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Intrinsic dendritic integration features of prefrontal layer 5 pyramidal cell subclasses

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Abstract

Prefrontal cortex (PFC) is an associative center in the brain and integrates various inputs to support cognition. Layer 5 pyramidal cells are themselves associative centers, as their dendrites span all cortical layers and sample multiple input streams. Backpropagating action potentials (bAPs) are an important mechanism for integrating synaptic inputs arriving at distinct dendritic locations. bAPs originating in the axon initial segment can depolarize the apical dendrite, activate voltage-gated currents that underlie dendritic processing and synaptic plasticity, and influence the integration of synaptic inputs arriving onto apical dendrites. How effectively bAPs depolarize apical dendrites depends on cell type, dendritic morphology, and the dendrite's passive and active properties. Here, we found that in a unique subclass of PFC layer 5 pyramidal cell defined by D3 dopamine receptor (D3R) expression, dendritic calcium responses to bAP stimuli were far greater for a burst of APs than expected from a linear sum of single AP-evoked events in mice of either sex. D3R-expressing neurons electrophysiologically resemble intratelencephalic, D1R-expressing pyramidal neurons but morphologically resemble pyramidal tract, D2R-expressing pyramidal neurons. In both D1R- and D2R-expressing cells, burst-evoked dendritic calcium events largely reflected a linear sum of individual AP responses. In D1R neurons, this was partially due to large conductance calcium-activated potassium (BK) channels, while in D2R neurons, both BK and hyperpolarization-activated cyclic nucleotide–gated channels contributed. These data demonstrate that the intrinsic dendritic excitability of PFC layer 5 pyramidal cells widely differs and suggest that nonlinear dendritic excitability in D3R-expressing neurons uniquely positions these cells within PFC circuits.

Significance Statement

Layer 5 pyramidal cells associate inputs from diverse information streams to shape behavior. Backpropagating action potentials (bAPs) enable the integration of synaptic inputs that arrive coincidentally on the basal and apical dendrites, but the extent to which bAPs depolarize the apical dendrites can vary across cell types and dendritic morphologies. In prefrontal cortex, layer 5 pyramidal cells can be distinguished based on expression of the D1, D2, or D3 dopamine receptor expression. Here, we examined intrinsic dendritic excitability across D1R, D2R and D3R-expressing neurons and found that bAP-associated dendritic calcium transients vary considerably across these three intermingled neuronal subtypes, suggesting that these pyramidal cell classes have unique roles in prefrontal cortex processing.

Introduction

Neocortical layer 5 pyramidal cells have dendrites that traverse all layers and are thought to act as coincidence detectors, firing in response to temporally convergent local and long-range synaptic input arriving onto different dendritic compartments from diverse sources (Larkum et al., 1999b; Harris and Shepherd, 2015; Burke et al., 2018; Anastasiades et al., 2021; Anastasiades and Carter, 2021; Shepherd and Yamawaki, 2021; Lafourcade et al., 2022). Coincidence detection is supported partly by backpropagating action potentials (bAPs), wherein APs originating at the axon initial segment depolarize dendritic regions, thus providing local signals that can shape dendritic integration and activity-dependent synaptic plasticity (Linden, 1999; Häusser et al., 2000; Kampa et al., 2006; Feldman, 2012; Ledergerber and Larkum, 2012). Additionally, bAPs can recruit dendritic voltage-gated calcium channels (Ca_Vs), further depolarizing membranes and contributing to local biochemical signaling. How effectively bAPs recruit dendritic Ca_Vs can depend on dendritic arborization, passive membrane properties, and the distribution of ion channels and receptors in those arbors (Magee and Carruth, 1999; Golding et al., 2001; Vetter et al., 2001; Tsay and Yuste, 2002; Stuart and Spruston, 2015). Differences in these properties imbue pyramidal cell classes with distinct integrative properties, which are thought to allow cells to respond preferentially to synaptic input arriving at differing frequencies and locations.

Prefrontal cortex (PFC) integrates local and long-range inputs from cortical and subcortical structures to support cognitive functions (Miller and Cohen, 2001; Parent et al., 2010; Euston et al., 2012; DeNardo et al., 2015; Collins et al., 2018). In mouse PFC, layer 5 pyramidal cell classes can be distinguished based on morphology, intrinsic properties, projection targets, or dopamine receptor expression patterns (Morishima and Kawaguchi, 2006; Dembrow et al., 2010, 2015; Gee et al., 2012; Seong and Carter, 2012; Lee et al., 2014; Clarkson et al., 2017; Collins et al., 2018). The morphology of D1 dopamine receptor (D1R)-expressing pyramidal cells is consistent with intratelencephalic neurons, with thin apical dendritic shafts, apical tufts with few branches, and a narrow layer 1 expansion. Cells that express D2R, by contrast, morphologically resemble the pyramidal tract subtype, with thick dendritic shafts, highly arborized tufts, and extensive layer 1 lateral projections (Morishima and Kawaguchi, 2006; Dembrow et al., 2010; Gee et al., 2012; Seong and Carter, 2012; Clarkson et al., 2017). Consistent with prior reports, these cells can also be separated into two classes based on intrinsic electrophysiological properties (Type 1: D1R+; Type 2: D2R+). Beyond D1R and D2R, layer 5 mouse PFC contains pyramidal cells that almost exclusively express D3R (Clarkson et al., 2017). We showed previously that D3R-expressing

pyramidal cells have their own electrophysiological signature (Type 3) and anatomical profile, with large apical arborizations (Clarkson et al., 2017).

These electrophysiological and anatomical distinctions suggest that layer 5 pyramidal cell subclasses may have differential dendritic integration features. To test this, we assessed dendritic calcium associated with bAPs in Type 1, 2, and 3 neurons. In Type 3 neurons, we found that bursts of APs resulted in supralinear dendritic calcium transients that were far greater than the linear sum expected from single APs. Conversely, Type 1 and 2 cells exhibited burst-related signals that more closely matched those expected from linear sums of single APs in most of the dendritic arbor; supralinearity was observed only in the distal-most apical tufts. By pharmacologically manipulating dendritic excitability, we found that supralinearities were restricted by recruitment of BK channels in Type 1 cells and a combination of BK and hyperpolarization-activated cyclic nucleotide–gated (HCN) channels in Type 2 cells. Calcium transients in Type 3 dendrites, by contrast, were largely insensitive to BK or HCN block. Instead, combined activation of dendritic Ca_V1 and Ca_V3 channels supported supralinearities in Type 3 cells. Our data demonstrate that PFC layer 5 pyramidal cell classes have different complements of ion channels expressed in their dendrites that result in distinct dendritic calcium responses and suggest that information flow across these three neuronal subtypes is specifically integrated and regulated.

Materials and Methods

Electrophysiology

All experiments were in accordance with guidelines set by the University of California, San Francisco Institutional Animal Care and Use Committee. Postnatal day (P) 28-67 wild type or transgenic mice on a C57Bl/6J background were used, and transgenic animals (D1-tdTomato, D3-Cre::Ai14) were genotyped by PCR. Transgenic mouse lines had the following research resource identifiers: D1-tdTomato, IMSR_JAX:016204; D3-Cre (KJ302), MMRRC_034696-UCD. Experiments were performed on mice of both sexes except in the case of D3-Cre animals, as Cre expression is linked to Y chromosome expression in this line.

Mice were anesthetized, and 250 µm-thick coronal slices containing prefrontal cortex were collected. Cutting solution contained (in mM): 87 NaCl, 25 $NaHCO_3$, 25 glucose, 75 sucrose, 2.5 KCl, 1.25 NaH_2PO_4, 0.5 $CaCl_2$ and 7 $MgCl_2$, bubbled with 5% CO_2/95%O_2. Following cutting, slices were incubated in the same solution for 30 min at 33°C, then at room temperature until recording. Recording solution contained the following (in mM): 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 25 glucose, bubbled with 5% CO_2/95% O_2. Osmolarity of the recording solution was adjusted to ~310 mOsm. Experiments were conducted at 31-34°C, and temperature was controlled using an in-line heater (Warner Instruments).

Patch electrodes were pulled from Schott 8250 glass (3-4 MΩ tip resistance) and filled with a solution containing the following (in mM): 113 K-gluconate, 9 HEPES, 4.5 $MgCl_2$, 14 Tris2-phosphocreatine, 4 Na_2-ATP, and 0.3 tris-GTP at ~290 mOsm and a pH of 7.2-7.25; 250 µM Fluo-5F and 20 µM Alexa Fluor 594 (Abcam) were added for all experiments to keep calcium buffering consistent.

Electrophysiological data were acquired using a Multiclamp 700B amplifier (Molecular Devices) and custom Igor-Pro (Wavemetrics) routines. All recordings were made using a quartz electrode holder (Sutter Instrument) to eliminate electrode drift within the slice. Data were acquired at 20 or 50 kHz and filtered at 3 kHz or 10 kHz, respectively. Pipette capacitance was compensated by 50% of the fast capacitance measured under gigaohm seal conditions in voltage-clamp prior to establishing a whole-cell configuration, and the bridge was balanced. Data were corrected for a 12 mV junction potential. Cells were held with constant bias current to -80 mV. For calcium imaging experiments, we evoked either an “intrinsic doublet” of APs, which is a pair of APs generated by neurons in response to 20 ms current steps with instantaneous frequencies >100 Hz, or a set of 3 APs at an instantaneous frequency of 50 Hz. In all experiments, calcium

signals evoked by these bursts were compared within-trial to the response to a single AP. The single AP was evoked first (stimulus: 2 nA somatic current for 2 ms), followed by doublets (20 ms, adjusted per cell to evoke doublets) or 3x50 Hz triplets (2 nA somatic current for 2 ms, 20 ms interstimulus interval) 1.3–1.8 sec later.

Neurons were visualized using Dodt contrast optics for conventional visually guided whole-cell recording or with 2-photon-guided imaging of reporter-driven tdTomato fluorescence overlaid on an image of the slice. Pyramidal cells in layer 5 were selected for whole-cell recording and subsequent division into "Types." Type 2 (presumed D2+) neurons were identified by their characteristic response to a -400 pA, 120 ms current step from rest, the lack of tdTomato fluorescence (i.e., if experiments were done in a D1-tdTomato or D3-Cre::Ai14 animal) if available, and confirmed with morphology. Negative current steps activate HCN channels, which are expressed at high levels in PT, D2R expressing neurons (Dembrow et al., 2010). Current steps result in characteristic voltage sag during and rebound overshoot following current offset. In prior work, we defined Type 2 neurons as all cells with peak rebound overshoot that occurred within 90 ms of current offset. This identified 74% of all D2+ cells (experiments done in a Drd2-GFP line) and only 4% and 6% of D1R and D3R expressing cells (Clarkson et al., 2017). For non-Type 2/D2R+ cell identification, many experiments were performed by targeting tdTomato fluorescence in D1-tdTomato or D3-Cre::Ai14 mice. In these cases, cells were further selected by confirming they had Type 1 or Type 3 electrophysiological properties. In a subset of cases, experiments were performed on presumed D1R and D3R expressing neurons using Type 1 and Type 3 classes electrophysiological criteria alone, as this typing scheme correlates with D1R and D3R expression in >90% of cases (Clarkson et al., 2017). Those cells that could not be Typed definitively were not included in any analysis.

Imaging

Two-photon imaging was performed as previously described (Bender and Trussell, 2009) using a Chameleon Ultra II laser (Coherent) tuned to 810 nm to identify tdTomato-positive cells or to 770 nm for calcium imaging. A 40x, 0.8 NA objective and 1.4 NA oil immersion condenser (Olympus) were used to capture fluorescence. Fluorescence was split into red and green channels using dichroic mirrors and bandpass filters (575 DCXR, ET525/70 m-2p, ET620/60 m-2p, Chroma OR T560LPXR, ET525/50, ET620/60; Chroma). Red fluorescence (Alexa Fluor 594)

was collected with R9110 photomultiplier tubes (PMTs, Hamamatsu) and green fluorescence (Fluo-5F) with H10770PA-40 PMTs.

Calcium imaging data were collected in linescan mode, where the laser was repeatedly scanned over a ~5 µm section of the dendrite at a rate of 0.5 kHz. Data were averaged over 10-20 trials for each imaging site and reported as $\Delta G/G_{sat}$ $(\Delta G/R)/(G/R)_{sat}*100$, where $(G/R)_{sat}$ was the maximal fluorescence in saturating 2 mM calcium. The peak of each calcium transient was calculated from an exponential fit to the fluorescence decay following stimulus offset. Importantly, single and burst calcium transients were collected in the same sweep for each trial. Expected burst calcium transients were calculated by fitting exponentials to the calcium transients for the single AP data and by summing three of those exponentials, offset by the AP timing. Analysis was done using custom MATLAB scripts.

We (unpublished) and others (Gulledge and Stuart, 2003) have noted run-down of calcium signals in dendrites over prolonged experimental time periods like those required for within-cell drug wash-in experiments. We therefore chose the alternative population approach, matching calcium imaging experiments to similar time periods after establishing whole-cell configurations for experiments in **Fig. 4-6**. Furthermore, ratios between single APs and bursts are always done in a "within-trial" design, where single APs and bursts are evoked within seconds of each other. This helps control for whole-cell related changes in signals.

Chemicals

All calcium imaging experiments were done in the presence of 10 µM DNQX, 10 µM R-CPP, and 25 µM picrotoxin (Tocris). DNQX was dissolved in DMSO (50 mM stock solution) and added to the recording solution for a final DMSO concentration of 0.02%. R-CPP was dissolved in water (10 mM stock solution), and picrotoxin was dissolved in ethanol (50 mM stock solution). Nifedipine and ZD 7288 (Tocris) were dissolved in DMSO to 100 mM stock and added to the recording solution for final concentrations of 10 µM in 0.01% DMSO and 25 µM in 0.025% DMSO, respectively. TTA-P2 (Alomone Labs) was dissolved in DMSO to 10 mM stock and added to the recording solution for a final concentration of 2 µM in 0.02% DMSO. Iberiotoxin (Alomone Labs) was dissolved in water (0.5 mM stock solution) and added to the recording solution for a final concentration of 100 nM. For iberiotoxin experiments, 0.1 mg/mL bovine serum albumin was added to the recording solution to minimize peptide preabsorption. The recording solution

reservoir and all piping, except for the 30-mm length of Tygon tubing fed through the recirculation peristaltic pump (Ismatec Reglo), were made of borosilicate glass.

Statistics

Sample sizes were chosen based on standards in the field. No assumptions were made about data distributions, and unless stated otherwise, two-sided, rank-based nonparametric tests were used. Significance was set for an alpha level of 0.05, and a Holm-Sidak correction was used for multiple comparisons when appropriate. Statistical analyses were performed using MATLAB and the Real Statistic Pack plugin for Microsoft Excel (Release 8.0).

Results

AP-evoked dendritic calcium dynamics in PFC layer 5 pyramidal cells

Layer 5 pyramidal cells in mouse medial prefrontal cortex (mPFC) differentially express D1, D2 and D3 dopamine receptors in largely non-overlapping populations (Clarkson et al., 2017). Dopamine receptor expression covaries with intrinsic electrophysiological characteristics, allowing for electrophysiological classification of likely D1, D2 or D3R-expressing neurons into three "types" (Type 1, 2 and 3) (Morishima and Kawaguchi, 2006; Dembrow et al., 2010; Gee et al., 2012; Seong and Carter, 2012; Clarkson et al., 2017). Type 1 intratelencephalic (IT; D1R+) and Type 2 pyramidal tract (PT; D2R+) pyramidal neurons have different dendritic branching patterns (Morishima and Kawaguchi, 2006; Dembrow et al., 2010; Gee et al., 2012; Seong and Carter, 2012; Clarkson et al., 2017). Although Type 3 neurons (D3R+) are a subclass of IT-projecting neuron, their apical dendrites arborize in ways that resemble Type 2 more than Type 1 neurons (Clarkson et al., 2017). These anatomical and electrophysiological differences suggest that, in these intermingled pyramidal cell classes, backpropagating action potentials (bAPs) may differentially depolarize the dendritic arbor and generate dendritic calcium events.

To test this, we performed whole-cell current-clamp recordings from layer 5 pyramidal cells in acute coronal slices containing mPFC in either WT, D1-tdTomato or D3-Cre::Ai14 transgenic mouse lines. Cell types were identified by a combination of electrophysiological signatures and co-expression of tdTomato (see methods). Within these pyramidal cell classes, we used two-photon laser-scanning microscopy to image bAP-associated calcium throughout the basal and apical dendritic arbors. In contrast to axonal propagation, bAPs decrement in amplitude as they move farther from the soma into the dendrite, often failing to depolarize distal dendritic structures like the apical tuft (Stuart et al., 1997; Golding et al., 2001; Waters and Helmchen, 2004). But when packaged in bursts of certain frequencies, bAPs can evoke dendritic calcium transients in the apical tuft (Larkum et al., 1999b, 1999a; Gulledge and Stuart, 2003; Pérez-Garci et al., 2006; Boudewijns et al., 2013). We compared the bAP-associated dendritic calcium for a single AP with the calcium associated with a burst of APs (3 APs, 50 Hz interspike frequency) (**Fig. 1A,B**). (**Fig. 1B**). In a subset of experiments (9/14 Type 1 neurons, 5/11 Type 2 neurons, 11/13 Type 3 neurons), we elicited what we term an "intrinsic doublet" of APs, which often occur at the onset of prolonged somatic current stimuli, with instantaneous frequencies exceeding 100 Hz (Type 1: 138.084 ± 14.312 Hz, Type 2: 162.587 ± 25.740 Hz, Type 3: 107.705 ± 18.805 Hz) (Fig. 1A inset, 1B). Both the doublets and 3 AP bursts evoked calcium influx throughout the dendritic tree of all three neuron classes, with the largest transients occurring in the basal dendrites (**Fig 1B**).

If each AP within a burst evokes a uniform increase in dendritic calcium, then the size of the burst-evoked calcium transient should equal the linear sum of single AP-evoked events, offset by intra-burst AP timing. This is the case for initiating and propagating APs in axons (Bender and Trussell, 2009). In dendrites, calcium transients can exceed those expected from a simple sum of APs, and bAP-mediated dendritic calcium can shape dendritic processing and synaptic plasticity (Larkum et al., 1999b; Linden, 1999; Häusser et al., 2000; Kampa et al., 2006; Feldman, 2012; Ledergerber and Larkum, 2012; Larkum, 2013). To determine how different pyramidal cell classes respond to bAP bursts, and if they are prone to evoke dendritic supralinearities, we calculated the ratio between observed calcium evoked by a burst and that expected from the linear sum of single AP-evoked calcium influx (**Fig. 1A, C)**. These ratios were largely linear in all Type 1 and 2 cells in basal dendrites (filled circles at negative distances from the soma in **Fig. 1C**), but were instead modestly supralinear in Type 3 basal dendrites. Similar relationships were found in the primary apical dendritic shaft and apical obliques. Upon entering the apical tuft (open circles), supralinearity increased in all cell types. Most strikingly, we found that single APs failed to evoke calcium influx at locations that responded robustly to bursts in Type 3 neurons, producing "infinite" observed/predicted ratios (**Fig. 1A, C**). There were far fewer "infinite" supralinear calcium events in Type 1 and 2 neurons and they occurred more distally from the soma relative to those in Type 3 neurons (**Fig. 1A, C**).

To better quantify the differences in dendritic calcium across cell types, we also plotted the calcium response distributions separately for the apical tufts, apical shafts/obliques, and basal dendrites (**Fig. 2A, B**). Instead of normalizing events to single AP predictions (**Fig. 1C**), we transformed data using a "1-(expected calcium/observed calcium)" function. Here, linear predictions would result in a value of 0, sublinear events would be <0 and supralinear events would be >0, with those producing "infinite" points in Fig. 1C at 1.0 (**Fig. 2A**). This analysis confirmed that Type 3 cell responses were more supralinear than Type 1 or 2 in basal dendrites (**Fig. 2B, C**; Type 1 median: 0.011, interquartile range (IQR): -0.029-0.087; Type 2 median: 0.022, IQR: -0.072-0.133; Type 3 median: 0.214, IQR: 0.124-0.260). In apical shafts/obliques, the calcium responses for Type 1s and 3s were statistically more nonlinear than Type 2s (**Fig. 2B, C**; Type 1 median: 0.138, IQR: 0.005-0.256; Type 2 median: 0.0290, IQR: -0.082-0.053; Type 3 median: 0.180, IQR: 0.131-0.322). The most overt differences were in the apical tufts, however, where the dendritic calcium events were all statistically significant from one another across cell classes. Type 2 neurons were the most linear. Type 1s had modest supralinearities, but in Type

3 neurons, the distribution was skewed markedly towards 1.0 (**Fig. 2C**; Type 1 median: 0.388, IQR: 0.169-0.643; Type 2 median: 0.068, IQR: -0.083-0.340; Type 3 median: 1, IQR: 0.499-1). These data demonstrate that the apical tufts of Type 3 neurons are tuned for nonlinear bAP-associated calcium transients and suggest that bursts are a privileged form of backpropagating signal in the apical tufts of Type 3 neurons, setting them apart from the Type 1 and 2 classes.

Enrichment of HCN channels in Type 2 neurons restricts their dendritic nonlinearities

Our results suggest that there are biophysical mechanisms that differentiate Type 1 and 2 neurons from Type 3 cells. Previous work has shown that thick-tufted PFC layer 5 pyramidal cells, corresponding to Type 2 neurons here, have a high density of hyperpolarization-activated cyclic nucleotide-gated channels (HCN) compared to other layer 5 pyramidal cells (Morishima and Kawaguchi, 2006; Dembrow et al., 2010; Gee et al., 2012; Seong and Carter, 2012; Clarkson et al., 2017). We therefore hypothesized that a lack of HCN channels may be one such biophysical mechanism that distinguishes Type 3 neurons from the other subtypes.

To test for HCN channel activity across cell types, we performed whole-cell current-clamp recordings from Type 1, 2, and 3 pyramidal cells in PFC. We measured the voltage response to a hyperpolarizing current step in the absence or presence of the HCN antagonist ZD 7288 (25 μM) (**Fig. 3A**). Consistent with other studies, ZD 7288 had the largest effect on Type 2 neurons (Dembrow et al., 2010), where steady-state voltage responses increased by 73 ± 8% (**Fig. 3A, B**). In Type 1 neurons, the increase was only 37 ± 8%, and relatively little increase (9 ± 3%) was observed in Type 3 neurons (**Fig. 3A, B**). As a second measure of HCN function, we assessed subthreshold membrane resonance with a sinusoidal current injection that linearly increased in frequency from 0 to 15 Hz over 15 s (**Fig. 3C**). Type 2 neurons were the only class to exhibit resonance (2.7 ± 0.2 Hz), consistent with higher HCN channel expression levels (Hu et al., 2002; Dembrow et al., 2010). This resonant peak was eliminated following ZD 7288 application, and the impedance at 0.5 Hz increased 66 ± 9% (**Fig. 3D**). In Type 1 neurons, peak impedance was observed at 0.5 Hz. Consistent with the results in **Fig. 3A, B**, ZD 7288 also increased the impedance amplitude at 0.5 Hz in these cells, but to a lesser degree (10 ± 2%) (**Fig. 3D**). In Type 3 neurons, ZD 7288 had no effect on the impedance amplitude at 0.5 Hz (-1 ± 4%) (**Fig. 3D**). These results suggest that Type 3 neurons have markedly lower HCN channel density as measured in the soma compared to Type 1 and Type 2 cells. Further, these data confirm that in

PFC, as in other brain regions, thick-tufted layer 5 (Type 2) pyramidal cells are especially enriched in HCN channels.

When expressed in apical dendrites, I_H decreases the local membrane time constant, impairing bAP summation and dendritic spike generation by shortening the decay phase between individual dendritic bAPs (Berger et al., 2003; Dembrow et al., 2015). Given the paucity of nonlinear dendritic calcium events and a higher density of HCN in Type 2 neurons (**Fig. 1, 3**), we hypothesized that blocking I_H might increase supralinearity of bAP burst-related dendritic calcium transients. To test this, we repeated the same experimental protocol as **Fig. 1** with Type 2 neurons in the presence of ZD 7288 (**Fig. 4A**). We found that calcium transients in the apical tuft were more supralinear (**Fig. 4A, B**). In fact, it appeared that dendritic calcium in response to a single AP was reduced (**Fig. 4A**), which may be related to a hyperpolarization of the dendritic membrane.

While HCN block did not result in any "infinite" calcium transients, all calculated observed/expected ratios were above the y = 1 line (**Fig. 4B**). HCN antagonism can hyperpolarize the dendrite but also increase axial resistance (Tsay et al., 2007), and in our experiments, this ultimately did not reduce the single bAP-associated calcium sufficiently to result in "infinite" calcium transients (i.e., cases where single APs fail to elicit detectable calcium influx). We compared the distribution of apical tuft calcium transients in the presence of ZD 7288 to those without (data from **Fig. 2B**) and found the distribution shifted significantly nonlinearly for Type 2 neurons but not Type 1 neurons with ZD 7288 application (**Fig. 4C**; Type 1 no drug median: 0.388, IQR: 0.169-0.643; Type 1 ZD7288 median: 0.558, IQR: 0.315-0.716; Type 2 no drug median: 0.068, IQR: -0.083-0.340; Type 2 ZD7288: 0.592, IQR: 0.484-0.693). In Type 3 neurons, there was a small but significant shift towards linearity in the distribution of apical tuft calcium transients (**Fig. 4C**; Type 3 no drug median: 1, IQR: 0.499-1; Type 3 ZD7288 median: 0.819, IQR: 0.614-0.954), suggesting that there may be HCN expression in Type 3 dendrites that is not measurable via somatic current clamp electrophysiology. Taken together, these experiments suggest that enrichment of HCN channels in Type 2, but not Type 1 or 3, pyramidal cells limits temporal windows for backpropagating AP integration in their dendrites.

BK channels limit dendritic nonlinearities in Type 1 and Type 2 neurons

Though Type 1 neurons appear to have some somatic HCN channel density, blocking HCN did not affect their dendritic calcium transients (**Fig. 4C**). This suggests that other channel classes contribute to limiting dendritic supralinearities in these neurons. Type 1 neurons can

generate high frequency AP doublets at the onset of a positive current step (Clarkson et al., 2017). Paradoxically, high frequency firing can be supported in part by potassium channels that result in large AP afterhyperpolarization, as this hyperpolarization allows sodium channels to recover from inactivation more quickly (Jaffe and Brenner, 2018). In many cell classes, these afterhyperpolarizations are supported by large/big-conductance calcium-activated potassium (BK) channels. Previous work from rat somatosensory cortex showed that BK channels are present along the dendrites of layer 5 pyramidal cells (Benhassine and Berger, 2005) and that BK block increased dendritic calcium transients in both layer 5a and 5b pyramidal cells (Benhassine and Berger, 2009; Grewe et al., 2010; Bock and Stuart, 2016). To test whether BK activation limits bAP burst supralinearities in Type 1 and 2 neurons, we again compared single bAPs to bursts, but now in the presence of the selective BK inhibitor iberiotoxin (100 nM). Consistent with a major role for BK channels in regulating dendritic calcium influx, BK block increased the number of nonlinear and "infinite" calcium events in both Type 1 and Type 2 compared to the no drug condition (data from **Fig. 2B**) (**Fig. 5A-C**; Type 1 no drug median: 0.388, IQR: 0.169-0.643; Type 1 IBTX median: 0.865, IQR 0.661-1; Type 2 no drug median: 0.068, IQR: -0.083-0.340; Type 2 IBTX median: 0.502, IQR: 0.155-0.863). Interestingly, iberiotoxin had no effect on Type 3 neuron linearity ratios (Type 3 no drug median: 1, IQR: 0.499-1; Type 3 IBTX median: 1, IQR: 0.664-1). These data suggest that BK channels contribute to limiting dendritic depolarization and restricting nonlinear calcium transients in Type 1 and Type 2 neurons, but not Type 3 neurons.

Ca_V1 and Ca_V3 channels support nonlinear dendritic calcium signals in the apical tufts of Type 3 neurons

Data described above suggest that Type 3 dendrites have little I_H and BK to limit dendritic depolarization and thus are primed to generate supralinear dendritic calcium signals. But what channels contribute to the generation of dendritic calcium supralinearities in Type 3 neurons is unclear (**Fig. 1**). Ca_V1 and Ca_V3 channels have voltage-dependent properties and kinetics that can support burst dynamics in multiple cell types (Williams and Stuart, 1999; Lacinova et al., 2008; Cain and Snutch, 2010). Previous work in neocortical pyramidal cells showed that Ca_V1 channels contribute to bAP boosting near the base of the apical tuft (Seamans et al., 1997), and apical dendrites appear enriched with Ca_V3 channels (McKay et al., 2006). Therefore, we tested whether these channel classes contribute to dendritic bAP-evoked calcium signaling by blocking them individually or together with the Ca_V1 inhibitor nifedipine (10 μM) and the Ca_V3 inhibitor TTA-P2 (2 μM).

Ca_V1 and Ca_V3 antagonists had differential effects on bAP-evoked dendritic calcium. In separate experiments, we tested the effects of antagonizing Ca_V1 and Ca_V3 alone or in combination. When examining the overall event amplitude, burst-associated calcium events were not appreciably different following Ca_V1 antagonism, whereas Ca_V3 antagonism alone or in combination with Ca_V1 antagonism consistently reduced both single bAP and burst-based bAP calcium signals, often resulting in no detectable calcium transients (**Fig. 6A**; baseline single AP $\Delta G/G_{sat}$ median: 0.235, IQR: 0.089-0.663; nifedipine single AP $\Delta G/G_{sat}$ median: 1.318, IQR: 0.566-2.012; TTA single AP $\Delta G/G_{sat}$ median: 0, IQR: 0-0.310; nifedipine and TTA single AP $\Delta G/G_{sat}$ median: 0, IQR: 0-0; baseline burst $\Delta G/G_{sat}$ median: 7.566, IQR: 2.585-7.620; nifedipine burst $\Delta G/G_{sat}$ median: 7.469, IQR: 5.515-9.483; TTA burst $\Delta G/G_{sat}$ median: 1, IQR: 0-1.975; nifedipine and TTA burst $\Delta G/G_{sat}$ median: 0, IQR: 0-4.378). When examining supralinear calcium responses in the dendritic tuft, there were fewer "infinite" responses, where a dendritic site had no calcium signal in response to a single bAP but responded robustly to a bAP burst, with Ca_V1 or Ca_V3 antagonism alone or in combination (**Fig. 6B**; no drug median: 1, IQR: 0.499-1; nifedipine median: 0.480, IQR: 0.248-0.791; TTA median: 0.699, IQR: 0.508-0.886; nifedipine and TTA median: 0.878, IQR: 0.544-1). However, these histograms do not capture the full effect of Ca_V3 or combined Ca_V1 and Ca_V3 antagonism. In the apical tuft, Ca_V3 antagonism alone was sufficient to eliminate both single bAP and burst bAP events at 19 of 46 (41%) sites imaged (**Fig. 6C**). Similarly, 17 of 35, or 49%, of sites had no detectable calcium when both Ca_V1 and Ca_V3 were blocked together. Taken together, our data suggest that these two channel classes contribute much of the bAP-evoked calcium in the apical dendrites of Type 3 neurons, and that a smaller component is likely mediated by the Ca_V2 class (Almog and Korngreen, 2009; Blömer et al., 2024). These data suggest that Ca_V3 channels, which can activate at membrane potentials more hyperpolarized than other Ca_Vs, are critical for the initial generation of dendritic calcium signals. Once initiated, Ca_V1 channels can provide additional calcium influx, giving rise to larger events, especially when bAPs occur in bursts.

Discussion

Here, we show that backpropagating action potential (bAP)-evoked calcium transients vary across three classes of layer 5 pyramidal cell in prefrontal cortex (PFC). This work builds on prior studies that examined bAP-evoked dendritic calcium signals in PFC layer 5 cells, independent of cell class (Seamans et al., 1997; Gulledge and Stuart, 2003), or dividing between IT and PT classes (Dembrow et al., 2015). Here, we extend these approaches to Type 3 PFC layer 5 pyramidal cell (Clarkson et al., 2017) and further identify mechanisms that support supralinear responsiveness across cell types.

Marked generation of supralinearities in apical dendritic calcium transients set Type 3 neurons apart from Types 1 and 2 classes. Supralinear calcium transients were modest in Type 1 neurons and were present only in the most distal apical tufts of Type 2 neurons. In Type 1 neurons, BK potassium channels acted to restrict dendritic depolarization, and in Type 2 neurons, a combination of BK and HCN channel activity limited bAP integration and dendritic nonlinearities. By contrast, concerted activation of Ca_V1 and Ca_V3 calcium channels contributed to the nonlinear calcium signals in Type 3 neurons. These properties may allow these different types of pyramidal cells to integrate information in unique ways.

Dendritic calcium in Type 3 PFC layer 5 pyramidal neurons

Our results suggest that the apical dendrites of Type 3 neurons are in a position to generate supralinear calcium signals in part due to little I_H and BK currents that in other cell types limit dendritic depolarization (Berger et al., 2003; Benhassine and Berger, 2009; Grewe et al., 2010; Dembrow et al., 2015; Bock and Stuart, 2016) and that Ca_V1 and Ca_V3 calcium channels mediate the much of the calcium influx (**Fig. 6**). This is consistent with other studies demonstrating the presence of these calcium channels in the apical dendrites of pyramidal cells in rat neocortex (Markram and Sakmann, 1994; Markram et al., 1995). Nonlinear calcium events were not blocked completely with Ca_V1 and Ca_V3 channel block alone, suggesting that there are other calcium sources. Ca_V2 is also expressed in the dendrites of pyramidal cells (Markram et al., 1995), and Ca_V1 and $Ca_V2.2$ channels together appear to cover the dendritic arbor. Ca_V1 channels reside more in the somatic and proximal apical dendritic regions, whereas $Ca_V2.2$ calcium channels are located throughout the apical dendrites (Westenbroek et al., 1990, 1992). In our data, blockade of Ca_V3 more effectively dampened apical dendritic nonlinearities than Ca_V1 blockade (by blocking overall calcium entry), consistent with Ca_V1 localization closer to the soma. Recently

published work showed that in mouse somatosensory cortex, Ca_V2 calcium channels interact with BK in layer 5 pyramidal cells (Blömer et al., 2024). Taken with our findings, these results suggest that in Type 3 neurons, Ca_V2 channels may provide additional calcium influx rather than interacting with BK in the apical dendrites. We did not test for complete blockade of Ca_V1, Ca_V2, and Ca_V3 channels here as our aim was not to identify all possible dendritic calcium sources but rather to identify which Ca_Vs are crucial for dendritic nonlinear calcium events in Type 3 neurons. Blocking the majority of dendritic calcium channels would render us unable to measure dendritic calcium.

Functional implications

These pyramidal cell subclasses also express different combinations of neuromodulatory receptors that undoubtedly shape dendritic integration. In rodent PFC, dopaminergic fibers are concentrated in deeper cortical layers, while norepinephrinergic axons are concentrated in layer 1 (Miner et al., 2003), D1R family signaling was shown previously to interact with both Ca_V1 and $Ca_V2.2$ channels to change the amplitude and duration of dendritic calcium potentials in PFC (Yang et al., 1999), although another study showed that PFC layer 5 pyramidal cell bAPs are insensitive to dopaminergic modulation (Gulledge and Stuart, 2003). Previous work from our lab showed that arrestin-mediated signaling via D3R can modulate Ca_V3 calcium channels in the axon initial segment of Type 3 neurons (Schamiloglu et al., 2023). This modulation can in turn reduce bursting activity in multiple cell types, including Type 3 neurons (Clarkson et al., 2017). Thus, modulation of AP initiation can in turn have effects on dendritic integration, especially in neurons like Type 3 pyramidal cells where bAP bursts evoke very different dendritic responses than single APs. This may have marked effects on how such neurons integrate apical dendritic inputs through activity-dependent plasticity mechanisms. It is unknown where D3Rs are expressed on PFC layer 5 pyramidal cells. However, given our findings in the axon initial segment, it is also possible that D3R signaling via arrestin-3 also modulates Ca_V3 in the dendrites of these pyramidal cells (Schamiloglu et al., 2023).

Our results suggest that backpropagating signals, and bursts in particular, are an important neuronal signal in PFC; however, this is predicated on the idea that bursts backpropagate in a similar manner in vivo. While the location of prelimbic and infralimbic cortex deep along the frontal lobe medial wall makes it difficult to interrogate tuft calcium dynamics in vivo directly, studies in sensory cortices suggest that spikes backpropagate to a similar extent in

vivo as in slice, and that bursts can evoke tuft calcium signals in vivo (Helmchen et al., 1999; Waters et al., 2003). Further, backpropagation can be enhanced during network up states that PFC networks enter in response to dopaminergic signals (Lewis and O'Donnell, 2000; Waters and Helmchen, 2004). In future studies, it will be critical to study dendritic integration in PFC neurons in vivo to determine when AP bursts and bAPs are recruited during PFC-dependent learning tasks.

Limitations of this study

Previous work in rodent somatosensory cortex found that only bursts of APs above a critical frequency resulted in supralinear increases in apical dendritic calcium (Larkum et al., 1999a; Kampa et al., 2006). Our study focused on bursts of 3 APs at 50 Hz, although we also tested intrinsic doublets. These doublets had instantaneous frequencies that varied by cell type (Type 1: 138.084 ± 14.312 Hz, Type 2: 162.587 ± 25.740 Hz, Type 3: 107.705 ± 18.805 Hz), but, remarkably, tended to mirror results obtained with 3x50 Hz stimulation (**Fig. 1B**). It is possible that Type 1, 2, and 3 neurons have different critical frequencies or a critical minimum number of APs whereby bursts of APs can generate supralinear bAPs. However, our results demonstrate that at least in our tested frequency regimes, there are fundamental differences between Type 3 and Type 1/2 neurons.

Intracortical inputs innervate PFC as a series of parallel fibers in layer 1 and synapse onto the apical dendritic tufts of layer 5 pyramidal cells (Yang et al., 1999). As an associative hub, PFC receives diverse long-range inputs from brain regions including thalamus and hippocampus (Parent et al., 2010; DeNardo et al., 2015; Collins et al., 2018) as well as local inhibitory inputs (Lee et al., 2014). Type 1 and 2 classes receive different proportions of synaptic inputs from different regions and those inputs have distinct synaptic properties (Lee et al., 2014; Dembrow et al., 2015). Inputs onto Type 3 neurons have not been explicitly studied. While our study focuses on the intrinsic bAP-evoked dendritic calcium properties across PFC layer 5 cell classes, in future studies, it will be important to understand how those intrinsic properties combine with incoming synaptic input. Calcium influx induced by bAPs is required for spike-timing dependent plasticity, a biologically relevant form of synaptic plasticity that depends on the timing relationship between presynaptic inputs and postsynaptic bAPs (Feldman, 2012) as well as behavioral timescale synaptic plasticity, recently observed in mPFC (Caya-Bissonnette et al., 2023). Supralinear

dendritic calcium in Type 3 neurons may strengthen or weaken inputs relative to their Type 1 and 2 neighbors depending on the specific inputs, timing, and concurrent neuromodulatory inputs.

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Author contributions

S.S., R.L.C., and K.J.B. designed research; S.S., R.L.C., and K.J.B. collected the data; S.S., N.S.S, A.T.L., R.L.C., and K.J.B. analyzed data; S.S. and K.J.B. wrote the paper.

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660

Figure Legends

Figure 1: AP-evoked dendritic calcium dynamics in PFC layer 5 pyramidal cells

A: Calcium transients for single action potentials (APs, cyan) and bursts (3x50 Hz, black) at the imaging sites in the apical tufts indicated by red circles for Type 1, 2, and 3 pyramidal cells in layer 5 of PFC. Red overlays ("Expected Burst Ca^{2+}") are exponential fits of single AP-associated calcium transients (blue), offset and summed based on the timing of APs in the burst. Note that in the Type 1 and Type 2 examples, the imaging sites are more distal from the soma compared to the Type 3 example, but the calcium transients are more linear. Type 3 inset shows the analogous experiment for an intrinsic doublet (green) rather than a stimulated burst.

B: Single AP (cyan), intrinsic doublet (green), and 3x50 Hz burst (black) calcium for each cell class, binned by dendritic distance from the soma (50 μm bins). Symbols are mean ± SEM. Basal dendrites have negative distance values. Horizontal gray bars denote 2*SD of optical signal (noise threshold). All cells were identified using a "Typing" classifier as previously described (Clarkson et al., 2017). For the intrinsic doublet data, Type 1: 62 sites from 9 cells in 7 mice; Type 2: 38 sites from 5 cells in 4 mice; Type 3: 54 sites from 11 cells in 6 mice.

C: Observed 3x50 calcium (peak of black trace in A) normalized to expected calcium transient (peak of red exponential in A). Linear calcium events are at y=1. "Infinite" values are locations in which only bursts, not single APs, evoked local calcium influx. Gray shading delineates "infinite" calcium events. Closed circles are individual imaging sites in basal dendrites or apical main/obliques. Open circles are in apical tufts. Type 1: 85 imaged sites from 14 cells in 11 mice; Type 2: 85 imaged sites from 11 cells in 8 mice; Type 3: 72 imaged sites from 13 cells in 10 mice. 2/12 and 3/11 Type 1 and Type 2 neurons in which apical tufts were imaged had infinite calcium events compared to 9/12 Type 3 neurons.

Figure 2: Type 3 neurons have significantly more nonlinear dendritic calcium transients

A: Example calcium transients in a Type 3 cell for single action potentials (APs, cyan) and bursts (3x50 Hz, black) at the two imaging sites indicated by red circles. Red overlays denote the expected burst calcium. The calcium transient is linear, where 1-(expected/observed) is close to 0, in the proximal apical shaft and is supralinear, where 1-(expected/observed) is close to 1, in the distal tuft.

B: The distribution of sublinear, linear, and supralinear calcium events in the apical tuft (top row), apical shaft (middle), and basal dendrites (bottom row) across Type 1, 2, and 3 pyramidal neurons. Same data as in Fig. 1C, now plotted as 1-(expected calcium transients normalized to observed calcium transients), such that linear transients have values close to 0, sublinear events are <0, and supralinear calcium events are >0. Events in which only bursts, not single APs, evoked local calcium influx have values of 1. Type 1 apical tuft: 33 sites from 10 cells in 8 mice; Type 2 apical tuft: 36 sites from 9 cells in 6 mice; Type 3 apical tuft: 41 sites from 12 cells in 9 mice. Type 1 apical shaft: 25 sites from 11 cells in 9 mice; Type 2 apical shaft: 29 sites from 10 cells in 5 mice; Type 3 apical shaft: 14 sites from 5 cells in 4 mice. Type 1 basal: 27 sites from 11 cells in 9 mice; Type 2 basal: 21 sites from 11 cells in 7 mice; Type 3 basal: 17 sites from 6 cells in 5 mice.

C: Overlaid calcium event distributions across cell classes for the apical tuft (top), apical shaft (middle), and basal dendrites (bottom). Type 1 is in red, Type 2 in green, and Type 3 in purple. In the apical tuft, all three distributions were significantly different from one another. Apical tuft: Type 1 vs. Type 2, $p = 0.002$; Type 1 vs. Type 3, $p < 0.001$; Type 2 vs. Type 3, $p < 0.001$; apical shaft: Type 1 vs. Type 2, $p = 0.004$; Type1 vs. Type 3, $p = 0.388$; Type 2 vs. 3, $p < 0.001$; basal dendrites: Type 1 vs. Type 2, $p = 0.950$; Type 1 vs. Type 3, $p < 0.001$; Type 2 vs. Type 3, $p < 0.001$. Kruskal-Wallis tests with Mann-Whitney U tests *post hoc* (Holm-Sidak corrections).

Figure 3: Type 2 neurons are enriched in HCN channels

A: Hyperpolarizing voltage responses before (black) and after HCN block by 25 μM ZD 7288 (green).

B: Steady-state voltage in ZD 7288 (from A), normalized to baseline value. Measurements made immediately before current offset. Circles are single cells. Bars are mean ± SEM. Type 1: 37 ± 8%, n = 6 cells from 3 mice; Type 2: 73 ± 8%, n = 7 cells from 4 mice; Type 3: 9 ± 3%, n = 7 cells from 3 mice, $p<0.001$ between all classes, Newman-Keuls test. 100%

C: Sinusoid current injection (top) and resultant subthreshold voltage response across Type 1, 2, and 3 cells before and after ZD 7288.

D: Impedance amplitude profiles across cell types before and after ZD 7288 (from C). Shadows are SEM. At 0.5 Hz, peak impedance was increased by ZD 7288 by 10 ± 2% in Type 1 neurons ($p < 0.001$, paired t-test comparing within-cell impedance at 0.5 Hz), 66 ± 9% in Type 2 neurons ($p < 0.0001$, paired t-test), and -1 ± 4% in Type 3 neurons ($p = 0.9$, paired t-test). All cells in the figure were identified using the classifier.

Figure 4: HCN channels restrict dendritic nonlinearities in Type 2 neurons

A: Calcium transients for single action potentials (APs, cyan) and bursts (3x50 Hz, black) at the imaging sites indicated by the red circles in two Type 2 cells, one in the absence of drug (left) and the other in the presence of the HCN channel blocker ZD 7288. As in **Fig. 1**, red overlays denote the expected burst Ca^{2+} and are exponential fits of single AP-associated calcium transients (blue), offset and summed based on the timing of APs in the burst.

B: Observed calcium normalized to expected calcium transient in the presence of ZD 7288. Linear calcium events are at y=1. Closed circles are individual imaging sites in apical main shafts. Open circles are in apical tufts. 64 imaged sites from 8 cells in 6 mice.

C: Comparison of the distribution of sublinear, linear, and supralinear calcium events in the apical tufts of Type 1, 2, and 3 neurons without (gray, same data as Fig. 2B) and with HCN block (green). Type 1, $p = 0.165$ (n = 33 no drug sites from 10 cells in 8 mice, n = 36 ZD 7288 sites from 9 cells in 6 mice); Type 2, $p <0.001$ (n = 36 no drug sites from 9 cells in 6 mice, n = 31 ZD 7288 sites from 8 cells in 7 mice); Type 3, $p = 0.027$ (n = 41 no drug sites from 12 cells in 9 mice, n = 27 ZD 7288 sites from 7 cells in 4 mice); Kolmogorov-Smirnov test. 78% of Type 1 cells and 57% of

Type 3 cells were identified by fluorescence and confirmed with the classifier. The remainder were identified using the classifier.

Figure 5: BK channels limit dendritic nonlinearities in Type 1 and Type 2 neurons

A**:** Calcium transients for single action potentials (APs, cyan) and bursts (3x50 Hz, black) at the imaging sites indicated by the red circles in two Type 1 cells, one in the absence of drug (left) and the other in the presence of the BK channel blocker iberiotoxin. As in **Fig. 1** and **5**, red overlays denote the expected burst Ca^{2+}.

B: Observed calcium normalized to expected calcium transient in the presence of iberiotoxin. Linear calcium events are at y=1. “Infinite” values are locations in which only bursts, not single APs, evoked local calcium influx. Gray shading delineates “infinite” calcium events. Closed circles are individual imaging sites in apical main shafts. Open circles are in apical tufts. 53 imaged sites from 6 cells in 5 mice.

C: Comparison of the distribution of sublinear, linear, and supralinear calcium events in the apical tufts of Type 1 neurons without (gray, same data as Fig. 2B) and with BK block (orange). Events in which only bursts, not single APs, evoked local calcium influx have values of 1. Type 1, $p < 0.001$ (n = 33 no drug sites from 10 cells in 8 mice, n = 28 IBTX sites from 6 cells in 5 mice); Type 2, $p < 0.001$ (n = 36 no drug sites from 9 cells in 6 mice, n = 31 IBTX sites from 6 cells in 4 mice); Type 3, $p = 0.633$ (n = 41 no drug sites from 12 cells in 9 mice, n = 29 IBTX sites from 7 cells in 4 mice); Kolmogorov-Smirnov test. 100% of Type 1 cells and 14% of Type 3 cells were identified by fluorescence and confirmed with the classifier. The remainder were identified using the classifier.

Figure 6: Ca_V1 and Ca_V3 channels support nonlinear dendritic calcium signals in the apical tufts of Type 3 neurons

A: The distribution of apical tuft (200-450 µm from the soma) calcium transient amplitudes for single (top) or burst of APs (bottom) across baseline, nifedipine, TTA, or nifedipine and TTA conditions. For single AP: baseline vs. nifedipine, $p = 0.015$; baseline vs. TTA: $p = 0.018$; baseline vs. nifedipine and TTA: $p < 0.001$; nifedipine vs. TTA: $p < 0.001$; nifedipine vs. TTA and nifedipine: $p < 0.001$; TTA vs. nifedipine and TTA: $p = 0.016$. For burst: baseline vs. TTA: $p = 0.002$; baseline vs. nifedipine and TTA: $p < 0.001$; nifedipine vs. TTA: $p < 0.001$; nifedipine vs. TTA and nifedipine: $p < 0.001$. Kruskal-Wallis tests with Mann-Whitney U tests *post hoc* (Holm-Sidak corrections). Baseline: 25 sites from 9 cells in 7 mice; nifedipine: 25 sites from 8 cells in 4 mice; TTA: 32 sites from 11 cells in 7 mice; nifedipine and TTA: 28 sites from 7 cells in 5 mice. This analysis was performed on a subset of data where experimental conditions allowed for direct comparisons of absolute calcium signal.

B: Distribution of calcium events in the apical tuft of Type 3 neurons at baseline or in the presence of nifedipine (purple), TTA-P2 (green), or nifedipine and TTA-P2 (red). Note that we did not perform statistics on these data because the histograms do not include all of the dendritic sites where there was no detectable calcium to a single or burst of APs. Baseline: n = 41 no drug sites from 12 cells in 9 mice; nifedipine: 47 sites from 11 cells in 5 mice; TTA: 28 sites from 12 cells in 6 mice; nifedipine and TTA: 18 sites from 6 cells in 4 mice.

C: Observed calcium normalized to expected calcium transient at baseline (black, same data as Fig. 1B) or in the presence of the Ca_V1 channel blocker nifedipine (purple), the Ca_V3 channel blocker TTA-P2 (green), or both nifedipine and TTA-P2 (red). Linear calcium events are at y=1. "Infinite" values are locations in which only bursts, not single APs, evoked local calcium influx, and "no signal" values are imaged sites in which no calcium was observed for either a single AP or burst. Both are delineated by gray shading. Closed circles are individual imaging sites in apical main shafts. Open circles are in apical tufts. Baseline, n = 57 sites from 13 cells in 10 mice; nifedipine, n = 85 sites from 14 cells in 6 mice; TTA-P2: n = 87 imaged sites from 15 cells in 7 mice; nifedipine and TTA-P2: n = 53 sites from 17 cells in 5 mice. All cells were identified by fluorescence and confirmed with the classifier.