, 2010). Therefore, we predicted that Ca^{2+} signaling in dopamine neurons of the VTA would display a more linear relationship with firing rate than those of the SNc. In the VTA, measured Ca^{2+} signals closely resembled linear, predicted signals (paired t test of measured vs predicted Ca^{2+} signals: 1–2 Hz, $p = 0.32$; 2–3 Hz, $p = 0.88$; 3–4 Hz, $p = 0.63$; >4 Hz, $p = 0.10$; $n = 3$ –9 for each frequency, 8 dendrites). Furthermore, the ratio between measured and predicted signals at elevated rates (>3 Hz) was significantly smaller in dopamine neurons of the VTA compared with those of the SNc (Fig. 6H) (ratio for SNc, 2.86 ± 0.19 , $n = 28$; VTA, 1.53 ± 0.25 , $n = 8$; $p = 0.0025$, unpaired t test). These data further suggest that supralinear Ca^{2+} signaling in the SNc is due to activation of low-threshold Ca^{2+} channels as tonic firing rate increases.

Past work in dopamine neurons has shown that bAPs lead to Ca^{2+} -induced Ca^{2+} release from internal stores (Cui et al., 2007), which raises the question of whether Ca^{2+} stores contrib-

ute to the supralinearity shown here. To test this, we measured changes in dendritic Ca^{2+} signaling with increases in tonic firing rate following application of cyclopiazonic acid (CPA) to inhibit the sarcoendoplasmic reticulum Ca^{2+} -ATPase (SERCA). Consistent with past studies (Cui et al., 2007), we observed a reduction in AP-evoked Ca^{2+} influx at low rates of tonic firing (<2 Hz) following application of CPA (35 ± 7.3% reduction in peak, $n = 7$). However, at elevated firing rates, we still observed dramatic, supralinear increases in total dendritic Ca^{2+} signals, comparable to control conditions. As a result, we observed an increase in the ratio of measured/predicted Ca^{2+} influx following application of CPA (Fig. 6H) (2.84 ± 0.43 to 4.54 ± 0.44 , $p = 0.038$, paired t test). This is possibly because in addition to blocking Ca^{2+} -induced Ca^{2+} release, inhibition of SERCA pumps impairs buffering of cytosolic Ca^{2+} during ongoing neuronal activity. Regardless, the presence of the Ca^{2+} supralinearity following antagonism of SERCA pumps demonstrates that Ca^{2+} -induced Ca^{2+} release is not required for frequency-dependent Ca^{2+} supralinearity.

Supralinear dendritic Ca^{2+} signaling during burst firing

Dopamine neurons fire phasic high-frequency bursts of APs (>10 Hz) in response to reward-relevant stimuli (Grace and Bunney, 1984a; Overton and Clark, 1997; Schultz et al., 1997). We next asked whether SNc neurons display supralinear dendritic Ca^{2+} signaling during burst firing (Fig. 7). Bursts were evoked by current injections (100–200 pA) on a background of tonic firing (average burst rate, 21.3 ± 1.2 Hz, $n = 8$). Ca^{2+} signals recorded during the tonic firing preceding the burst

were used to generate linear predictions of dendritic Ca^{2+} signals (Fig. 7A). Burst-evoked Ca^{2+} signals were similar in amplitude throughout the dendrite (Fig. 7B). Furthermore, Ca^{2+} influx evoked by burst firing was consistently supralinear at all distances tested (Fig. 7C,F; ratio of measured/predicted signals = 1.41 ± 0.08 , $n = 8$).

Generation of bursts *in vivo* takes place in response to excitatory synaptic input and may involve ionic mechanisms distinct from those mediating bursts evoked by somatic current injection (Paladini and Roeper, 2014). Therefore, we imaged dendritic Ca^{2+} signals during stimulation-evoked burst firing. Stimulation of excitatory synaptic inputs at 100 Hz for 200 ms on a background of tonic firing generated transient, high-frequency burst firing (average burst rate, 15.4 ± 0.6 Hz, $n = 6$) and concurrent subthreshold depolarization (Fig. 7D). Measurement of Ca^{2+} influx in the contralateral dendrites revealed that backpropagation of synaptically evoked bursts produces supralinear Ca^{2+} influx (ratio of measured/predicted signals = 1.77 ± 0.16 ; $n = 6$; Fig. 7D,F).

As with tonic firing, the Ca^{2+} supra-linearity during burst firing may result from subthreshold depolarization and activation of L-type channels. Consistent with this hypothesis, bursts evoked from a fixed potential (-65 mV; 20 or 25 Hz) generated Ca^{2+} signals that were not supralinear (ratio of measured/predicted $\text{Ca}^{2+} = 0.87 \pm 0.06$, $n = 9$, $p = 8.5e - 5$, unpaired t test vs control; Fig. 7E,F). Interestingly, Ca^{2+} signals in six of nine cells were sublinear, indicating that bAP failures may occur during high-frequency bursting. bAPs during high-frequency bursting in CA1 pyramidal cells have been shown to fail due to accumulated Na^+ channel inactivation (Jung et al., 1997). Last, nifedipine significantly reduced the supra-linearity observed during current-evoked bursts (Fig. 7F; ratio of measured/predicted signals: 1.08 ± 0.05 , $n = 7$; $p = 0.0052$, unpaired t test vs step current-evoked bursts). Therefore, much like fast tonic firing, phasic bursts lead to supralinear Ca^{2+} influx in the dendrites by activation of low-threshold L-type Ca^{2+} channels.

Strong electrotonic coupling of soma and dendrites

Motivated by the observation that small somatic depolarizations evoke significant dendritic Ca^{2+} influx (Fig. 4), we next tested the strength and reach of somato-dendritic coupling in dopamine neurons. Past experiments using direct dendritic recordings (Häusser et al., 1995) suggest that tight electrotonic coupling exists between soma and dendrites of SNc neurons. As an alternative approach, we assayed the effect of the somatic membrane potential on synaptic conductances located along the dendrite using simultaneous two-photon glutamate uncaging and Ca^{2+} imaging. Slices were bathed in ACSF containing 3 mM MNI-glutamate. We blocked Na^+ -dependent APs and Ca^{2+} -dependent subthreshold membrane oscillations by application of 500 nM TTX and 10 μM nifedipine. We could more reliably measure increases in dendritic Ca^{2+} and somatic EPSPs by uncaging at four spots along a ~ 10 μm stretch of dendrite (0.5 ms pulse width, 1 ms interpulse interval; Fig. 8A,B). This paradigm was repeated during interleaved trials in which the somatic membrane potential was adjusted to -67 , -57 , or -47 mV with holding current (Fig. 8B).

An example experiment (Fig. 8B) shows Ca^{2+} signals evoked by glutamate uncaging at three different locations along the dendrite of a dopamine neuron. At a

Figure 6. L-type Ca^{2+} channels contribute to supralinear dendritic Ca^{2+} signals. **A**, The firing rate of dopamine neurons was altered by injection of holding current in the presence of nifedipine. **B**, AP-evoked Ca^{2+} signals during 1.3 Hz firing were averaged and fit as in Figure 3B. **C**, Predicted Ca^{2+} signals (black) were generated for the firing rates displayed in **A**. Blue lines represent the average values of the predicted Ca^{2+} signals. **D**, Dendritic Ca^{2+} signals (black) recorded at the firing rates displayed in **A**. Red lines indicate the average Ca^{2+} signal over the course of the scan. **E**, Summary plot of measured (red) and predicted (blue) Ca^{2+} signals plotted against the rate of tonic firing in the presence of nifedipine, and measured Ca^{2+} signals in control conditions (black). Light symbols are individual points; dark symbols are binned averages and SEM for all dendrites. Lines are lines of best fit to each dataset. The slopes of the lines of best fit were as follows: control measured data = $3.7\% \text{ G/G}_s$ per Hz; nifedipine-treated measured data = $2.2\% \text{ G/G}_s$ per Hz; nifedipine-treated predicted data = $1.3\% \text{ G/G}_s$ per Hz. Predicted data varies from the line of best fit due to variability in the size and kinetics of the AP-evoked Ca^{2+} transients between cells. **F**, Plot of the ratio of measured/predicted Ca^{2+} signals against firing rate. Pink lines represent data from individual nifedipine-treated dendrites. Red line represents binned averages and SEM for nifedipine data. Black line represents data for control neurons (replotted from Fig. 3F). **G**, Average ratio of measured/predicted Ca^{2+} signals for trials during which the frequency of tonic firing > 3 Hz. Error bars are SEM. Individual points shown in white. Lines between control and nifedipine points represent paired data. **H**, Average ratio of measured/predicted Ca^{2+} signals before and after application CPA. Open symbols represent mean and SEM.

Figure 7. Bursts of APs generate supralinear dendritic Ca^{2+} signals. **A**, Example of measured dendritic Ca^{2+} (red) and linear predictions (black) during a burst of APs evoked by a 300 ms, 100 pA current injection into the soma. Dashed line in somatic voltage recording represents -60 mV. The amplitude of the dendritic Ca^{2+} influx in response to burst firing was measured as the average value between 200 and 300 ms of the somatic current injection, indicated by gray circles. Inset, AP-evoked Ca^{2+} transients during tonic firing (gray), average AP-evoked Ca^{2+} transient (red), and line of best fit to the average (black). **B**, Plot of amplitude of dendritic Ca^{2+} evoked by a burst of APs against the distance from the soma at which the Ca^{2+} signal was measured. Individual dendrites displayed in gray; binned averages and SEM displayed in black. **C**, Plot of the ratio of measured dendritic Ca^{2+} signal to predicted dendritic Ca^{2+} signal evoked by a burst against distance from the soma at which the Ca^{2+} signal was measured. Individual dendrites displayed in gray; binned averages and SEM displayed in black. **D**, Example of measured (red) and predicted (black) dendritic Ca^{2+} signals (top) during a burst of action potentials (middle), evoked by synaptic stimulation of a contralateral dendrite (bottom). **E**, Example of measured and predicted dendritic Ca^{2+} signals (top) in response to a burst of APs evoked from a fixed voltage (-65 mV, middle) by brief current injections (bottom). Dashed line in the top represents line of best fit to a single AP-evoked Ca^{2+} transient. **F**, Box plots of the ratio of measured/predicted Ca^{2+} signal in response to a burst of APs in indicated conditions. Middle lines represent median value, boxes indicate middle 50% of data, and whiskers indicate maximum and minimum values. Circles represent average ratio calculated for individual dendrites.

distance of 120 μm from the soma, we found that holding the soma at -47 mV dramatically increased in the amplitude of the uncaging-evoked dendritic Ca^{2+} signals relative to signals recorded at -67 mV. Remarkably, somatic depolarization enhanced uncaging-evoked Ca^{2+} signals in even more distal regions of the dendrite. At 252 or 342 μm away from the soma, somatic depolarization from -67 to -47 mV resulted in a more than two-fold increase in uncaging-evoked Ca^{2+} signal. Because TTX and nifedipine were included in the external solution, enhancement of the uncaging-evoked Ca^{2+} signals at depolarized potentials is likely due solely to passive signal propagation and voltage-dependent Mg^{2+} unblock of NMDARs at the dendritic site of uncaging.

Plotting the ratio of the peak uncaging-evoked Ca^{2+} signals recorded at different somatic holding potentials in nine dendrites from six cells, we found that dendritic Ca^{2+} influx was between 1.3 and 2.9-fold greater when the soma was held at -47 mV than when held at -67 mV (Fig. 8C). This enhancement of glutamate-evoked dendritic Ca^{2+} influx by depolarization of the soma was observed throughout the distal dendrites, up to 480 μm from the soma (Fig. 8C). Ratios of Ca^{2+} signals are significantly >1 as determined by one-sample t test (0–50 μm , $p = 2.9 \times 10^{-5}$; 50–100 μm , $p = 1.9 \times 10^{-5}$; 100–200 μm , $p = 1.3 \times 10^{-5}$; 200–300 μm , $p = 0.011$; 300–480 μm , $p = 0.017$). This demonstrates that dendritic synaptic receptors are exquisitely sensitive to steady-state changes in the somatic membrane potential.

In a separate set of experiments, we measured dendritic Ca^{2+} influx in response to somatic step depolarizations and found that amplitude of dendritic Ca^{2+} signals was preserved with distance throughout the distal dendrites (Fig. 9A,B). Together, these results further emphasize the robust electrotonic coupling of the soma and dendrites of SNc dopaminergic neurons.

Tonic firing rate tunes the rate of NMDA receptor-mediated burst output
Dopamine neurons fire synaptically driven bursts on a background of tonic firing. Our results show that fast tonic firing in dopamine neurons is accompanied by steady depolarization of the nonspike voltage (Fig. 5A,B). Furthermore, subthreshold depolarization of the soma enhances NMDAR-mediated Ca^{2+} signals throughout the dendrite (Fig. 8). Com-

binning these observations, we hypothesized that the tonic firing rate of dopamine neurons will influence the strength of synaptic inputs throughout the proximal and distal dendrites.

To test the interaction between tonic and burst firing, synaptic inputs were activated using small diameter theta-glass stimulation electrodes ($\sim 5 \mu\text{m}$ tip diameter) placed within $10 \mu\text{m}$ of a dendrite, at distances between 57 and $194 \mu\text{m}$ from the soma (mean = $105 \pm 16 \mu\text{m}$ from soma). Stimulating local inputs at 100 Hz for 200 ms routinely evoked bursts (>13 Hz) from tonically active neurons (Fig. 10A). We then tested the effect of background firing rate (from 0 to 6.5 Hz) on the strength of synaptically evoked burst responses.

Figure 10B shows somatic voltage recordings from an example neuron arranged according to the rate of background tonic firing. The rate of the synaptically driven burst responses dramatically increased when the rate of background firing was elevated (Fig. 10B,C). Specifically, synaptic stimulation during 1.1 Hz tonic firing resulted in a 21.5 Hz burst (Fig. 10C, left). However, the same synaptic stimulus during faster tonic firing at 5.7 Hz resulted in a $\sim 100\%$ increase in the burst firing response to 43.1 Hz (Fig. 10C, right). A plot of the rate of burst responses against the range of background firing rates tested for this cell is provided in Figure 10D. The slope of the best linear fit to the data show that the burst firing response increased by 3.5 Hz for every 1 Hz increase in background firing rate.

Strikingly, the influence of the background firing rate on the burst firing rate was substantially reduced following blockade of NMDARs ($50 \mu\text{M}$ D-AP5; Fig. 10B,C, red traces). This reduction in the burst response was even more clear in the plot of the burst versus background rates (Fig. 10D, red symbols), which shows a dramatic reduction in the slope to 0.84 Hz/Hz. Interestingly, the remaining burst output in the presence of D-AP5, presumably due to AMPA receptor mediated conductances, was still enhanced during firing at elevated background rates. In eight neurons, the relationship between burst and background tonic firing in the presence of D-AP5 was 1.22 ± 0.22 Hz/Hz, a significant decrease from 3.35 ± 0.33 Hz/Hz measured under control conditions ($p = 2.0 \times 10^{-5}$, paired t test; Fig. 10E). Here, the slope of 1 Hz/Hz (Fig. 10D, dashed lines) would be predicted under conditions where a fixed stimulus leads to a set increase in firing rate that is independent of background rate.

Last, the relationship between burst and tonic firing was observed during local stimulation of synapses in the distal dendrites. Synaptic stimulation of the distal dendrites ($>100 \mu\text{m}$) tended to

Figure 8. Depolarization of the soma enhances synaptic strength throughout the dendrites of SNc dopamine neurons. *A*, Maximum intensity projection of the recorded neuron. Colored bars represent sites of linescan Ca^{2+} imaging and glutamate uncaging (indicated by red inverted triangles). *B*, Dendritic Ca^{2+} signals evoked by two-photon glutamate uncaging (timing of uncaging indicated by red vertical line) at specified somatic voltages. Color of traces corresponds to sites of scans shown in *A*. Dashed lines represent baseline Ca^{2+} levels. *C*, Plot of the ratio of the uncaging-evoked dendritic Ca^{2+} signal at -47 mV to Ca^{2+} signal evoked at -67 mV against the distance from the soma. Gray plots represent individual dendrites; black plot represents averages and SEM binned according to distance.

evoke weaker burst firing or require stronger stimulus intensities, consistent with results from past studies (Blythe et al., 2009). Despite the reduced strength of bursting in the most distally stimulated recording ($194 \mu\text{m}$), increases in the background firing rate still enhanced synaptically driven burst responses (slope of burst to tonic fit, 3.19 Hz/Hz). Coupled with the results in Figure 8, showing that somatic depolarization enhances NMDAR-mediated Ca^{2+} signals throughout the distal dendrites, these results demonstrate that strong electrotonic coupling of the dendrites equalizes somatic influence over proximally and distally located synaptic inputs in dopamine neurons.

In total, these results demonstrate that the gain of synaptically driven burst firing is remarkably sensitive to the rate of back-

Figure 9. Electrotonic coupling of soma and dendrites. **A**, Examples of dendritic Ca^{2+} signals (top) in response to somatic voltage steps (bottom) measured 76 and 235 μm from the soma. **B**, Plot of the amplitude of dendritic Ca^{2+} signal in response to a voltage step from -60 to -50 mV against the distance from the soma at which dendritic Ca^{2+} was measured. Individual dendrites plotted in gray. Averages and SEM for six cells binned according to distance plotted in black.

ground firing, which tunes the strength of burst output in a manner that depends upon NMDAR activation.

Discussion

The bulk of studies examining dendritic integration have focused on pyramidal neurons in either hippocampus or cortex. However, firing in midbrain dopamine neurons differs significantly from pyramidal neurons in an important way. Unlike pyramidal neurons that require synaptic input to drive cells past AP threshold, dopamine neurons fire spontaneously as a result of intrinsic conductances located in and around the soma. For this reason, EPSPs occur on a background of ongoing firing, and thus are more frequently influenced by AP-evoked conductances. Despite the importance of this question, relatively little is known about the functional role of background tonic firing in influencing dendritic excitability and shaping synaptically evoked firing responses in spontaneously active neurons. Here, we demonstrate a previously unrecognized role of background firing rate in tuning dendritic excitability and modulating the gain of synaptic responses in SNc neurons.

Supralinear dendritic Ca^{2+} signaling in SNc dopamine neurons

Our findings show that approximately one-half of the dendritic Ca^{2+} influx during fast tonic firing (4–6 Hz) originates from activation of low-threshold Ca^{2+} channels. Three observations support this claim. First, evoking spikes from fixed membrane potentials results in a $\sim 50\%$ reduction in averaged Ca^{2+} signals as compared with control values (control Ca^{2+} signals at 4.5–6 Hz = 16.4% vs fixed at 5 Hz = 8.1%). Second, in the absence of APs, voltage steps to subthreshold potentials (between -60 and -50 mV) evoke Ca^{2+} signals that are $\sim 50\%$ of the amplitude of Ca^{2+} signals during fast tonic firing (Ca^{2+} signal evoked by voltage step from -70 to -55 mV = 8.5%, vs average Ca^{2+} signal during 4.5–6 Hz tonic firing = 16.4%). Third, the average Ca^{2+} signals recorded during 3.5–4.5 Hz tonic firing in nifedipine-treated cells is reduced by 51% compared with control cells (G/G_s , control, $15.8 \pm 1.5\%$ vs nifedipine, $7.7 \pm 1.7\%$, $p = 0.00015$, unpaired t test). By contrast, nifedipine showed a smaller effect (29.7% reduction) on the peak of AP-evoked Ca^{2+} signals. Therefore, these results show that a large fraction of the dendritic Ca^{2+} signal generated by fast tonic firing in SNc dopamine neurons originates from influx through low threshold L-type Ca^{2+} channels.

Recent work has identified Ca^{2+} influx, likely through $\text{Ca}_v1.3$ channels, as a potential source of vulnerability and selective death

of SNc dopamine neurons in Parkinson's disease (Chan et al., 2007). Recordings in dissociated cells seem at odds with this view, however, showing that the bulk of Ca^{2+} entry ($>80\%$) during low-frequency pacemaking occurs during their characteristically broad APs (half-width, 1.5–5 ms; Puopolo et al., 2007). During low tonic firing (<2 Hz), our results corroborate the findings in dissociated neurons. On the other hand, during fast tonic firing, at rates much more likely to be encountered in vivo (>3 Hz), we find that strong activation of L-type channels leads to a substantial increase in Ca^{2+} influx in SNc neurons. Notably, we did not observe supralinear Ca^{2+} influx at elevated firing rates (>3 Hz) in VTA dopamine neurons, which are mostly spared in Parkinson's disease. These findings are consistent with past work showing reduced involvement of subthreshold Ca^{2+} currents in VTA neurons (Khaliq and Bean, 2010). In the context of the Ca^{2+} hypothesis of Parkinson's disease, our results reveal that the role of low threshold L-type channels in Ca^{2+} signaling, and subsequent Ca^{2+} overload, may be even more prominent than has been appreciated in past studies of SNc dopamine neurons.

Strong electrotonic coupling of soma to distal dendrites

Our results demonstrate tight electrotonic coupling between the soma and dendrites of SNc dopamine neurons, similar to earlier work using direct dendritic recordings (Häusser et al., 1995; Gentet and Williams, 2007). However, the functional consequences of tight dendritic coupling had not been tested. Here, we use glutamate uncaging and Ca^{2+} imaging to gain access to small-caliber distal dendrites. We show that somatic depolarization from -67 mV to -47 mV leads to a ~ 50 – 150% enhancement of NMDA receptor mediated Ca^{2+} signals, even at distances nearly 500 μm distal to the soma. Therefore, our findings demonstrate that the somatic voltage exerts a powerful influence over dendritic conductances due in large part to strong electrotonic coupling of the soma and dendrites.

Modeling studies show that strong electrotonic properties and simple geometry of the dendrites of SNc neurons, which exhibit little branching, favor reliable backpropagation (Vetter et al., 2001). Consistent with this, our results show little attenuation of bAP-evoked Ca^{2+} signals with distance, up to 450 μm from the soma. Olfactory bulb mitral cells are similar in form and function, with dendrites that branch minimally and show reliable backpropagation over long distances (Bischofberger and Jonas, 1997; Chen et al., 1997; Christie and Westbrook, 2003; Djuricic et al., 2004). In contrast, pyramidal neurons in the hippocampus and neocortex show significantly more branching and significant attenuation of spike-evoked Ca^{2+} signals at distances <200 μm from the soma (Waters et al., 2003; Gasparini et al., 2007, 2011). Dentate gyrus neurons branch closely to the soma and not much beyond, but exhibit strong attenuation of bAP-evoked Ca^{2+} signals likely due to only a modest expression of voltage-gated Na^+ channels (Krueppel et al., 2011). Therefore, bAPs invade the distal dendrites of dopamine neurons more extensively than pyramidal neurons, likely due to their simple morphology.

The extent to which steady-state and slow voltage changes are passively propagated through the dendrites depends chiefly upon the specific membrane resistivity. For example, direct dendritic

recordings from CA1 neurons show 50% attenuation of steady-state voltage deflections 238 μm from the soma (Golding et al., 2005). Correspondingly, the input resistances of CA1 neurons tend to be low at $\sim 50 \text{ M}\Omega$ on average. By contrast, our glutamate uncaging results suggest that SNc dopamine neurons are much more compact (Fig. 7), consistent with direct recordings that show little voltage attenuation up to 200 μm from the soma (Häusser et al., 1995). The tight coupling is likely explained by the unusually high membrane resistance of dopamine neurons that range in our recordings from 143 to 583 $\text{M}\Omega$ (mean = 302 $\text{M}\Omega$, data not shown). We suggest that the strong electrotonic coupling of the dendrites to the soma may serve to equalize the soma's influence (particularly at subthreshold voltages, where membrane resistance is high) on synaptic inputs throughout the dendritic tree.

As an additional consequence of the tight coupling, neurotransmitters that alter the membrane conductance could dramatically influence the extent to which small changes in the somatic membrane potential are faithfully reported to the dendrites. For example, in SNc dopamine neurons, activation of D_2 -dopamine receptors or GABA_B receptors increases the membrane conductance of dopamine neurons via activation of G-protein-coupled inward-rectifier K-channels (Lüscher and Slesinger, 2010; Ford, 2014). Similarly, previous work in pyramidal neurons shows that shunting inhibition resulting from activation of dendritic GABA_A receptors can significantly influence dendritic excitability and also produce failures in bAPs (Zhou and Antic, 2012). In the case of SNc dopamine neurons, inhibitory inputs play a particularly important physiological role as nearly 70% of synaptic inputs onto SNc neurons are GABA_A ergic (Tepper and Lee, 2007; Lobb et al., 2010). In future experiments, it will be important to examine the effect of local dendritic shunting from GABA_A receptors on propagation of steady-state somatic signals to the distal dendrites.

Influence of tonic firing on synaptically evoked bursts

Studies of DA neuron firing *in vivo* have observed a weak correlation between average firing rate and burst firing (quantified as the percentage of APs contained in bursts; Grace and Bunney, 1984a; Marinelli and McCutcheon, 2014). However, the consequences of changes in background firing rate on burst firing within a cell have not been fully examined. We have demonstrated that elevations in the rate of tonic firing increase synaptic gain due to

Figure 10. Elevation of background firing rate enhances synaptically evoked bursts. **A**, Schematic of experiment in which background firing rate was altered by tonic current injection at the soma while presynaptic inputs were stimulated (100 Hz, 200 ms) using a bipolar theta glass electrode. **B**, Example of somatic recordings of burst firing in response to local synaptic stimulation (indicated by gray bars) in control conditions (black traces, left) and after blockade of NMDA receptors (red traces, right). Traces are arranged by increasing background firing rate. Asterisks indicated traces examined in **C**. **C**, Examples of burst firing from **B** at indicated frequencies in control conditions and in the presence of D-AP5. Dashed lines indicate -60 mV . **D**, Plot of burst frequency versus background frequency in control conditions (black) and in the presence of D-AP5 (red) for the cell in **B**. Solid lines are lines of best fit to each condition. Dashed lines are theoretical plots in which every 1 Hz increase in background firing rate leads to a 1 Hz increase in burst firing rate. **E**, Summary plot of the slopes of the lines of best fit as in **D** for eight neurons, in control conditions and after application of D-AP5. Empty circles are mean $\pm$ SEM.

subthreshold membrane depolarization and increased activation of NMDARs. These results are important because they suggest that relatively weak inputs which may not be sufficient to generate bursts themselves could promote burst firing by increasing

the rate of tonic firing. The electrotonically compact nature of SNc dopamine neurons could allow such cooperativity to take place over large stretches of a dendrite or even between dendrites. Similarly, slow neuromodulators that alter the background activity of dopamine neurons, such as nicotine or cannabinoids (French et al., 1997; Zhang et al., 2009; Li et al., 2011), may change the propensity of dopamine neurons to generate bursts in response to synaptic inputs associated with rewarding stimuli.

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