Dopamine Inhibition Differentially Controls Excitability of Substantia Nigra Dopamine Neuron Subpopulations through T-Type Calcium Channels

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While there is growing appreciation for diversity among ventral tegmental area dopamine neurons, much less is known regarding functional heterogeneity among the substantia nigra pars compacta (SNc) neurons. Here, we show that calbindin-positive dorsal tier and calbindin-negative ventral tier SNc dopaminergic neurons in mice comprise functionally distinct subpopulations distinguished by their dendritic calcium signaling, rebound excitation, and physiological responses to dopamine D2-receptor (D2) autoinhibition. While dopamine is known to inhibit action potential backpropagation, our experiments revealed an unexpected enhancement of excitatory responses and dendritic calcium signals in the presence of D2-receptor inhibition. Specifically, dopamine inhibition and direct hyperpolarization enabled the generation of low-threshold depolarizations that occurred in an all-or-none or graded manner, due to recruitment of T-type calcium channels. Interestingly, these effects occurred selectively in calbindin-negative dopaminergic neurons within the SNc. Thus, calbindin-positive and calbindin-negative SNc neurons differ substantially in their calcium channel composition and efficacy of excitatory inputs in the presence of dopamine inhibition.

Key words: calbindin; dendrites; dopamine; substantia nigra; t-type calcium channels; two-photon imaging

Significance Statement

Substantia nigra dopaminergic neurons can be divided into two populations: the calbindin-negative ventral tier, which is vulnerable to neurodegeneration in Parkinson's disease, and the calbindin-positive dorsal tier, which is relatively resilient. Although tonic firing is similar in these subpopulations, we find that their responses to dopamine-mediated inhibition are strikingly different. During inhibition, calbindin-negative neurons exhibit increased sensitivity to excitatory inputs, which can then trigger large dendritic calcium transients due to strong expression of T-type calcium channels. Therefore, SNc neurons differ substantially in their calcium channel composition, which may contribute to their differential vulnerability. Furthermore, T-currents increase excitation efficacy onto calbindin-negative cells during dopamine inhibition, suggesting that shared inputs are differentially processed in subpopulations resulting in distinct downstream dopamine signals.

Introduction

Dopamine neurons play a critical role in a variety of basic behaviors, including voluntary movement, reward learning, and aversion. Early studies considered midbrain dopamine neurons to be a fairly homogenous population, categorizing them broadly accord-

ing to their location within the substantia nigra pars compacta (SNc) or the neighboring ventral tegmental area (VTA). However, recent studies have revealed heterogeneity within these nuclei. Specifically, dopamine neurons of the VTA have been shown to differ in molecular composition (Liu et al., 2014; Poulin et al., 2014), projection pattern (Fallon and Moore, 1978; Aransay et al., 2015), input pattern (Geisler and Zahm, 2005; Ogawa et al., 2014; Beier et al., 2015; Menegas et al., 2015), and electrophysiological characteristics (Neuhoff et al., 2002; Ford et al., 2006; Lammel et al., 2008; Margolis et al., 2008; Zhang et al., 2010; Hnasko et al., 2012). By contrast, much less is known regarding functional heterogeneity among SNc dopaminergic neurons.

The SNc can be divided into two anatomical regions, the dorsal and ventral tiers (Gerfen et al., 1987). The dorsal-most layer of the SNc, or the dorsal tier, contains dopamine neurons that ex-

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press the Ca^{2+} -binding molecule calbindin. The ventral tier lies directly above the substantia nigra reticulata and contains many calbindin-negative (Calb $^{-}$) dopaminergic neurons. These anatomically and biochemically defined subpopulations have been further distinguished by their susceptibility to neurodegeneration in Parkinson's disease. In particular, Calb $^{-}$ SNc neurons of the ventral tier are highly vulnerable to cell death in the brains of Parkinson's patients, while calbindin-positive (Calb $^{+}$) neurons of the dorsal tier tend to be resilient (Yamada et al., 1990; Damier et al., 1999). In addition, neurons of the dorsal and ventral tiers differ in their projection patterns: ventral tier neurons project to the dorsal striatum, where they preferentially innervate the patch compartments, while the dorsal tier neurons may innervate the matrix or possibly project to extrastriatal regions (Gerfen et al., 1987; Prensa and Parent, 2001; Bentivoglio and Morelli, 2005; Matsuda et al., 2009; Dopeso-Reyes et al., 2014). A significant physiological study reported differences in the hyperpolarization cation current (I_h ; Neuheff et al., 2002). A separate study of cultured lateral and medial mesencephalic dopamine neurons (putative SNc/Calb $^{-}$ and VTA/Calb $^{+}$ cells) found that SNc/Calb $^{-}$ neurons show increased mitochondrial stress (Dryanovski et al., 2013). Given the importance of these cell types under normal and pathological conditions, a clearer understanding of the physiological characteristics that distinguish these two cell types is necessary.

Dopamine neurons undergo autoinhibitory responses to somatodendritically released dopamine (Beckstead et al., 2004; Gantz et al., 2013). Somatic and dendritic D2-autoreceptors activate G-protein-coupled inwardly rectifying potassium (GIRK) channels that hyperpolarize neurons, resulting in the inhibition of tonic firing (Lacey et al., 1990). Studies of dendritic excitability in SNc neurons have shown that dopamine gates action potential (AP) backpropagation (Gentet and Williams, 2007). Specifically, GIRK-mediated hyperpolarization recovers A-type potassium channels, which inhibit spike backpropagation, and compartmentalizes dendritic signaling. Furthermore, direct voltage-clamp recordings of somatic voltage-gated calcium (Ca^{2+}) currents show that dopamine modulation results in inhibition of high-threshold Ca^{2+} channels (Cardozo and Bean, 1995). Together, these results suggest that the role of dopamine is to cause inhibition of dendritic excitability, but direct dendritic Ca^{2+} imaging has not previously been performed.

Here, our experiments reveal that dopamine inhibition surprisingly leads to an enhancement of dendritic responses to excitation as well as enhancement of dendritic Ca^{2+} signals selectively in Calb $^{-}$ ventral tier SNc dopamine neurons. This is due to a recruitment of subthreshold T-type Ca^{2+} channels expressed at higher densities in Calb $^{-}$ neurons. In addition to large all-or-none regenerative events, we show that moderate hyperpolarization produced by D2-receptor activation results in the graded amplification of depolarization. Therefore, we present the first evidence that the Ca^{2+} channel composition differs between the dorsal tier Calb $^{+}$ and ventral tier Calb $^{-}$ SNc subpopulations. In addition, this study provides clear physiological evidence that dorsal tier Calb $^{+}$ and ventral tier Calb $^{-}$ SNc dopaminergic neurons comprise functionally distinct subpopulations distinguished by their responses to dopamine D2-receptor autoinhibition.

Materials and Methods

Animal use. All animal handling and procedures were approved by the Animal Care and Use Committee for the National Institute of Neurological Disorders and Stroke at the National Institutes of Health. *Tyrosine hydroxylase-GFP (TH-GFP; C57B6 background)* mice and *calbindin-cre/*

Td-tomato mice [*Calb1-2A-dgCre-D/Ai9(RCL-tdT)*; Zeng, 2013] of either sex were used at age postnatal day (P) 13–P24 except where adult (>6 week old) mice are specified. The *calbindin-cre* mice express a destabilized cre selectively in calbindin-containing neurons. To stabilize the cre and drive expression of td-tomato, intraperitoneal injections of trimethoprim (Sigma-Aldrich) were given at 170 $\mu\text{g/g}$ (Sando et al., 2013). Young mice were always given one injection, while some adult mice were given the same dose of trimethoprim injections on 2 consecutive days. These mice were used for electrophysiology and Ca^{2+} imaging 3–10 d after injection. Costaining for calbindin in these mice showed that not all stained neurons were td-tomato-positive (~50%). However, to increase the likelihood of patching Calb $^{-}$ dopamine neurons in these mice, we chose neurons located at the border of the compacta and reticulata, where Calb $^{+}$ neurons are not present (Fig. 1A). Because of this, we considered all td-tomato-labeled neurons to be Calb $^{+}$ and unlabeled neurons to be largely Calb $^{-}$. Wild-type (Swiss-Webster) mice were used where specified.

Slicing and electrophysiology. Mice were anesthetized with isoflurane and decapitated. For young mice, brains were extracted to ice-cold modified ACSF containing (in mM) the following: 250 glycerol, 2.5 KCl, 2 MgCl_2 , 2 CaCl_2 , 1.2 NaH_2PO_4 , 10 HEPES, 21 NaHCO_3 , and 5 glucose. Sagittal slices were cut 300 μm thick and incubated for 30 min in heated (34°C) ACSF containing (in mM) the following: 125 NaCl, 25 NaHCO_3 , 1.25 NaH_2PO_4 , 3.5 KCl, 10 glucose, 5 HEPES, 1 MgCl_2 , and 2 CaCl_2 . Adult mice were anesthetized with isoflurane and transcardially perfused with ice-cold modified ACSF containing (in mM) the following: 198 glycerol, 2.5 KCl, 1.2 NaH_2PO_4 , 20 HEPES, 25 NaHCO_3 , 10 glucose, 10 MgCl_2 , 0.5 CaCl_2 , 5 Na-ascorbate, 3 Na-pyruvate, and 2 thiourea. Sagittal slices were cut 200 μm thick and incubated for 30 min in heated (34°C) ACSF containing (in mM) the following: 92 NaCl, 30 NaHCO_3 , 1.2 NaH_2PO_4 , 2.5 KCl, 35 glucose, 20 HEPES, 2 MgCl_2 , 2 CaCl_2 , 5 Na-ascorbate, 3 Na-pyruvate, and 2 thiourea. Slices were removed to room temperature and used 30 min to 6 h later. Whole-cell recordings were made using borosilicate pipettes (2–7 M Ω) filled with internal recording solution containing (in mM) the following: 122 KMeSO $_3$, 9 NaCl, 1.8 MgCl_2 , 4 Mg-ATP, 0.3 Na-GTP, 14 phosphocreatine, 9 HEPES, 0.3 Fluo-5F (K_D = ~2.3 μM), and 0.05 AlexaFluor 594 hydrazide adjusted to a pH value of 7.35 with KOH. Recordings for *post hoc* immunohistochemistry used internal solution replacing Fluo-5F with 0.45 EGTA and 0.09 CaCl_2 . Current-clamp recordings were manually bridge-balanced. Liquid junction potential (−8 mV) was not corrected. All experiments in adult mice, all voltage-clamp experiments, and all experiments involving synaptic stimulation or rebound firing were conducted heated (30–32°C). The remaining experiments were at room temperature.

Synaptic stimulation was evoked with theta-glass electrodes pulled on a vertical puller and filled with ACSF. Electrodes were placed near, but not against, dendrites. Stimulations were evoked using an Isoflex, amplitudes ranging from 1 to 90 V. Dopamine iontophoresis was conducted using sharp microelectrodes pulled to 40–80 M Ω resistance containing 1 M dopamine and placed at the slice surface directly above the soma of the recorded cell. Internal solution for iontophoresis experiments contained 9 mM BAPTA. In parallel experiments without intracellular BAPTA, quinpirole (10–50 μM) was puffed onto wild-type neurons using a pneumatic picopump (WPI). In cases when dopamine iontophoresis or quinpirole puff did not hyperpolarize the neuron $\geq$ −60 mV, the recording was not included in analysis (n = 3 of 18, 4 of 10 neurons respectively). Voltage-ramp experiments were conducted in the presence of 0.5 μM TTX. Series resistance was $\leq$ 25 M Ω and was compensated 40–60%.

Two-photon calcium imaging. Two-photon Ca^{2+} imaging was performed using previously published procedures (Hage and Khaliq, 2015). Briefly, cells were imaged on a two-photon microscope (Brucker) with a Mai Tai ultrafast Ti:sapphire laser (Spectra-Physics) tuned to 810 nm. A 575 nm dichroic long-pass mirror was used to split the fluorescence signal through 607/45 nm and 525/70 nm filters to the multialkali photomultiplier tubes (Hamamatsu). Ca^{2+} signals were quantified as the ratio of green (Ca^{2+} sensitive) to red (stable) signal. The green/red (G/R) ratio was normalized by the ratio of saturated (2 mM Ca^{2+}) green (Gs) to red signal measured daily via a pipette placed at the surface of the

Figure 1. AP characteristics of Calb+ and Calb- neurons. **A**, *Calbindin-cre/Td-tomato* mouse (red) stained for calbindin (cyan) and TH (green). **B**, Example cell-attached spontaneous firing recordings for a young Calb+ (blue) and Calb- (yellow) cell. Thirty seconds of spontaneous firing was recorded in young Calb- ($n = 12$) and Calb+ ($n = 17$) neurons at room temperature (RT; left) and in adult Calb- ($n = 9$) and Calb+ ($n = 4$) heated to 30–32°C (right). * $p = 0.018$, young RT; * $p = 0.034$, adult heated. **C**, Overlaid averaged phase plot of AP waveform during tonic pacemaking in adult mice heated to 30–32°C for Calb- (yellow) and Calb+ (blue) subpopulations (left). Shading represents $\pm$ SEM. AP activation threshold (AP thresh) and width at half-height (AP width) did not differ between subpopulations ($n = 13$ for each group). AP height differed slightly, * $p = 0.034$. **D**, Capacitance and resistance measurements from adult Calb+ ($n = 18$) and Calb- ($n = 27$) neurons at 30–32°C. ** $p = 0.00032$.

slice. Cells were filled with dye for ≥ 10 min before imaging. Using EGTA-clamped Ca^{2+} concentrations (calculated with maxchelator; <http://web.stanford.edu/~cpatton/maxc.html>), we calculated the K_D of Fluo-5F as ~ 418 nM at 33°C and ~ 711 nM at room temperature. We quantified our

nonlinearity (NL) values for each temperature [$NL = dG/R_{ideal}V_{sReal}/(G/R - G/R_{min})$] where $dG/R_{ideal}V_{sReal}$ is the difference between the linear approximation and the exact relationship between $[\text{Ca}^{2+}]$ and G/R signal under our experimental conditions (Yasuda et al., 2004), and found that

G/Gs signals of 0.4, 0.3, and 0.2 resulted in nonlinearities of 44, 25, and 10% at 33°C and 51, 30, and 13% at room temperature (22°C). These data suggest that a number of our largest Ca^{2+} signals were imaged under conditions of partial dye saturation and likely indicate even larger than reported increases in dendritic Ca^{2+} concentration (Regehr and Atluri, 1995; Maravall et al., 2000).

Immunohistochemistry and confocal imaging. In slices from *TH-GFP* mice, cells were filled for ≤ 6 min to prevent calbindin and aldehyde dehydrogenase 1 family, member A1 (Aldh1a1) washout (Müller et al., 2005), after which the pipette was carefully removed. Slices were then fixed in 4% PFA in PBS overnight at 4°C. Then slices were washed 1×10 min in PBS, then 1×10 min in PBS containing 0.1% Triton-X (PBST). Slices were permeabilized in PBST containing 10% normal donkey serum for 90 min at room temperature and then incubated in primary antibody in PBST overnight on shaker at room temperature. After 6×10 min washes in PBST, slices were incubated in secondary antibody for 3 h on shaker at room temperature. After 6×10 min washes in PBS, slices were mounted with Vectashield in square frame seals (Bio-Rad) on glass slides and coverslipped. Primary antibodies were used at the following concentrations: rabbit anti-calbindin (Swant), 1:1000; sheep anti-TH (Novus Biologicals), 1:1000; rabbit anti-Aldh1a1 (Abcam), 1:400. Secondary antibodies were used at the following concentrations: donkey anti-rabbit Cy5 (Jackson ImmunoResearch), 1:50; donkey anti-sheep Alexa488 (Invitrogen), 1:1000. Images were acquired through $20\times$ and $10\times$ objectives on a spinning-disk confocal microscope.

Drugs. All salts were from Sigma-Aldrich. Fluo5F and Alexa594 (Life Technologies), gabazine, D-AP5, dopamine, and D-serine were dissolved in deionized water. TTA-P2 (Alomone), ML-218 (Tocris Bioscience), sulpiride, quinpirole, picrotoxin, nifedipine, CGP55845 (Tocris), NBQX, and cyclopiazonic acid (CPA) were dissolved in DMSO.

Analysis. Analysis was conducted in Igor (Wavemetrics). Mann–Whitney *U* tests were used to compare two samples. Wilcoxon signed-rank tests were used for paired comparisons. For multiple comparisons, Kruskal–Wallis tests determined significance of the dataset and *post hoc* Mann–Whitney *U* tests determined significance between groups. Data in text is reported as mean $\pm$ SEM and error bars on graphs are indicated as $\pm$ SEM. Box plots show medians, 25 and 75% (boxes) percentiles, and 10 and 90% (whiskers) percentiles.

Results

Low-threshold Ca^{2+} depolarizations occur in Calb $^{-}$, but not Calb $^{+}$, SNc neurons

Substantia nigra dopamine neurons fire spontaneously at characteristically low rates (< 5 spikes/s) in *in vivo* experiments and in brain slices. Previous *in vivo* (Brown et al., 2009) and *in vitro* studies (Neuhoff et al. 2002) showed that Calb $^{-}$ SNc dopamine neurons fire tonically at slightly higher rates than Calb $^{+}$ SNc neurons. Therefore, we compared spontaneous activity in Calb $^{+}$ and Calb $^{-}$ SNc neurons from a *calbindin-cre* mouse line (Zeng, 2013) crossed with the *Ai9 td-tomato* line [*Calb1-2A-dgCre-D/Ai9(RCL-tdT)*; Fig. 1A]. In sagittal brain slices, cell-attached recordings of spontaneous firing in SNc neurons showed that Calb $^{-}$ neurons fire at slightly higher rates in juvenile (Calb $^{+}$, 0.87 ± 0.07 Hz, $n = 17$ cells from 11 mice; Calb $^{-}$, 1.23 ± 0.13 Hz, $n = 12$ cells from 11 mice, $p = 0.018$) and adult mice (Calb $^{+}$, 0.59 ± 0.16 Hz, $n = 4$ cells from 4 mice; Calb $^{-}$, 1.6 ± 0.29 Hz, $n = 9$ cells from 6 mice, $p = 0.034$; Fig. 1B). Whole-cell recordings of APs during pacemaking showed that threshold, width, and height were similar between these two subpopulations (Fig. 1C,D). These results show that SNc neuron subpopulations are relatively uniform in their tonic firing properties.

Upon hyperpolarization, however, striking differences between Calb $^{+}$ and Calb $^{-}$ neurons were apparent. To test the effect of hyperpolarization on excitability of Calb $^{+}$ and Calb $^{-}$ neurons, we evoked a 20 Hz AP triplet after holding over a range of hyperpolarized membrane potentials for 900 ms. Surprisingly, we found

that a large afterdepolarization developed at hyperpolarized potentials in the Calb $^{-}$, but not Calb $^{+}$, neurons (Fig. 2B, top). Two-photon Ca^{2+} imaging revealed a simultaneous dramatic increase in dendritic Ca^{2+} (Fig. 2B, bottom). When plotted against the baseline membrane voltage, the area under the curve (AUC) of the triplet-evoked depolarization and the dendritic Ca^{2+} amplitude increase steeply with hyperpolarization in the Calb $^{-}$, but not the Calb $^{+}$, neuron (Fig. 2C).

On average, the AUC of depolarizations measured from -80 mV was larger in Calb $^{-}$ neurons (4.8 ± 0.63 mV $\cdot$ s, $n = 10$ cells from 8 mice) than Calb $^{+}$ neurons (2.24 ± 0.27 mV $\cdot$ s, $n = 26$ cells from 11 mice; Mann–Whitney *U* test, $p = 0.0067$). Likewise, simultaneously imaged dendritic Ca^{2+} signals were considerably larger in Calb $^{-}$ neurons (0.3 ± 0.051 dG/Gs) than in Calb $^{+}$ neurons (0.063 ± 0.016 dG/Gs; Mann–Whitney *U* test, $p = 0.0024$; Fig. 2D, top). We observed a similar pattern in adult mice (30 – 32°C) where the AUC of depolarizations evoked by single-current injections was significantly larger in Calb $^{-}$ neurons (1.6 ± 0.18 mV $\cdot$ s, $n = 27$ cells from 14 mice) than in Calb $^{+}$ neurons (0.48 ± 0.08 mV $\cdot$ s, $n = 18$ cells from 11 mice, Mann–Whitney *U* test, $p = 0.000023$), and the Ca^{2+} amplitude was larger in Calb $^{-}$ neurons (0.15 ± 0.024 dG/Gs), than in Calb $^{+}$ neurons (0.046 ± 0.014 dG/Gs; Mann–Whitney *U* test, $p = 0.00019$; Fig. 2D, bottom).

In Figure 2E, we plotted dendritic Ca^{2+} signals against the AUC for individual cells measured from baseline voltages closest to -80 mV. In a set of separately recorded unlabeled wild-type neurons (Fig. 2E, open symbols), we found a strong correlation between the size of the low-threshold depolarization (AUC) and the dendritic Ca^{2+} signal [Pearson's *R* value (*Pr*) = 0.71, $n = 128$, $p = 0.00001$]. Unlabeled wild-type neurons and the Calb $^{-}$ neurons cover a range of values, while Calb $^{+}$ neurons are clustered and more likely to exhibit low AUC and low Ca^{2+} amplitudes (Fig. 2E). Consistent with Neuhoff et al. (2002), our findings indicate that the Calb $^{+}$ neurons of the dorsal tier are a physiologically distinct subpopulation of the SNc dopamine neurons.

Aldh1a1-positive SNc dopamine neurons display low-threshold depolarizations

Recently, Aldh1a1 has been shown to be critical for GABA corelease from dopamine neurons (Kim et al., 2015), and was identified as a potential marker for vulnerable SNc dopamine neurons (Liu et al., 2014; Poulin et al., 2014). Additionally, molecular profiling studies show that Aldh1a1-positive SNc neurons are located in the ventral tier and are mostly Calb $^{-}$ (Anderegg et al., 2015; Poulin et al., 2014), suggesting that the Aldh1a1 $^{+}$ and Calb $^{-}$ SNc neurons may share similar physiological characteristics. To compare Aldh1a1 $^{+}$, Calb $^{+}$, and Calb $^{-}$ subpopulations under matched experimental conditions, we recorded dopamine neurons from *TH-GFP* mice and tested for Calb1 or Aldh1a1 expression with *post hoc* immunostaining (Fig. 3A,B). In preliminary experiments, we observed a time-dependent washout of calbindin during whole-cell recordings similar to reports in hippocampal neurons of the dentate gyrus (Müller et al., 2005). Therefore, to minimize washout and enable *post hoc* identification, recordings were limited to ≤ 6 min.

Comparing low-threshold depolarizations between these three cell populations, we found a significant effect of immunostaining category (Calb $^{-}$, Calb $^{+}$, Aldh1a1 $^{+}$; Kruskal–Wallis test, $H = 11.42$, $p = 0.0026$). Similar to results from *Calb1-Cre* mice, Calb $^{-}$ neurons identified with immunostaining showed prominent low-threshold depolarizations (AUC, 4.6 ± 0.3 mV $\cdot$ s, $n = 22$ cells from 5 mice) while the Calb $^{+}$ neurons did not (AUC,

Figure 2. Hyperpolarization reveals differences between Calb+ and Calb- SNc neurons. **A**, Two-photon image SNc dopamine neuron with patch pipette. Inset, Higher-magnification image of region in red box. Scan path through a spine and dendrite indicated by white line. **B**, Low-threshold afterdepolarizations in response to a 20 Hz AP triplet occurs with hyperpolarization in Calb- neurons (left traces), but not Calb+ neurons (right traces). Differences are reflected in membrane potential (above) and dendritic calcium (Ca^{2+}) amplitudes (below) in young mice. **C**, AUC (above) and Ca^{2+} (below) amplitude plotted by baseline V_m for the example neurons. **D**, Comparison between Calb- and Calb+ AUC and Ca^{2+} amplitude from membrane potential closest to -80 mV in each cell in neurons from young (Calb- $n = 10$; Calb+ $n = 27$) and adult (Calb- $n = 26$; Calb+ $n = 18$) mice. **E**, Ca^{2+} amplitude plotted by AUC measured at membrane potential closest to -80 mV for Calb+ and Calb- cells. Unlabeled neurons from wild-type mice (wt; open squares) span a wide range of values, demonstrating heterogeneity in unlabeled SNc neurons.

$2.7 \pm 0.39 \text{ mV}^*s$, $n = 13$ cells from 6 mice; Mann-Whitney U test $p = 0.0024$). Interestingly, we found that Aldh1a1+ neurons also displayed low-threshold depolarizations (AUC, $4.5 \pm 0.3 \text{ mV}^*s$, $n = 10$ cells from 3 mice) that were significantly larger than those of the Calb+ neurons (AUC, $2.7 \pm 0.39 \text{ mV}^*s$, $n = 13$ cells from 6 mice; Mann-Whitney U test, $p = 0.0015$), but indistinguishable from those measured in Calb- neurons (AUC, $4.6 \pm 0.3 \text{ mV}^*s$, $n = 22$ cells from 5 mice; Mann-Whitney U test, $p = 0.7$; Fig. 3C–E).

SNc dopamine neurons are characterized by a depolarizing voltage sag that is most prominent in Calb- SNc neurons. This indicates a higher density I_h (Neuhoff et al., 2002). We found that the voltage sags in Aldh1a1+ neurons ($17.9 \pm 2.0 \text{ mV}$, $n = 10$ cells from 3 mice; Mann-Whitney U test, $p = 5.7e-5$) and Calb- neurons ($18.5 \pm 0.11 \text{ mV}$, $n = 31$ cells from 13 mice; Mann-Whitney U test, $p = 4.9e-11$) were significantly larger than in Calb+ neurons ($7.1 \pm 0.07 \text{ mV}$, $n = 34$ cells from 17 mice). Sag amplitude data collected from neurons stained *post hoc* did not

differ from those collected from the *calbindin-cre* mice, and were therefore pooled (Fig. 3F, G). Our comparison of low-threshold depolarizations and sag potentials demonstrate that Aldh1a1+ SNc neurons share similar characteristics with the Calb- SNc neurons. Therefore, our functional data supports results from genetic sequencing studies (Poulin et al., 2014) that indicate that Aldh1a1+ and Calb- SNc neuron subpopulations may partially overlap.

Hyperpolarization increases sensitivity to synaptic frequency

To test whether low-threshold depolarizations could be activated synaptically, we recorded cells in the presence of picrotoxin ($50 \mu\text{M}$) or gabazine ($10 \mu\text{M}$) to isolate excitatory inputs. Excitatory synaptic stimulation evoked low-threshold depolarizations, which could be eliminated by blocking NMDA and AMPA receptors (data not shown). To test the relative effect of synaptic input frequency at hyperpolarized and depolarized potentials, we delivered four synaptic stimuli over a range of frequencies (28–200

Figure 3. Calb[−] and Aldh1a1⁺ SNc dopaminergic neurons display similar characteristics. **A**, Sagittal section of the substantia nigra. Genetically labeled TH-positive dopamine neurons (green neurons) were filled with Alexa594 (red) and stained for calbindin (magenta). **B**, Sagittal section of the substantia nigra immunostained for aldehyde dehydrogenase (cyan). **C**, Example traces of low-threshold depolarization in TH-positive neurons that were identified as Calb⁺ (blue), Calb[−] (yellow), and Aldh1a1⁺ (red) using *post hoc* immunostaining. **D**, AUC as a function of membrane potential in *post hoc* stained neurons. cb⁺, Calb⁺ neurons; cb[−], Calb[−] neurons; ald⁺, Aldh1a1⁺ neurons. **E**, AUC measured from membrane voltage closest to -80 mV for each cell in Calb⁺, Calb[−], and Aldh1a1⁺ neurons. $*p < 0.01$. **F**, Example traces from Calb⁺, Calb[−], and Aldh1a1⁺ neurons showing voltage sag measurement. Black arrow indicates minimum V_m . **G**, Both Aldh1a1⁺ ($n = 10$) and Calb[−] ($n = 31$) neurons have significantly larger voltage sags than Calb⁺ ($n = 34$) neurons when measured from the minimum V_m closest to -100 mV for each cell. $***p < 0.0001$ $****p < 1e-9$.

Hz) using a theta-glass stimulating electrode. For each cell, the location of the stimulating electrode and the stimulation strength were the same at hyperpolarized and depolarized potentials and for all stimulation frequencies. To ensure accurate comparisons and prevent spontaneous firing, we applied holding current and adjusted the strength of the stimulus to below AP threshold levels. Simultaneously, Ca^{2+} was imaged in dendritic segments at an average distance of $42 \pm 12 \mu\text{m}$ from the site of stimulation. Figure 4A shows one cell's response to synaptic stimulation when held first at a depolarized potential (-58 mV; green traces) and then at a hyperpolarized potential (-70 mV; black traces). At depolarized potentials, we observed a slight subthreshold depolarization, likely due to NMDA receptor activation. However, at hyperpolarized potentials, where NMDA receptors are more

strongly blocked by Mg^{2+} , low-threshold depolarizations were observed only in response to high stimulation frequencies. In recordings from five wild-type neurons, which displayed low-threshold depolarizations, the frequency required for a low-threshold depolarization ranged from 40 to 66 Hz. Averaging these neurons, we found that hyperpolarization enables a non-linear dependence on input frequency. The size of the response to 66 Hz synaptic stimulation at depolarized potentials (average baseline $V_m = -63 \pm 3$ mV; average AUC, 1.2 ± 0.4 mV*s) was significantly smaller than at hyperpolarized potentials (average baseline $V_m = -77 \pm 2.7$ mV; average AUC, 3.0 ± 0.2 mV*s, $n = 5$ cells from 5 mice, Mann–Whitney U test, $p = 0.008$), as was the dendritic Ca^{2+} signal (average dG/G_s , depolarized, 0.019 ± 0.005 dG/G_s ; hyperpolarized,

Figure 4. Synaptically generated low-threshold depolarizations depend on input frequency. **A**, Example traces from an SNc neuron given four excitatory synaptic stimulation pulses at different frequencies from depolarized (top) and hyperpolarized (middle) potentials. Bottom, Corresponding calcium traces. **B**, Overlay of responses for all frequencies in **A**. **C**, Size of low-threshold depolarizations (AUC) versus stimulation frequency. Note, AUC increases nonlinearly with input frequency at hyperpolarized (black) but not depolarized (green) potentials. **D**, Same as **C**, but for Ca^{2+} amplitude. Data are presented as mean $\pm$ SEM.

0.16 ± 0.03 dG/Gs; Mann–Whitney U test, $p = 0.008$). These results show that the effect of hyperpolarization on dopamine neurons is to increase temporal summation of synaptic input, demonstrating that integration of synaptic input is substantially different at hyperpolarized potentials than at depolarized potentials.

T-type Ca^{2+} channels are necessary for synaptically driven and current injection-evoked low-threshold depolarizations

We have shown that the Calb $^{-}$ and Calb $^{+}$ neurons of the SNc display striking differences when activated from hyperpolarized potentials. The particular characteristics of the low-threshold depolarization, such as the requirement for hyperpolarization and the accompanying large dendritic Ca^{2+} transient, are consistent with events generated by T-type Ca^{2+} channels, as has been observed in the thalamus (Jahnsen and Llinás, 1984; Connelly et al., 2015) and cerebellum (Aizenman and Linden, 1999). In dopamine neurons, early studies reported that a subset of neurons could generate low-threshold spikes (Llinás et al., 1984; Kang and

Kitai, 1993a), and the presence T-type Ca^{2+} channels in dopamine neurons has been confirmed through staining (Dufour et al., 2014) and voltage-clamp recordings (Kang and Kitai, 1993b; Poetschke et al., 2015; Philippart et al., 2016).

To test whether T-type Ca^{2+} channels were necessary for the low-threshold depolarization, TTA-P2 (1 μM), a selective T-type channel blocker (Shipe et al., 2008; Dreyfus et al., 2010), was bath-applied to wild-type cells, which displayed low-threshold depolarizations, while low-threshold depolarizations were synaptically generated once every 20 s. In young wild-type mice, stimulation was delivered (four pulses, 100 Hz) to generate a low-threshold depolarization from a hyperpolarized holding potential. When measured 6–8 min after TTA-P2 application, the size of the synaptically evoked low-threshold depolarization was greatly reduced [control, $95.8 \pm 6.6\%$, $n = 8$ (five cells, four young mice; three cells, two adult mice); TTA-P2, $48.4 \pm 7.9\%$, $n = 5$ cells from five mice; Mann–Whitney U test, $p = 0.0062$] as was the corresponding dendritic Ca^{2+} signal (control, $75.9 \pm 9.1\%$; TTA-P2, $13.1 \pm 2.2\%$; Mann–Whitney U test, $p = 0.0016$; Fig. 5A).

Figure 5. T-type Ca^{2+} channels are necessary for low-threshold depolarizations. **A**, Example traces showing synaptically evoked low-threshold depolarizations (top; scale bars: 5 mV, 100 ms) and dendritic Ca^{2+} signals (bottom; scale bars: 0.025 dG/Gs , 100 ms) before (black) and after (red) TTA-P2 application. **B**, Time course for drug application on AUC, Ca^{2+} , and first EPSP amplitude compared with no-drug control ($n = 8$, 5 young, 3 adult). **C**, Summarized data from shaded area in **B**. ****** $p < 0.01$ * $p < 0.05$. **D**, Example low-threshold depolarization (top; scale bars: 20 mV, 100 ms) and dendritic Ca^{2+} signal (bottom; scale bars: 0.05 dG/Gs , 100 ms) before drug application (black) and after TTA-P2 application (red) in young mice. **E**, Time course for drug effect on low-threshold depolarization (AUC, top) and dendritic Ca^{2+} signal (bottom), compared with no-drug control. cntrl, Control; nif, Nifedipine; CPA, Cyclopiazonic acid; Cs+, Cesium. **F**, Summarized data from shaded area in **E**. ****** $p < 0.01$ * $p < 0.05$.

Because immunostaining studies suggest that T-type channel expression may change during development (Dufour et al., 2014), we repeated this experiment in adult *TH-GFP* mice. Stimulations for each cell were tuned to generate a low-threshold depolarization from hyperpolarized potentials (10–200 ms, 100–350 Hz). No-drug control experiments measured at 6–8 min did not differ between young ($n = 5$ cells from 4 mice) and adult ($n = 3$ cells from 2 mice) mice, and were therefore pooled. In adult

mice, TTA-P2 greatly reduced the dendritic Ca^{2+} signal ($16.9 \pm 6.5\%$, $n = 5$ cells from 4 mice; Mann–Whitney *U* test vs control, $p = 0.0031$) and the AUC ($50.5 \pm 11.5\%$; Mann–Whitney *U* test vs control, $p = 0.0062$). A separate selective inhibitor of T-type channels, ML218 (Xiang et al., 2011), also reduced the dendritic Ca^{2+} signal ($18.3 \pm 2.3\%$, $n = 6$ cells from 5 mice; Mann–Whitney *U* test vs control, $p = 0.00067$) and the AUC ($64.0 \pm 11.1\%$; Mann–Whitney *U* test vs control, $p = 0.020$). Before these *post*

Figure 6. Calbindin washout does not affect low-threshold depolarization. **A**, AUC in response to 20 Hz triplet measured in young mice at membrane voltage closest to -80 mV at two different time points measured from the time whole-cell configuration was achieved (break in). Time from break in did not alter the AUC for either Calb $-$ or Calb $+$ neurons. **B**, AUC from Calb $+$ neurons measured early (<5 min from break in) and late (>15 min from break in) differ from Calb $-$ (average time from break in, 12.6 ± 0.7 min). * $p < 0.01$. Bar graph shows mean $\pm$ SEM.

hoc comparisons, a Kruskal–Wallis test was run on all drug conditions and showed that drug application had a significant effect on AUC (Kruskal–Wallis test, $p = 0.0039$) and dendritic Ca^{2+} (Kruskal–Wallis test, $p = 0.00013$; Fig. 5B,C). Importantly, neither TTA-P2 nor ML218 altered the amplitude of the first EPSP in adult or young mice (Kruskal–Wallis test, $p = 0.66$), indicating that T-type Ca^{2+} channels have little influence on presynaptic vesicle release at excitatory synapses onto SNc neurons (Fig. 5B).

To examine possible contributions of other channels, we conducted a battery of pharmacological tests on triplet-evoked low-threshold depolarizations. As before, TTA-P2 reduced the dendritic Ca^{2+} signal (control, $61.4 \pm 6.5\%$, $n = 5$; TTA-P2, $6.5 \pm 2.3\%$, $n = 6$; Mann–Whitney U test, $p = 0.004$) and AUC (control, $96.5 \pm 5.1\%$; TTA-P2, $67.3 \pm 7.8\%$; Mann–Whitney U test, $p = 0.009$; Fig. 5D). Because I_h is stronger in Calb $-$ neurons than in Calb $+$ neurons (Neuhoff et al., 2002), we tested whether cesium (Cs^+), a blocker of I_h , would reduce the low-threshold depolarization. Cs^+ (2 mM) had no significant effect on the low-threshold depolarization (control, $96.5 \pm 5.1\%$, $n = 5$ cells from 5 mice; Cs^+ , $98.8 \pm 11.1\%$, $n = 5$ cells from 4 mice; Mann–Whitney U test, $p = 0.42$) or the Ca^{2+} transient (control, $61.4 \pm 6.5\%$; Cs^+ , $43.1 \pm 12.9\%$; Mann–Whitney U test, $p = 0.22$; Fig. 5E).

SNc neurons express a high density of L-type Ca^{2+} currents, which are active during subthreshold voltages (Chan et al., 2007; Puopolo et al., 2007; Hage and Khaliq, 2015). However, a high concentration of nifedipine (10 μM), sufficient to block 80% of the Cav1.3 subunit from a -80 mV membrane potential (Helton et al., 2005), had no significant effect on the low-threshold depolarization (control, $96.5 \pm 5.1\%$, $n = 5$ cells from 5 mice; nifedipine $89.3 \pm 5.5\%$, $n = 5$ cells from 4 mice; Mann–Whitney U test, $p = 0.31$) or on the amplitude of the dendritic Ca^{2+} signal (control, $61.4 \pm 6.5\%$; nifedipine, $56.2 \pm 6.6\%$; Mann–Whitney U test, $p = 0.69$). This result may seem surprising because SNc neurons contain low-threshold L-type channels (Cav1.3), which contribute to the Ca^{2+} oscillations that underlie tonic pacemaking (Chan et al., 2007; Guzman et al., 2009; Khaliq and Bean, 2010). However, the membrane potential during the low-threshold depolarization peaks at ~ -55 mV, where the L-type current is small and only starting to activate (see Fig. 8). Our

findings agree with those of previous work reporting that the L-type controlled pacemaker oscillation is a separate phenomenon from the low-threshold depolarization (Kang and Kitai, 1993a; Wilson and Callaway, 2000). Finally, we found that depleting internal Ca^{2+} stores with CPA (10 μM) did not affect the low-threshold depolarization (control, $96.5 \pm 5.1\%$, $n = 5$ cells from 5 mice; CPA, $91.2 \pm 2\%$, $n = 5$ cells from 3 mice; Mann–Whitney U test, $p = 0.22$) or the dendritic Ca^{2+} signal (control, $61.4 \pm 6.5\%$; CPA, $71.1 \pm 5.5\%$; Mann–Whitney U test, $p = 0.31$; Fig. 5E,F). Before conducting the above *post hoc* tests, a Kruskal–Wallis test run on all drug conditions determined that drug treatment had a significant effect on both AUC and Ca^{2+} amplitude (Kruskal–Wallis test, AUC, $H = 11.1$, $p = 0.02$; Ca^{2+} signal, $H = 15.8$, $p = 0.0008$; Fig. 5E,F).

The defining difference between the Calb $+$ and Calb $-$ neurons is their expression of the calbindin molecule. Because calbindin binds to Ca^{2+} , it may contribute to their differential ability to generate these calcium-dependent low-threshold depolarizations. To examine this possibility, we took advantage of the fact that calbindin washes out of the neuron during whole-cell recording with a time constant of 9.7 min (Müller et al., 2005). We analyzed the AUC for Calb $+$ and Calb $-$ neurons at different times relative to the time whole-cell configuration was achieved. We found no correlation between time from break in and the AUC for either Calb $-$ ($Pr = 0.1$, $n = 20$, $p = 0.67$) or Calb $+$ ($Pr = 0.16$, $n = 41$, $p = 0.32$) neurons (Fig. 6A), and there was no difference between the AUC of Calb $+$ neurons at early time points (<5 min, 1.9 ± 0.5 mV*s, $n = 7$ cells from 6 mice) compared with late time points (>15 min, 1.7 ± 0.4 mV*s; $n = 9$ cells from 6 mice; Mann–Whitney U test, $p = 0.68$; Fig. 6B). However, the AUC at both early and late time points in Calb $+$ neurons is significantly different from the AUC of Calb $-$ neurons (average 12.6 ± 0.7 min from break in, 4.7 ± 0.6 mV*s, $n = 10$ cells from 8 mice; Mann–Whitney U test vs Calb $+$ early, $p = 0.0046$; vs Calb $+$ late, $p = 0.0015$).

These results indicate that, while calbindin may play an important physiological role in these subpopulations, the ability of Calb $-$ neurons to generate low-threshold depolarizations is not directly due to their lack of calbindin.

T-type Ca^{2+} channels influence rebound spike timing

Previous work in SNc dopamine neurons has shown that I_h and the A-type potassium (I_A) currents can control the timing of rebound APs (Amendola et al., 2012). To test whether T-type Ca^{2+} channels also could play a role in rebound spike timing, we injected hyperpolarizing current steps for 1 s and measured the time to first spike (rebound delay) upon release. Comparing TTA-P2 application to time-matched no-drug control experiments, we found that TTA-P2 significantly increased the duration of the rebound delay 6–8 min after application (control, $103 \pm 2.3\%$, $n = 6$ cells from 4 mice; TTA-P2, $138 \pm 13.6\%$, $n = 7$ cells from 4 mice; Mann–Whitney U test, $p = 0.0023$), and substantially reduced the rebound dendritic Ca^{2+} signal (control, $86 \pm 8.9\%$; TTA-P2, $49 \pm 7.9\%$; Mann–Whitney U test, $p = 0.014$; Fig. 7A–C). To ensure that this increase in rebound delay was not due to a change in I_h , the voltage sag was also monitored and was not affected by TTA-P2 (control, $92 \pm 13\%$; TTA-P2, $99 \pm 8.2\%$; Mann–Whitney U test, $p = 0.84$; Fig. 7B, bottom). These results show that, in addition to I_h and I_A , T-type Ca^{2+} channels contribute significantly to rebound spike timing by shortening the delay to first spike.

Given the subpopulation differences in T-type channels, we next compared dendritic Ca^{2+} signals during rebound activity in Calb+ and Calb– dopamine neurons. We released cells from a range of hyperpolarized voltages and measured rebound delays and dendritic Ca^{2+} signals (Fig. 7D–F). The rebound delay from strongly hyperpolarized potentials (-75 to -85 mV) was significantly shorter in Calb– neurons (0.23 ± 0.1 s, $n = 6$ cells from 5 mice) than in Calb+ neurons (1.41 ± 0.29 s, $n = 7$ cells from 5 mice; Mann–Whitney U test, $p = 0.0023$), and the rebound Ca^{2+} signal was significantly larger in Calb– neurons [0.3 ± 0.04 dG/Gs, $n = 13$ dendrites from 6 cells from 5 mice) than in Calb+ neurons (0.07 ± 0.01 dG/Gs, $n = 22$ dendrites from 8 cells from 5 mice; Mann–Whitney U test, $p = 2.7 \times 10^{-9}$). Importantly, at more physiologically relevant levels of hyperpolarization (-65 to -70 mV), the rebound delay was significantly shorter in Calb– (0.18 ± 0.02 s, $n = 4$ cells from 4 mice) than in Calb+ neurons (0.83 ± 0.23 s, $n = 8$ cells from 5 mice; Mann–Whitney U test, $p = 0.016$), and the rebound Ca^{2+} signal was still larger in Calb– neurons (0.2 ± 0.03 dG/Gs, $n = 12$ dendrites from 6 cells from 5 mice) than in Calb+ neurons (0.043 ± 0.006 , $n = 21$ dendrites from 8 cells from 5 mice; Mann–Whitney U test, $p = 6.8 \times 10^{-8}$; Fig. 7E,F). These results show that Calb– neurons experience large dendritic Ca^{2+} signals when rebounding from both strong and mild hyperpolarized potentials and more readily resume normal spiking after a pause.

In vivo studies have shown rebound spiking (Fiorillo et al., 2013) and rebound Ca^{2+} (Lerner et al., 2015) in subsets of SNc dopamine neurons. This rebound activation may be due to intrinsic properties, as explored above, or it may be due to a shift from synaptic inhibition to synaptic excitation. To test the effectiveness of synaptic stimulation in driving rebound bursts, we used a theta-glass electrode to locally stimulate excitatory synapses (100 Hz, 250 ms, 0.5 ms pulse width) as the cell was released from hyperpolarization (Fig. 7H). Recordings were conducted in the presence of gabazine, CGP, sulpiride, and D-serine. Stimulation intensity was adjusted to generate a burst (3–10 spikes) during spontaneous firing and was the same strength at all hyperpolarization voltages (Fig. 7G). The number of spikes during the rebound (500 ms after release of current) was significantly attenuated by hyperpolarization in Calb+ neurons [depolarized, -50 to -60 mV, 4.3 ± 0.72 spikes; hyperpolarized, -70 to -85 mV, 0.74 ± 0.36 spikes; Wilcoxon signed rank test, $n = 8$ cells (from 5

mice) paired, $p = 0.0078$], but not in Calb– neurons [depolarized, 3.9 ± 0.45 spikes; hyperpolarized, 3.4 ± 0.8 spikes; Wilcoxon signed rank test, $n = 6$ cells (from 5 mice) paired, $p = 0.69$; Fig. 7I]. These results support a role for T-type currents in the maintenance of excitability during inhibition as has been observed in the thalamus (Deleuze et al., 2012; Hong et al., 2014). T-type Ca^{2+} channels may act as a compensation mechanism to help maintain the responsiveness of Calb– dopamine neurons to excitatory synaptic stimulation during periods of hyperpolarizing inhibition.

T-type current density differs between Calb+ and Calb– neurons

Our findings above show that T-type Ca^{2+} channels can cause regenerative low-threshold depolarizations in Calb– neurons and that they contribute to the timing of AP output when cells are released from sustained hyperpolarization. However, it is unclear how long the cell must be hyperpolarized to recover T-type channels from inactivation. Furthermore, a comparison of voltage-clamp recorded T-type currents in Calb+ and Calb– SNc dopamine neurons would provide direct mechanistic insight into the differences in the ability of these neuronal subpopulations to generate low-threshold depolarizations. Therefore, we compared T-type currents recorded from Calb+ and Calb– neurons in voltage clamp. We applied slow voltage ramps delivered from -70 to -40 mV (400 ms) to activate T-type ramp currents in adult Calb+ and Calb– SNc neurons (Fig. 8A). The raw current traces evoked by these slow voltage ramps show that the total current contains a prominent low-threshold component and was net inward in many Calb– neurons. The inward component increased in amplitude with the duration of the -70 mV recovery step (Fig. 8B). Plotting the leak-subtracted current by voltage, the inward peak falls between -50 and -55 mV, consistent with a low-threshold T-type Ca^{2+} current (Fig. 8C).

We repeated the ramp protocol in the presence of TTA-P2 and used subtraction to isolate the component of the total current due to T-type channels. Figure 8E shows example TTA-P2-sensitive currents for Calb+ and Calb– neurons for holding durations of 100 ms (left) and 26 s (right). To compare recovery of T-type ramp currents in Calb+ and Calb– neurons, we plotted the averaged current amplitude between -52 and -54 mV for each trace against the duration of the recovery step. The amplitude of the T-type current increased with the duration of the recovery step in the Calb– neurons, but not in the Calb+ neurons (Fig. 8D). Comparing the current densities (pA/pF), we found a significant difference between Calb+ (-0.02 ± 0.04 pA/pF; $n = 6$ cells from 4 mice) and Calb– (-0.43 ± 0.12 ; $n = 6$ cells from 5 mice) neurons after the 600 ms recovery step (Mann–Whitney U test, $p = 0.002$) and at the maximal (26 s) recovery duration (Calb+, -0.17 ± 0.08 pA/pF; Calb–, -0.72 ± 0.17 pA/pF; Mann–Whitney U test, $p = 0.009$). However, after a 100 ms recovery step, the Calb+ (-0.04 ± 0.09 pA/pF) and Calb– (-0.17 ± 0.09 pA/pF) TTA-P2-sensitive currents did not show a significant difference (Mann–Whitney U test, $p = 0.18$; Fig. 8F). These results demonstrate that Calb+ and Calb– neurons have different densities of T-type current, and that subpopulation differences are apparent even with mild, subsecond hyperpolarizations.

To compare T-type activation to L-type activation in this voltage range, we applied nifedipine ($10 \mu\text{M}$) to adult TH-GFP neurons, which exhibited low-threshold depolarizations, during the voltage ramp protocol. Nifedipine-sensitive currents were clear at more depolarized voltages (> -50 mV), and did not depend on the holding duration. By contrast, TTA-P2-sensitive currents

Figure 7. T-type Ca^{2+} channels contribute to rebound spike timing. **A**, Rebound spikes (top) and rebound Ca^{2+} (bottom) before (black) and after (red) TTA-P2 application. Scale bars: top, 20 mV, 100 ms; bottom, 0.05 dG/Gs, 100 ms. **B**, Time course of TTA-P2 application for rebound delay (top), rebound Ca^{2+} (middle), and sag amplitude (bottom). n RD, Normalized rebound delay; n Ca^{2+} , normalized Ca^{2+} amplitude; n sag, normalized sag amplitude. **C**, Summarized data from shaded areas in **B**. $**p < 0.01$, $*p < 0.05$. **D**, Example rebound spikes (top) and rebound Ca^{2+} (bottom) from membrane potentials close to -80 mV for a Calb+ and a Calb- neuron. Scale bars: top, 20 mV, 100 ms; bottom, 0.05 dG/Gs, 100 ms. **E**, Amplitude of Ca^{2+} signal versus baseline voltage in Calb- (yellow symbols) and Calb+ (blue symbols) neurons. $**p < 0.01$. **F**, Rebound delay versus baseline voltage for Calb+, but not Calb-, neurons. Statistics reported in the text are from shaded areas. $*p < 0.05$, $**p < 0.01$. **G**, Example traces showing rebound burst responses to stimulation at different levels of hyperpolarization for Calb+ and Calb- neurons. Shaded area represents rebound time period (500 ms). Scale bars: 20 mV, 200 ms. **H**, Rebound burst protocol. **I**, Average number of rebound spikes versus baseline voltage. Statistics reported in text compare hyperpolarized potentials (hyperpol) to depolarized potentials (depol). Data are presented as mean $\pm$ SEM.

Figure 8. T-type Ca^{2+} channels are activated by voltage ramps. **A**, Voltage-clamp ramp protocol. Four hundred millisecond ramps from -70 to -40 mV were run after varying the duration of the recovery step (-70 mV). **B**, Raw current traces from times shown in **A**. **C**, Same currents as in **B**, but leak subtracted and plotted by voltage. **D**, Amplitude of TTA-P2-subtracted current plotted by duration of recovery step. Maximal holding duration was the intersweep interval of 26 s. Data presented as mean $\pm$ SEM. **E**, Example TTA-P2-subtracted traces from Calb+ and Calb- neurons after 100 ms (left) and 26 s (right) durations of -70 mV holding potential. **F**, Summary of current density (Idens; pA/pF) amplitude from TTA-P2-sensitive currents. $*p < 0.01$. **G**, Example trace from a TH-GFP mouse showing the nifedipine-sensitive (nif-sst; $10 \mu\text{M}$) and TTA-P2-sensitive (TTA-P2-sst; $1 \mu\text{M}$) ramp currents from the same cell (left). Current traces from six TH-positive neurons, which displayed low-threshold depolarizations, binned by voltage and averaged. Ramp currents were evoked after 100 ms recovery step. **H**, Same as **G**, but with ramp currents evoked after 26 s recovery step. Data are from adult mice and are presented as mean $\pm$ SEM.

were present at more hyperpolarized voltages, where they increased in amplitude following longer holding durations at -70 mV (Fig. 8G,H). These results confirm that the low-threshold component of this ramp current is due to T-type rather than L-type current, and demonstrate a clear difference in sensitivity to holding duration and activation voltage between these two currents in SNc dopamine neurons.

Dopamine-mediated hyperpolarization differentially effects Calb+ and Calb- neurons

Dopamine neurons release dopamine from their dendrites and soma. Somatodendritically released dopamine opens potassium (GIRK) channels by activating D2-dopamine receptors, hyperpolarizing dopamine neurons (Beckstead et al., 2004; Dragicevic et al., 2014). Previous studies have demonstrated that Ca^{2+} can inhibit D2-mediated GIRK currents and can modulate D2-receptor desensitization (Kramer and Williams, 2016; Gantz et

al., 2015; Poetschke et al., 2015; Dragicevic et al., 2014). We tested whether D2-receptors recruitment of GIRK currents is sufficient to hyperpolarize neurons and enable low-threshold depolarizations under weak Ca^{2+} buffering condition (300 micromolar Fluo5F). Quinpirole (10 – $50 \mu\text{M}$), a D2-receptor agonist, was puffed onto SNc neurons. During quinpirole application (1 – 3 min), a low-threshold depolarization was evoked by the AP triplet (Fig. 9A, top), and a large dendritic Ca^{2+} signal was observed (Fig. 9A, bottom). As the cell depolarized, the size of the low-threshold depolarization and the Ca^{2+} amplitude decreased, leaving depolarizations that occurred in a more graded manner. In an average of 6 wild-type neurons (from 4 mice), quinpirole-mediated hyperpolarization increased AUC and dendritic Ca^{2+} amplitude (Fig. 9B). Cells where quinpirole application failed to hyperpolarize the neurons below -60 mV were not used. Plotting the AUC or the Ca^{2+} amplitude by membrane potential reveals that quinpirole-mediated hyperpolarization increased

both AUC and dendritic Ca^{2+} amplitude for a number of cells (Fig. 9C). These results show that activation of endogenous D2-dopamine receptors can hyperpolarize dopamine neurons sufficiently to recover T-type currents.

D2-receptor mRNA is more strongly expressed in the SNc than the VTA (Hurd et al., 1994) and the GIRK currents activated by dopamine appear larger in the SNc than in the VTA (Sharpe et al., 2014). However, it is not known whether the Calb+ and Calb− neurons of the SNc differ in their response to dopamine. Therefore, dopamine iontophoresis (8 s) was applied to the soma of Calb+ and Calb− neurons while the soma was repeatedly depolarized by small subthreshold current injection (10 pulses, 3 ms width, 200 pA, 5 ms interstimulus interval). Dopamine iontophoresis resulted in fast-acting membrane hyperpolarization in both subpopulations. Cells that did not hyperpolarize to ≥ -60 mV (1 of 10 Calb− and 2 of 8 Calb+ neurons) were not used. On average, the extent of dopamine-mediated hyperpolarization was similar in Calb+ (-67.8 ± 1.3 mV, $n = 6$ cells from 6 mice) and Calb− (-66.7 ± 1.1 mV, $n = 9$ cells from 6 mice) dopamine neurons (Mann–Whitney U test, $p = 0.39$). In the presence of dopamine, the subthreshold depolarizing current was sufficient to generate low-threshold depolarizations in Calb− neurons [AUC, 3.1 ± 0.5 mV*s, $n = 9$ cells from 6 mice], but not Calb+ neurons (AUC, 1.09 ± 0.09 mV*s, $n = 6$ cells from 6 mice; Mann–Whitney U test, $p = 0.0008$; Fig. 10A,B). Interestingly, the low-threshold depolarizations could occur in a graded manner, with depolarizing stimuli early in the iontophoresis protocol often showing “partial” low-threshold depolarizations (Fig. 10C), likely due to incomplete recovery of T-type channels.

Altogether, these results demonstrate that dopamine hyperpolarizes both Calb+ and Calb− neurons, but selectively increases the sensitivity of Calb− neurons to depolarizing input through recruitment of T-type Ca^{2+} channels.

Discussion

Calb+ and Calb− dopamine neurons are distinct SNc subpopulations

Here, we report that Ca^{2+} channel composition differs between the ventral tier Calb− and dorsal tier Calb+ neurons within the SNc, which results in differences in their physiological response to dopamine. We show that dopamine-mediated hyperpolarization results in the amplification of excitatory signals and enables low-threshold depolarizations and large dendritic Ca^{2+} signals selectively in the Calb− subpopulation. These low-threshold depolarizations can occur in a graded manner at moderately hyperpolarized potentials (-70 to -60 mV) due to recruitment of T-type Ca^{2+} channels. We show that T-type Ca^{2+} currents contribute to a rapid recovery of firing following hyperpolarization

Figure 9. D2-receptor activation recruits T-type channels in a graded manner. **A**, Example voltage (top) and Ca^{2+} (bottom) traces from quinpirole puff experiment. Quinpirole is applied at the 1 min mark. Note that both the voltage membrane potential and the baseline Ca^{2+} decrease in amplitude with quinpirole application. APs are truncated. **B**, Time course for effect of quinpirole puff on Ca^{2+} , AUC, and baseline membrane potential (BL). Data presented as mean $\pm$ SEM. **C**, All data points from each cell plotted against membrane voltage for Ca^{2+} amplitude (bottom) and AUC (top). Each cell is represented by a different color and symbol. AUC and Ca^{2+} increase with increased hyperpolarization in a graded manner.

in Calb− neurons. Voltage-clamp recordings of ramp currents reveal a high density of T-type current in Calb− neurons with relatively little present in Calb+ neurons. Altogether, these results show that Calb+ and Calb− SNc dopamine neurons differ in their response to dopamine D2-receptor autoinhibition due to differential expression of T-type current.

These findings support that idea that Calb+ and Calb− dopamine neurons within the SNc form functionally distinct populations that likely transmit separate dopamine signals to the striatum. Important prior studies that also support this idea have reported cellular differences in the expression of I_h (Neuhoff et al., 2002), as well as differences in tonic and burst firing during *in vivo* experiments (Schiemann et al., 2012). Further evidence for parallel signaling between dorsal and ventral tier SNc dopamine neurons comes from anatomical studies showing that the axonal projections of Calb− neurons tend to target patch compartments while Calb+ neurons prefer to innervate the matrix (Gerfen et al., 1987; Prensa and Parent, 2001; Bentivoglio and Morelli, 2005; Matsuda et al., 2009).

Our results clearly show subpopulation differences, with the Ca^{2+} amplitudes from Calb+ neurons forming a discrete cluster

Figure 10. Dopamine differentially modulates Calb+ and Calb- neurons. **A**, Example traces of activity in two Calb- (yellow) and one Calb+ (blue) SNc dopamine neurons during dopamine iontophoresis. Depolarizing input trains (10 pulses, 200 pA) were applied periodically to cells (I inject). **B**, AUC for all Calb+ (blue) and Calb- (yellow) neurons during dopamine iontophoresis plotted by baseline membrane potential (Baseline). **C**, AUC and baseline voltage (bl) for Calb+ (blue) and Calb- (yellow) neurons measured at different time points from start of iontophoresis. Data are presented as means $\pm$ SEM. Error bars fall within symbols. **D**, Example traces from cell 2 at an early (yellow) and a later time point (orange). Blue trace is from a Calb+ cell. Open arrows correspond to time points indicated in **C**.

of data points (Fig. 2E). However, the data from Calb- neurons tended to span a wider range of values in their low-threshold depolarization amplitudes and dendritic Ca^{2+} signals (Fig. 2E), suggesting that additional diversity may exist within the Calb- subpopulation. The possibility that additional subpopulations exist is supported by *in vivo* experiments showing that lateral and medial dopamine neurons exhibit differences in ATP-sensitive potassium (K_{ATP}) channel-dependent bursting (Schiemann et al., 2012) and responses to aversive stimuli (Matsumoto and Hikosaka, 2009; Lerner et al., 2015). These studies did not investigate responses in the Calb+ and Calb- subpopulations (but see Brown et al., 2009), but found that the lateral SNc neurons were excited by aversive stimuli, while medial SNc neurons were inhibited. Interestingly, Lerner et al. (2015) found that inhibited

neurons displayed a subsequent rebound Ca^{2+} signal. Fiorillo et al. (2013) compared rebound activity after aversive pauses between dorsal and ventral SNc neurons and found that rebound excitation occurred selectively in the ventral SNc neurons, suggesting that the ventral Calb- neurons are more likely to rebound *in vivo*.

In addition to the differential response to rewarding and aversive stimuli, behavioral studies have shown that subsets of dopamine neurons were activated or inactivated at movement onset (Jin and Costa, 2010; Dodson et al., 2016). This work has been corroborated by Ca^{2+} imaging studies both in mice and in flies that show that separate subsets of dopamine neurons are selectively activated by either reward or movement (Cohn et al., 2015; Howe and Dombeck, 2016). Future work is needed to determine

whether the subpopulations defined by differential *in vivo* activity correspond to the Calb $^-$ and Calb $^+$ neurons, or whether they represent further diversity within the Calb $^-$ subpopulation.

Interactions of T-type Ca^{2+} currents with I_h

We have shown that T-type current density is larger in Calb $^-$ SNc neurons than Calb $^+$ SNc neurons, and that this difference profoundly alters these subpopulations' responses to excitation from hyperpolarized potentials. However, other hyperpolarization-dependent currents, such as I_h , also differ between these subpopulations (Fig. 3F; Neuhoﬀ et al., 2002). While T-type current and I_h may seem to perform similar functions in their strengthening of rebound firing (Fig. 7), studies in other neuronal cell types have described opposing roles for T-type currents and I_h in control of dendritic excitability. For example, dendritic T-type current broadens the time window for synaptic integration (Egger, 2008) while I_h shortens it (Magee, 1998). In CA1 cells, I_h inhibits excitability of the distal dendrites by depolarizing the membrane potential, which leads to inactivation of distally located T-type channels (Tsai et al., 2007). Although I_h has been shown to limit dendritic excitability in SNc dopamine neurons (Engel and Seutin, 2015), we show that dopamine D2-autoreceptor inhibition can provide sufficient hyperpolarization to recover T-type Ca^{2+} currents and enable ampliﬁcation of excitatory inputs in Calb $^-$ neurons.

Functional role for T-type Ca^{2+} channels in Calb $^-$ SNc neurons

In considering the function of the high density of T-type current in Calb $^-$ SNc neurons, it is important to understand the physiological mechanisms that can hyperpolarize dopamine neurons to strongly recruit T-type currents and enable low-threshold depolarizations. SNc dopamine neurons can be hyperpolarized by incoming inhibitory signals, activating both GABA $_A$ and GABA $_B$ receptors, by D2-receptor autoinhibition, and through Katp channels, which will hyperpolarize them during periods of metabolic stress. Because the chloride reversal potential in dopamine neurons is relatively depolarized (-62 to -69 mV; Grace and Bunney, 1985; Gulácsi et al., 2003), GABA $_A$ inhibition most likely occurs through a shunting mechanism rather than through direct hyperpolarization. Therefore, as in the cerebellum (Alviña et al., 2008; Zheng and Raman, 2009), GABA $_A$ is not likely to strongly recruit T-type channels or facilitate low-threshold depolarizations. Alternatively, dopamine D2-autoreceptors and GABA $_B$ receptors can potentially hyperpolarize SNc dopamine neurons. Both D2 and GABA $_B$ receptors couple to GIRK channels that can drive the membrane potential close to the potassium reversal potential. We demonstrate in Figures 9 and 10 that D2-receptor activation can generate sufficient hyperpolarization to effectively engage T-type current and enable regenerative low-threshold depolarizations. Physiologically, dopamine is released through dendrodendritic synapses between dopamine neurons (Chen and Rice, 2002; Beckstead et al., 2004; Gantz et al., 2013) and a global increase in dopaminergic tone could be achieved through blocking dopamine reuptake with cocaine (Lacey et al., 1990). Therefore, GIRK-dependent hyperpolarization represents a physiological mechanism for the effective recruitment of T-type Ca^{2+} channels.

In addition to showing that strong hyperpolarization can effectively recover T-type channels from inactivation and enable regenerative Ca^{2+} events, we propose that T-type currents may contribute to the activity of Calb $^-$ SNc neurons when partially recovered from inactivation. Our voltage-clamp recordings dem-

onstrate the presence of a T-type Ca^{2+} channel-mediated ramp current active during voltage ramps from -70 to -40 mV in Calb $^-$ SNc dopamine neurons (Fig. 8). These findings are consistent with data from the thalamus (Dreyfus et al., 2010) and locus ceruleus (Matschke et al., 2015) reporting observations of a T-type “window current,” which is a small, subthreshold current thought to result from overlap of activation and inactivation curves. In locus ceruleus neurons, T-type window currents in conjunction with current from L-type Ca^{2+} channels have been shown to contribute to the robustness of pacemaking (Matschke et al., 2015). Although our experiments in SNc dopamine neurons do not directly test the role of T-type currents in pacemaking, two existing studies show clear effects of pharmacological block of T-type channels on spontaneous firing in SNc neurons. Wolfart and Roeper (2002) found that T-type channels contribute to the regularity of pacemaking in cells from juvenile mice through coupling to calcium-activated potassium channels, while Poetschke et al. (2015) found that block of T-type current slowed pacemaking by 30% in adult dopamine neurons. Our voltage-clamp results are consistent with a role for T-type channels in spontaneous pacemaking, but predict that such a role would be limited to the Calb $^-$ SNc subpopulation.

Conclusion

Our work shows that the Calb $^+$ and Calb $^-$ SNc dopamine neurons represent distinct subpopulations that differ in their Ca^{2+} channel composition. The Calb $^-$ SNc neurons have a paradoxical increase in dendritic excitability in response to D2-mediated hyperpolarization due to their higher density of T-type Ca^{2+} current. The higher expression of both T-type current and I_h allows Calb $^-$ neurons to recover more quickly from periods of inhibition and may facilitate rebound bursts. By contrast, Calb $^+$ neurons will be strictly inhibited by hyperpolarization and likely require strong excitatory synaptic activity to burst or to return to tonic firing. These differential responses to hyperpolarization may reflect differences in function. For example, if dopamine released from the Calb $^-$ neurons is important for movement, the ability to quickly return to tonic firing after or during a period of inhibition may be essential for maintaining tonic levels of dopamine in the striatum. Similarly, if dopamine from Calb $^+$ neurons is important for signaling rewarding or aversive events, the ability to be effectively silenced by inhibition may be critical for the timing of learning signals.

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