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RECEIVED 11 December 2023

ACCEPTED 24 January 2024

PUBLISHED 14 February 2024

CITATION

Blömer LA, Giacalone E, Abbas F, Filipis L,
Tegolo D, Migliore M and Canepari M (2024)
Kinetics and functional consequences of BK
channels activation by N-type Ca^{2+}
channels in the dendrite of mouse
neocortical layer-5 pyramidal neurons.
Front. Cell. Neurosci. 18:1353895.
doi: 10.3389/fncel.2024.1353895

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Kinetics and functional consequences of BK channels activation by N-type Ca^{2+} channels in the dendrite of mouse neocortical layer-5 pyramidal neurons

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The back-propagation of an action potential (AP) from the axon/soma to the dendrites plays a central role in dendritic integration. This process involves an intricate orchestration of various ion channels, but a comprehensive understanding of the contribution of each channel type remains elusive. In this study, we leverage ultrafast membrane potential recordings (V_m) and Ca^{2+} imaging techniques to shed light on the involvement of N-type voltage-gated Ca^{2+} channels (VGCCs) in layer-5 neocortical pyramidal neurons' apical dendrites. We found a selective interaction between N-type VGCCs and large-conductance Ca^{2+} -activated K^+ channels (BK CAKCs). Remarkably, we observe that BK CAKCs are activated within a mere 500 μs after the AP peak, preceding the peak of the Ca^{2+} current triggered by the AP. Consequently, when N-type VGCCs are inhibited, the early broadening of the AP shape amplifies the activity of other VGCCs, leading to an augmented total Ca^{2+} influx. A NEURON model, constructed to replicate and support these experimental results, reveals the critical coupling between N-type and BK channels. This study not only redefines the conventional role of N-type VGCCs as primarily involved in presynaptic neurotransmitter release but also establishes their distinct and essential function as activators of BK CAKCs in neuronal dendrites. Furthermore, our results provide original functional validation of a physical interaction between Ca^{2+} and K^+ channels, elucidated through ultrafast kinetic reconstruction. This insight enhances our understanding of the intricate mechanisms governing neuronal signaling and may have far-reaching implications in the field.

KEYWORDS

N-type voltage-activated Ca^{2+} channel, BK Ca^{2+} -activated K^+ channel, dendrite, action potential, neocortical layer-5 pyramidal neuron, neuron modelling

1 Introduction

In neocortical pyramidal neurons, action potentials (APs) actively propagate back into the dendritic tree (Stuart and Sakmann, 1994; Stuart et al., 1997), where they elicit transient elevations of intracellular Ca^{2+} concentration (Markram et al., 1995; Schiller et al., 1995). Analyses performed in dissociated pyramidal neurons (Stewart and Foehring, 2000) and in Layer-5 (L5) pyramidal neurons from brain slices (Markram et al., 1995; Almog and Korngreen, 2009) have shown that all high-voltage activated (HVA) VGCCs, namely L-type (Ca_v1), P/Q-type ($\text{Ca}_v2.1$), N-type ($\text{Ca}_v2.2$) and R-type ($\text{Ca}_v2.3$), contribute to the AP-mediated dendritic Ca^{2+} transient, but low-voltage activated (LVA) VGCCs (T-type, Ca_v3) may also contribute (Talley et al., 1999). Following the Ca^{2+} transient, Ca^{2+} -binding proteins can be activated by indistinct cytosolic Ca^{2+} elevation (Ghosh and Greenberg, 1995), and in this case these proteins are equally activated by Ca^{2+} ions from any contributing source. Alternatively, the proteins are selectively activated by a physically-coupled Ca^{2+} source. In this case, the protein experiences a larger Ca^{2+} elevation in a nanodomain adjacent to the Ca^{2+} source. This type of multi-protein structure is characterized in the synaptic cleft (Gandini and Zamponi, 2022).

Voltage-gated Ca^{2+} channels (VGCCs) in pyramidal neuron dendrites can target Ca^{2+} -activated K^+ channels (CAKCs), in particular SK and BK CAKCs (Sah and Davies, 2000). Both channels can be coupled with a Ca^{2+} source (Vierra and Trimmer, 2022), but it was suggested that BK CAKCs must localize closer to the Ca^{2+} source to ensure reliable Ca^{2+} -dependent activation because they have lower affinity for Ca^{2+} (Fakler and Adelman, 2008; Shah et al., 2022). In cell-attached patches from isolated CA1 hippocampal pyramidal neurons it was shown that L-type VGCCs activate exclusively SK CAKCs whereas N-type VGCCs activate BK CAKCs (Marrion and Tavalin, 1998). In contrast, whole-cell patch-clamp recordings from freshly dissociated neocortical pyramidal neurons showed that BK CAKCs are activated both by L-type and N-type VGCCs (Sun et al., 2003), consistently with the findings that both L-type VGCCs (Grunnet and Kaufmann, 2004) and N-type VGCCs (Loane et al., 2007) molecularly co-assemble with BK CAKCs. From the functional side, both CAKCs participate in shaping the AP, but with different kinetics (Sah and Davies, 2000) as also reproduced by computer modeling (Almog and Korngreen, 2014). Specifically, SK CAKCs regulate the AP waveform during the medium and late phase of the AP re-polarization with a variability that depends on the different Ca^{2+} source and neuronal types (Bond et al., 2004; Villalobos et al., 2004; Pedarzani and Stocker, 2008). Interestingly, dendritic excitability is impacted by SK CAKC activation in unpredictable manner (Bock et al., 2019). In contrast, BK CAKCs, which are functionally expressed in the dendrites L5 neocortical pyramidal neurons (Kang et al., 1996; Benhassine and Berger, 2005) regulate the AP in the early phase of re-polarization (Sun et al., 2003) when the membrane potential (V_m) is depolarized, consistently with the voltage dependence of the channel (Vergara et al., 1998; Cui, 2010).

The close interaction between the Ca^{2+} source and the BK CAKC implies the activation of the K^+ channel at sub-millisecond time scale (Berkefeld et al., 2006). In the present study, performed in L5 pyramidal neurons of the somatosensory cortex, we used

ultrafast optical measurements of V_m (Popovic et al., 2015) and Ca^{2+} currents (Jaafari et al., 2014) to investigate in parallel the kinetics of the dendritic back-propagating AP (bAP) and that of the associated Ca^{2+} current. We found that BK CAKCs are selectively activated by N-type VGCCs. Yet, whereas the peak of the Ca^{2+} current is delayed by $>500 \mu\text{s}$ with respect to the bAP peak, consistently with the kinetics of activation and deactivation of VGCCs in the order of 1 ms (Kay and Wong, 1987), the activation of BK CAKCs occurs in the first 500 μs , providing a negative feedback to the cytosolic Ca^{2+} elevation (Shah et al., 2022). We built a realistic NEURON model showing that experimental results could be reproduced by the only when N-type VGCCs and BK CAKCs were physically interacting.

2 Materials and methods

2.1 Brain slices preparation and maintenance

All experiments were performed in the “Laboratory of Interdisciplinary Physics” and mice manipulations previous to euthanasia were done in accordance with European Directives 2010/63/UE on the care, welfare and treatment of animals. Specifically, procedures were reviewed by the ethics committee affiliated to the animal facility of the university (D3842110001). Mice (C57BL/6j, 21-35 postnatal days old) purchased from Janvier Labs (Le Genest-Saint-Isle, France) were anesthetised by isoflurane inhalation and decapitated to extract the entire brain. Neocortical slices (350 μm thick) were prepared as described in recent previous reports (Filipis and Canepari, 2021; Montnach et al., 2022; Filipis et al., 2023), using a Leica VT1200 vibratome (Wetzlar, Germany). The extracellular solution contained (in mM): 125 NaCl, 26 NaHCO_3 , 1 MgSO_4 , 3 KCl, 1 NaH_2PO_4 , 2 CaCl_2 and 20 glucose, bubbled with 95% O_2 and 5% CO_2 . Slices were incubated at 37°C for 45 min and maintained at room temperature before being transferred to the recording chamber where the temperature was maintained at 32–34°C.

2.2 Electrophysiology and imaging

Slices with L5 pyramidal neurons in the somato-sensory cortex having the initial part of the apical dendrite parallel to the surface were used for the experiments. Patch-clamp (whole-cell) recordings were made using a Multiclamp 700A (Molecular Devices, Sunnyvale, CA) with a basic intracellular solution containing (in mM): 125 KMeSO_4 , 5 KCl, 8 MgSO_4 , 5 $\text{Na}_2\text{-ATP}$, 0.3 Tris-GTP, 12 Tris-Phosphocreatine, 20 HEPES, adjusted to pH 7.35 with KOH. To this basic solution, one or more indicators were added according to the type of optical recording. In V_m imaging experiments without concomitant Ca^{2+} recordings, cell membranes were loaded with the voltage-sensitive dye D-2-ANEPEQ (JPW1114, 0.2 mg/mL, Thermo Fisher Scientific) for 30 min using a first patch clamp recording and then re-patched a second time with dye free solution. In V_m imaging experiments with concomitant Ca^{2+} recordings, the intracellular solution in the second patch contained a Ca^{2+} indicator at the concentration of

2 mM which was either Cal-520FF (AAT Bioquest, Sunnyvale, CA, USA, see Blömer et al., 2021) in 2 cells or Fura2FF (Santa Cruz Biotechnology) in 6 cells. The choice of using, in some experiments, a UV-excitable indicator is motivated by the need to obtain, in the majority of cells, both V_m and Ca^{2+} signals with a combination of spectrally non-overlapping indicators (Vogt et al., 2011a). Finally, in exclusive Ca^{2+} imaging experiments, the Ca^{2+} indicator added to the intracellular solution at the concentration of 2 mM was either Oregon Green BAPTA-5N (Thermo Fisher Scientific) or the most sensitive low-affinity dye Cal-520FF. Ca^{2+} recordings started 30 min after achieving the whole cell configuration, a time necessary for full dye equilibration in the initial 200 μm segment of the apical dendrite. Somatic APs were elicited by short current pulses of 1.5–2.5 nA through the patch pipette in current clamp mode. Electrical somatic V_m transients were acquired at 20 kHz and the bridge associated with the current pulse was corrected offline by using the recorded injected current. Finally, the measured V_m was corrected for -11 mV junction potential. Experiments were performed using an imaging system described in several previous reports (Jaafari et al., 2014; Jaafari and Canepari, 2016; Ait Ouares et al., 2019; Ait Ouares and Canepari, 2020). This system is based on an Olympus BX51 microscope equipped with a 60X/1.0 NA Nikon objective where whole-field fluorescence illumination is provided either with an OPTOLED system (Cairn Research, Faversham, UK) or with an LDI-7 laser (89 North, Williston, VT). Fluorescence emission was demagnified by 0.5X and acquired with a NeuroCCD camera (Redshirt Imaging, Decatur, GA). Signals were sampled at 20 kHz (for 8 ms) with a resolution of 4X26 pixels except in V_m imaging calibration experiments where signals were sampled at 5 kHz with a resolution of 26X26 pixels (for 160 ms). In V_m imaging recordings, JPW1114 fluorescence was excited using the 528 nm line of an LDI-7 laser (89 North, Williston, VT) and the emitted fluorescence was long-pass filtered at >610 nm before being acquired. In Ca^{2+} imaging recordings, OG5N or Cal-520FF were excited by the 470 nm line of the OPTOLED and the emitted fluorescence was band-pass filtered at 530 ± 21 nm before being acquired. Finally, in Ca^{2+} imaging recordings with Fura2FF, fluorescence was excited by the 385 nm line of the OPTOLED and the emitted fluorescence was band-pass filtered at 510 ± 41 nm before being acquired.

2.3 Pharmacology of Ca^{2+} and K^+ channels

Channels blockers used in this study were either peptides (animal toxins), purchased from Smartox Biotechnology (Saint Egrève, France), or smaller organic molecules. Peptides, dissolved in water and used at the final concentration of 1 μM , were: ω -agatoxin-IVA (P/Q-type VGCC blocker), ω -conotoxin-GVIA (N-type VGCC blocker), snx-482 (R-type VGCC blocker), apamin (SK CAKC blocker) and iberiotoxin (BK CAKC blocker). The blockade of L-type VGCCs was achieved using 4-(2,1,3-Benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-3,5-pyridinecarboxylic acid methyl 1-methylethyl ester (isradipine, purchased from HelloBio, Bristol, UK) that was diluted in external solution at 20 μM concentration. To obtain an alternative blockade of N-type VGCCs we

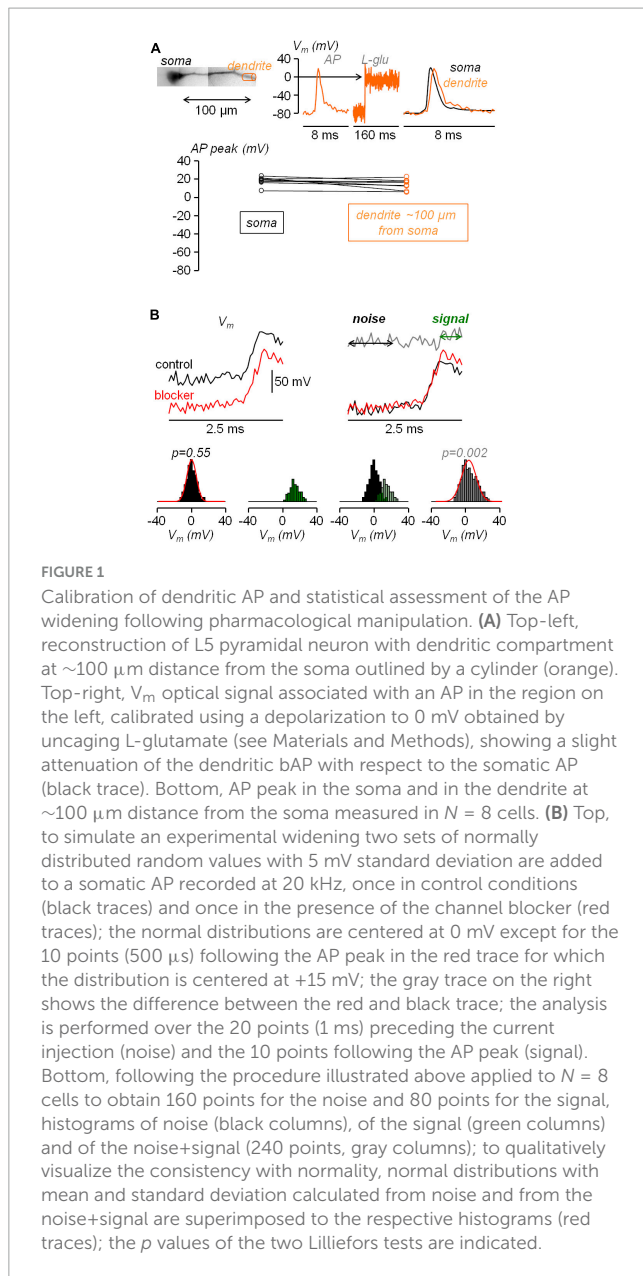
used *N*-[[4-(1,1-Dimethylethyl)phenyl]methyl]-*N*-methyl-L-leucyl-*N*-(1,1-dimethylethyl)-*O*-(phenylmethyl)-L-tyrosinamide (pd173212, purchased from TOCRIS, Bristol, UK) that was used at the final concentration of 5 μM . Finally, to block T-type VGCCs, we used a cocktail of two drugs (from TOCRIS) at the final concentrations of 30 and 5 μM , respectively: 3,5-dichloro-*N*-[[[(1 α ,5 α ,6-exo,6 α)-3-(3,3-dimethylbutyl)-3-azabicyclo[3.1.0]hex-6-yl]methyl]-benzamide-hydrochloride (ml218) and (1*S*,2*S*)-2-[2-[[3-(1*H*-Benzimidazol-2-yl)propyl]methylamino]ethyl]-6-fluoro-1,2,3,4-tetrahydro-1-(1-methylethyl)-2-naphthalenyl-cyclopropanecarboxylate-dihydrochloride (nnc550396). In all experiments, the blocker(s) was (were) locally delivered by gentle pressure application near the optical recording area using a pipette of 2–4 μm of diameter. The application lasted 2–4 min before recording in order to assure the stable blockade of the channel(s).

2.4 Data analysis and quantification

All data were from averages of 6–9 trials with identical somatic response in Ca^{2+} recordings, and of 4 trials with identical somatic response in V_m recordings. Raw data were converted in MATLAB format and analysed using custom-made code written in MATLAB. As a first step, fluorescence values including an AP were corrected for photo-bleaching using multi-exponential fits of fluorescence values in single trials without an AP. Then, the fractional change of fluorescence from the first image ($\Delta F/F_0$) was calculated over the mean fluorescence in regions of 20–40 μm located at ~ 100 μm from the soma. It is important to state that all the conclusions reported in this study were based on comparisons of signals under control conditions and signals after the blockade of one or more channels, for which a calibration of the size of the signals was not necessary. Nevertheless, to build realistic NEURON models, the V_m ($\Delta F/F_0$) associated with the bAP was converted into mV considering an attenuation of the somatic AP size, which was 4% on average. This value was obtained in a set of experiments in which the V_m ($\Delta F/F_0$) associated with the bAP was calibrated in mV using a previously reported method (Vogt et al., 2011b). As shown in the example of Figure 1A, the V_m $\Delta F/F_0$ signal associated with an AP was measured in the apical dendrite at ~ 100 μm from the soma. Then, in the presence of 1 μM tetrodotoxin to block APs and 50 μM cyclothiazide to inhibit AMPA receptors desensitization, L-glutamate was locally photo-released from 1 mM 4-Methoxy-7-nitroindolyl-caged-L-glutamate (MNI-glutamate, TOCRIS) using an OPTOLED pulse of 1 ms at 365 nm wavelength. As this procedure brings the dendritic V_m to the reversal potential of ionotropic glutamate receptors (i.e., to 0 mV), the associated V_m $\Delta F/F_0$ signal was used to calibrate the V_m $\Delta F/F_0$ signal associated with the bAP. This assessment was repeated in $N = 8$ cells obtaining a consistent result (Figure 1A). For the quantification of Ca^{2+} signals, the dendritic Ca^{2+} $\Delta F/F_0$ signal associated with the bAP, normalized to its asymptotic value, was initially fitted with a 4-sigmoid function $Y(t)$:

$$Y(t) = \prod_{j=1}^4 \frac{1}{1 + e^{-\varphi_j \cdot (t - \theta_j)}}$$

where t is time and φ_j and θ_j are the parameters to be determined by the fit. The time derivative of $Y(t)$ was then used



as measurement of the Ca^{2+} current (I_{Ca}). The maxima of $Y(t)$ and of its derivative were used to quantify the fractional change of the signals produced by the channel blockers.

2.5 Statistics

To assess the consistency of the experimental results obtained in groups of cells, three statistical tests were performed and in all tests we considered 0.01 as the threshold for significance.

1. To assess the effect of a channel blocker on the Ca^{2+} and I_{Ca} signals in the same group of experiments, the parametric paired t -test was performed on the signals maxima under two different conditions. In each cell, the maxima were measured in control conditions and in the presence of one or several channel blockers.

2. To assess the effect of a channel blocker on the Ca^{2+} and I_{Ca} signals in two different groups of experiments, the Wilcoxon

rank non-parametric test was performed on the fractional changes of signals maxima under two different conditions in the two groups of cells.

3. Finally, to establish the effect of a channel blocker on the shape of the dendritic AP, we assessed the widening of the AP over the photon noise of the recording using the following statistical analysis. Assuming that the photon noise is normally distributed, the distribution of the difference between the noise samples in the presence of the channel blocker and in control condition is also normal with standard deviation equal to $\sqrt{2}$ times the standard deviation of the noise. Thus the hypothesis to test was whether or not the addition of the sample differences at given intervals after the AP peak produces a distribution that deviates from normality. The rationale of the analysis is illustrated in the simulations reported in **Figure 1B**. In the example reported on the top of the panel, two sets of normally distributed values with 5 mV standard deviation (~ 15 mV peak-to-peak noise) were added to a somatic AP recorded at 20 kHz. This was done once to simulate the control conditions and once to simulate the presence of the channel blocker. The normal distributions were centered at 0 mV except for the 500 μs following the AP peak in the red trace for which the distribution was centered at +15 mV in order to mimic the widening. The distribution of the difference between the noise samples in the presence of the channel blocker and in control condition are also normal with standard deviation equal to $\sqrt{2}$ times the standard deviation of the original signals. The distribution of the difference between the noise + signal samples, however, deviates from normality since the two original distributions are centered at two different values. Thus, we repeated the simulations in $N = 8$ cells and we illustrate the histograms of the difference in the noise, in the signal and in the noise + signal (**Figure 1B**). To visualize the normal behavior of the noise difference and the deviation from normality of the noise + signal difference, we superimpose to the histograms the two normal distributions with mean and standard deviation calculated from the points. The test to obtain a quantitative assessment of the normal behavior is, in principle the Kolmogorov-Smirnov test. In practice, since in the experiments the noise varies from one cell to another, we opted for the stronger Lilliefors test, which is a generalization of the Kolmogorov-Smirnov test for unknown normal distributions (Lilliefors, 1967). The p values of this test for the above simulations are reported in **Figure 1B**. In the experiments, to establish the kinetics of the widening, the test was repeated over three distinct time intervals during the falling phase of the AP, namely the first 500 μs following the AP peak, the next 500 μs and the next 1.5 ms.

2.6 Computational modelling

All simulations were carried out using the NEURON simulation environment (v.8.2.0). Model and simulation files will be available in the ModelDB section of the Senselab database.¹ We used a 3D reconstructed morphology of a mouse L5 pyramidal neuron (morphology NMO_36595, Buchanan et al., 2012) downloaded from NeuroMorpho.org database (Ascoli et al., 2007), with uniform passive properties ($C_m = 1 \text{ mF/cm}^2$,

¹ <https://modeldb.science/2015410>

$R_m = 30 \text{ kohm/cm}^2$, $R_a = 160 \text{ ohm-cm}$) where we attached the axon morphology used in Filipis et al. (2023). Temperature was set at 33°C. The NEURON model, built based on the original model reported by Hallermann et al. (2012) was previously employed in our previous studies (Filipis and Canepari, 2021, Filipis et al., 2023). It was adapted to reproduce the somatic AP and dendritic bAP recordings used as a reference in this work. Active properties included two Na^+ currents, the $\text{Na}_v1.2$ and $\text{Na}_v1.6$ channels, five types of K^+ currents (a delayed rectifier conductance, A-type K^+ conductance, K_v7 conductance, SK Ca^{2+} -dependent K^+ conductance and BK SK Ca^{2+} -dependent K^+ conductance), a non-specific I_h current, Ca^{2+} conductance modelling including T-type, L-type, R-type, N-type and P/Q-type Ca^{2+} currents, and a simple Ca^{2+} -extrusion mechanism with a 20 ms time constant, to reproduce the measured Ca^{2+} transient decay. For T-type VGCCs, we used the model from Migliore et al. (2008). For R-type VGCCs, we used the model from Mandge and Manchanda (2018). For N-type, P/Q-type, and L-type VGCCs, we used the same model from Wimmer et al. (2010). For BK channel in dendrites, we modified the model in Filipis et al. (2023) to match experimental data on the bAP. In particular, we shifted the voltage dependent activation kinetic by + 10 mV and used a time constant with a sigmoidal form. All other channels were taken from Filipis et al. (2023). Relative spatial distributions of channel dendritic densities were in accordance with Migliore and Shepherd (2002) and Ramaswamy and Markram (2015); $\text{Na}_v1.2$ expression was ~50-fold lower compared to the axon initial segment (Kole et al., 2008); an inward current generated by HCN cation channels was also inserted with a density increasing exponentially to 50-fold in the distal apical dendrite compared to the soma (Harnett et al., 2015). To determine the absolute channel densities of the model, we initially used the calibrated voltage imaging recording in Figure 1A to reflect the action potential experimental traces in both the soma and the dendrite. Specifically, we first modified the Na^+ channels to match the somatic AP and we then modified Ca^{2+} and K^+ channels to match the dendritic bAP. After that, we refined the dendritic channel density for each individual type of VGCC and set a coupling between particular VGCCs and the BK CAKC. To mimic this coupling, we distinguished the components of Ca^{2+} influx from each individual VGCC and we introduced an affinity of the CAKC for a specific component multiplied a factor $\alpha > 0$ ($\alpha = 0$ means no coupling). In agreement with a study in neocortical pyramidal neurons (Sun et al., 2003), we established the coupling both for L-type and N-type VGCCs, using values of $\alpha = 3$ and $\alpha = 10$ to mimic “weak” and “strong” coupling, respectively. Finally, the pharmacological blockade of each channel in the experiments was mimicked by removing 90% of the channel density from the model.

3 Results

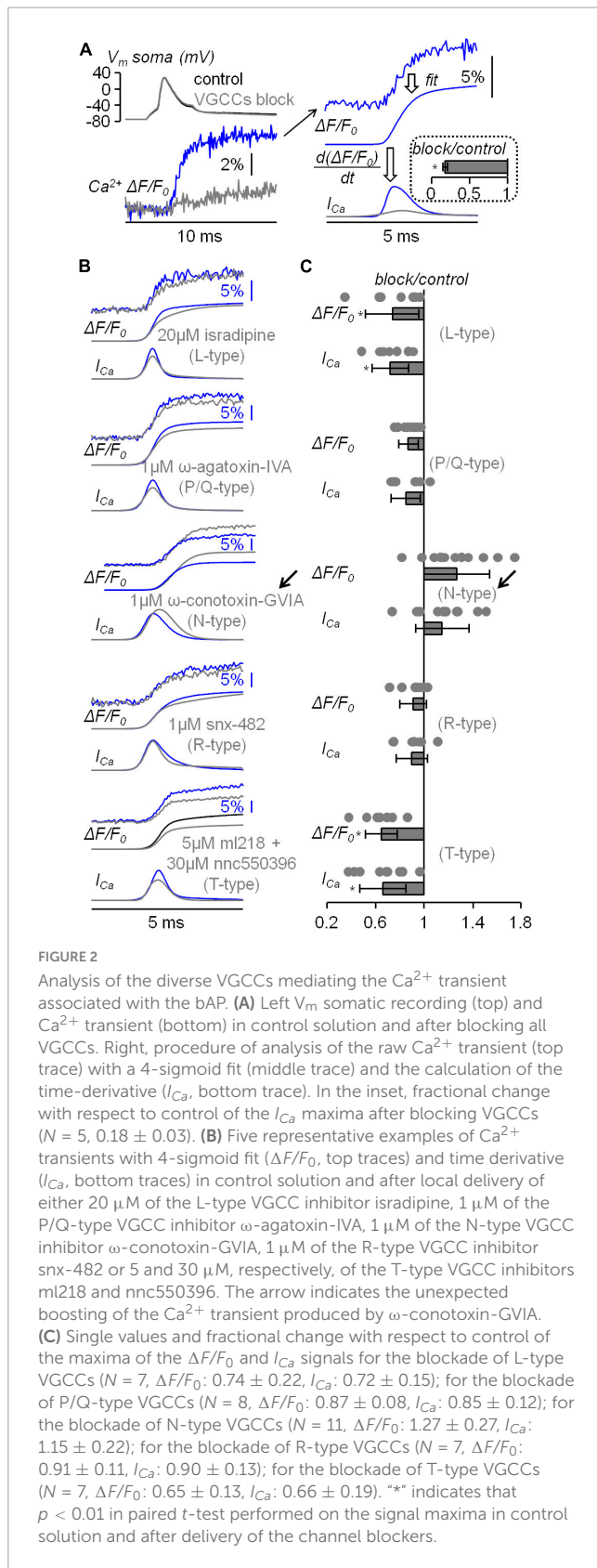
3.1 The dendritic Ca^{2+} influx associated with the bAP is mediated by diverse VGCCs

This study started with the investigation of the VGCCs that mediate the Ca^{2+} influx associated with the bAP in the apical dendrite at ~100 μm from the soma using the same approach

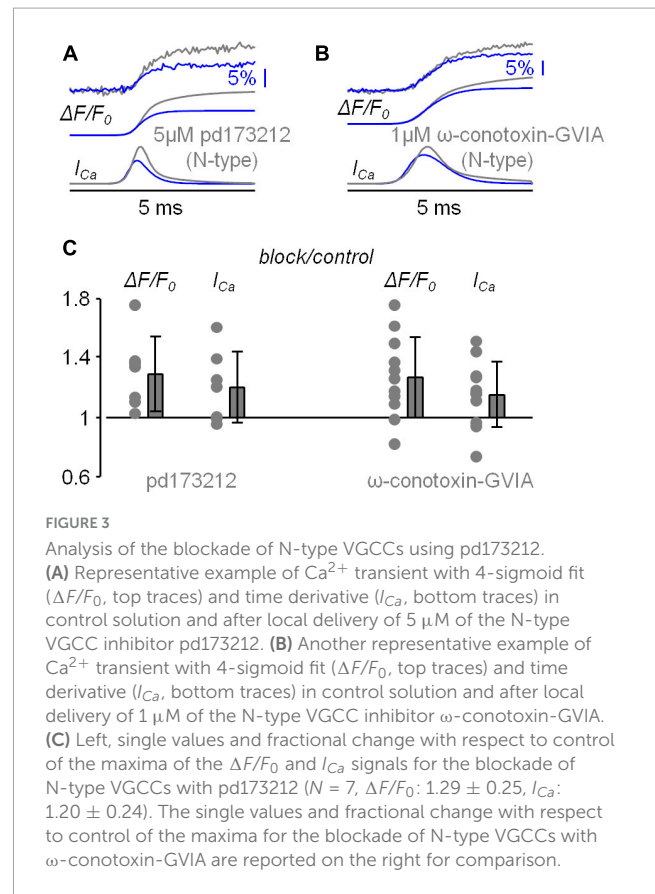
already utilised in CA1 hippocampal pyramidal neuron (Jaafari and Canepari, 2016). In the present experiments, L5 pyramidal neurons were loaded with 2 mM of a low-affinity Ca^{2+} indicator, either OG5N or the more sensitive Cal-520FF (Blömer et al., 2021), and Ca^{2+} transients ($\Delta F/F_0$ signals) were measured at 20 kHz by averaging fluorescence from dendritic segments of 20–40 μm length at ~100 μm from the soma. To investigate the contribution of the diverse VGCCs, we locally delivered one or several channel blockers using a pipette positioned near the recording region, as already done in other studies (Ait Ouaires et al., 2019; Filipis et al., 2023). Specifically, we blocked L-type VGCCs with 20 μM isradipine, P/Q-type VGCCs with 1 μM ω -agatoxin-IVA, N-type VGCCs with 1 μM ω -conotoxin-GVIA, R-type VGCCs with 1 μM of SNX482, and T-type VGCCs with 5 μM ML218 and 30 μM NNC550396. Figure 2A shows a Ca^{2+} transient associated with a bAP that was strongly attenuated by the cocktail of all VGCC blockers. Figure 2A also shows the analysis of the Ca^{2+} transient consisting in fitting the signal with a 4-sigmoid function and to calculate the time-derivative of the fit to extrapolate the kinetics of the Ca^{2+} current (I_{Ca}). As shown in the inset of Figure 2A, the cocktail of all VGCC blockers reduced the peak of the signal by ~80 % in 5 cells tested. We then applied this analysis on the Ca^{2+} transient recorded first in control solution and then after locally blocking each individual VGCC. The five representative examples reported in Figure 2B show that the blockade of L-type, P/Q-type, R-type and T-type VGCCs decreased, at different extent, the size of both $\Delta F/F_0$ and I_{Ca} signals, but surprisingly the blockade of N-type VGCCs increased the size of both $\Delta F/F_0$ and I_{Ca} signals. The test was repeated in 7–10 different cells for each channel blocker and the effect of the inhibitor was quantified by measuring the maximum of the $\Delta F/F_0$ and I_{Ca} signals (Figure 2C). Except for the blockade of N-type VGCCs, the individual blockade of each VGCC type produced on average a decrease of both $\Delta F/F_0$ and I_{Ca} signals, an effect that was more important for L-type and T-type VGCCs. In the case of N-type VGCCs, the blockade increased both $\Delta F/F_0$ and I_{Ca} signals in 8/11 cells tested. The unexpected result of locally delivering 1 μM ω -conotoxin-GVIA could be explained by the coupling of the N-type VGCC with a mechanism that boosts the Ca^{2+} influx associated with the bAP, or with the toxin binding to a different target. To discriminate between these two hypotheses, we repeated the analysis of the Ca^{2+} transient of Figure 2 by blocking the N-type VGCC with another selective inhibitor, namely pd173212 (Hu et al., 1999). As shown in the two representative examples of Figures 3A, B, local delivery of either 5 μM pd173212 or 1 μM ω -conotoxin-GVIA increased the size of both $\Delta F/F_0$ and I_{Ca} signals in a qualitatively similar manner. Altogether, the $\Delta F/F_0$ signal increased in 7 cells tested after delivery of pd173212, and the I_{Ca} signal increased in 4/7 cells tested after delivery of pd173212 (Figure 3C). We concluded that the blockade N-type VGCCs boosts the Ca^{2+} influx associated with the bAP.

3.2 N-type VGCCs are coupled with BK CAKCs

The boosting of the Ca^{2+} influx observed in Figures 2, 3 can be due to an enhancement of the depolarisation produced by the loss of Ca^{2+} influx via N-type VGCCs. It was shown in freshly



dissociated neocortical pyramidal neurons of the mouse that BK CAKCs are activated by N-type VGCCs (Sun et al., 2003). Thus, we repeated the analysis of the Ca^{2+} transient of Figures 2, 3 by blocking in sequence first BK CAKCs with local delivery of



$1 \mu\text{M}$ iberiotoxin and then BK CAKCs and N-type VGCCs together with local delivery of $1 \mu\text{M}$ iberiotoxin and $1 \mu\text{M}$ ω -conotoxin-GVIA. In the representative example of Figure 4A, the blockade of BK CAKCs increased both $\Delta F/F_0$ and I_{Ca} signals, but the further blockade of N-type VGCCs decreased both $\Delta F/F_0$ and I_{Ca} signals. Since L-type VGCCs also couple with BK CAKCs in many systems (Marcantoni et al., 2010; Vandael et al., 2010), we also performed experiments in which we blocked in sequence first BK CAKCs only and then BK CAKCs and L-type VGCCs together with local delivery of $1 \mu\text{M}$ iberiotoxin and $20 \mu\text{M}$ isradipine. As in the previous example, in the example of Figure 4B the blockade of BK CAKCs increased both $\Delta F/F_0$ and I_{Ca} signals and the further blockade of L-type VGCCs decreased both $\Delta F/F_0$ and I_{Ca} signals. These results were consistently obtained in $N = 9$ cells tested with blockade of N-type VGCCs and in $N = 7$ cells with blockade of L-type VGCCs. As shown in Figure 4C, the blockade of BK CAKCs boosted the Ca^{2+} transient similarly to the blockade of N-type VGCCs. In contrast, the blockades of N-type or of L-type VGCCs in the presence of iberiotoxin reduced the Ca^{2+} transient. To evaluate the difference between the results of blocking N-type or L-type VGCCs, either in control conditions or after blocking BK CAKCs, we performed a Wilcoxon rank non-parametric test on the groups of cells where a blocker was applied under different conditions, using $p < 0.01$ as discriminator to establish whether two groups were different. As shown in Table 1, the effects on both $\Delta F/F_0$ and I_{Ca} signals were similar when comparing the groups of cells where ω -conotoxin-GVIA, pd173212 or iberiotoxin were applied in control solutions, or when the effects of iberiotoxin were compared with the ensemble of cells where N-type VGCCs were

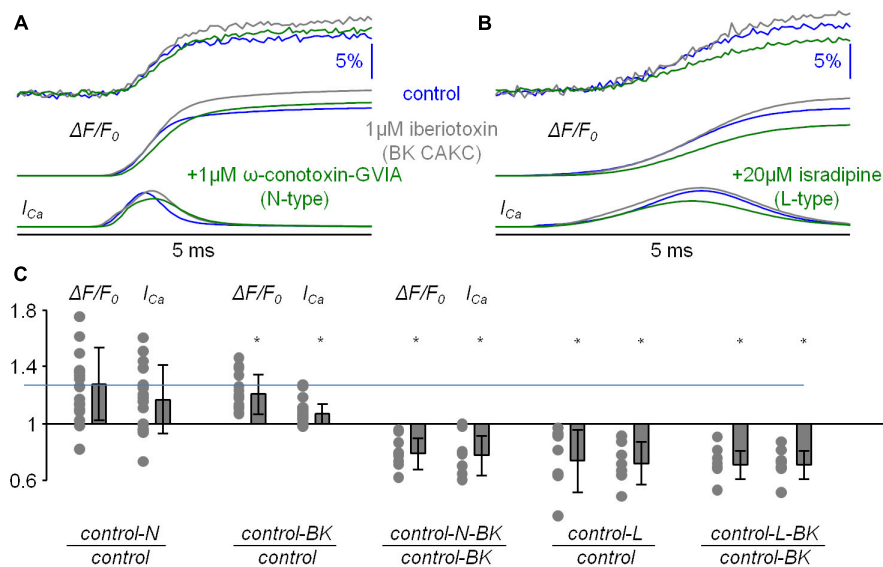


TABLE 1 Wilcoxon rank non-parametric test performed on the effects on Ca^{2+} transients produced by blocking N-type VGCCs, L-type VGCCs and BK CAKCs.

		$p \Delta F/F_0$	$p I_{Ca}$
ω -conotoxin-GVIA/control	pd173212/control	0.8601	0.7914
ω -conotoxin-GVIA/control	iberiotoxin/control	0.9998	0.2875
ω-conotoxin-GVIA/control	ω-conotoxin-GVIA/iberiotoxin	$4.74 \cdot 10^{-4}$	0.0024
pd173212/control	iberiotoxin/control	0.8371	0.7577
pd173212/control	ω-conotoxin-GVIA/iberiotoxin	$1.75 \cdot 10^{-4}$	0.0012
(ω -conotoxin-GVIA&pd173212)/control	iberiotoxin/control	0.9385	0.3681
(ω-conotoxin-GVIA&pd173212)/control	ω-conotoxin-GVIA/iberiotoxin	$6.71 \cdot 10^{-5}$	$4.26 \cdot 10^{-4}$
isradipine/control	isradipine/iberiotoxin	0.6875	0.9375

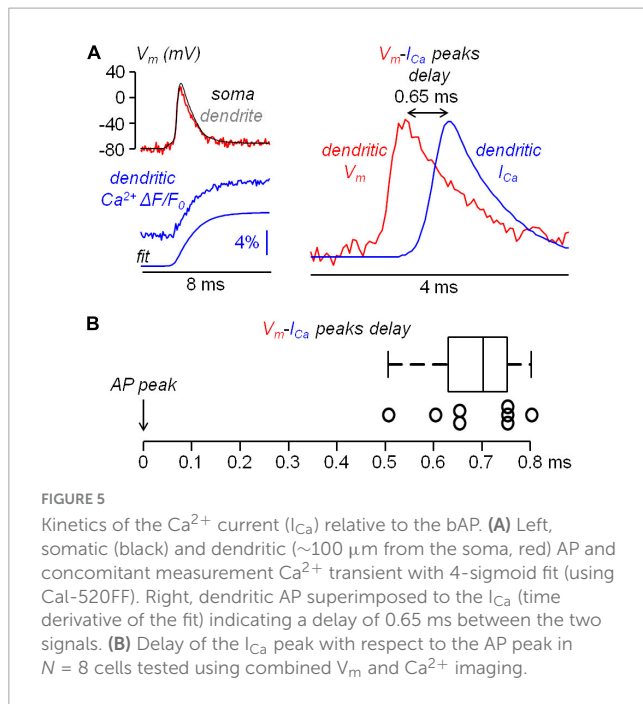
The considered groups are: delivery of $1 \mu\text{M}$ ω -conotoxin-GVIA in control conditions ($N = 11$ cells); delivery of $5 \mu\text{M}$ pd173212 in control conditions ($N = 7$ cells); delivery of either $1 \mu\text{M}$ ω -conotoxin-GVIA or $5 \mu\text{M}$ pd173212 in control conditions ($N = 18$ cells); delivery of $1 \mu\text{M}$ iberiotoxin in control conditions ($N = 9$ cells); delivery of $1 \mu\text{M}$ ω -conotoxin-GVIA in the presence of $1 \mu\text{M}$ iberiotoxin ($N = 9$ cells); delivery of $20 \mu\text{M}$ isradipine in control conditions ($N = 7$ cells); delivery of $20 \mu\text{M}$ isradipine in the presence of $1 \mu\text{M}$ iberiotoxin ($N = 7$ cells). In each line, the two groups compared are reported in the left columns and the p values for the $\Delta F/F_0$ and I_{Ca} signals are reported on the right columns. Tests where p was < 0.01 are reported in bold characters.

blocked in control solution. In contrast, the effects on both $\Delta F/F_0$ and I_{Ca} signals were different when comparing the cells where ω -conotoxin-GVIA was applied in the presence of iberiotoxin with the groups of cells where ω -conotoxin-GVIA or pd173212 were applied in control solution, or when the comparison was done with the ensemble of cells where N-type VGCCs were blocked in control solution. Finally, the effects on both $\Delta F/F_0$ and I_{Ca} signals were similar when comparing the groups of cells where isradipine was applied in control solutions or in the presence of iberiotoxin. Since the blockade of BK CAKCs prevents the boosting of the Ca^{2+} influx

when blocking N-type VGCCs, we concluded that this effect was due to the functional coupling of the two channels.

3.3 The peak of the Ca^{2+} current is delayed with respect of the bAP peak by $>500 \mu\text{s}$

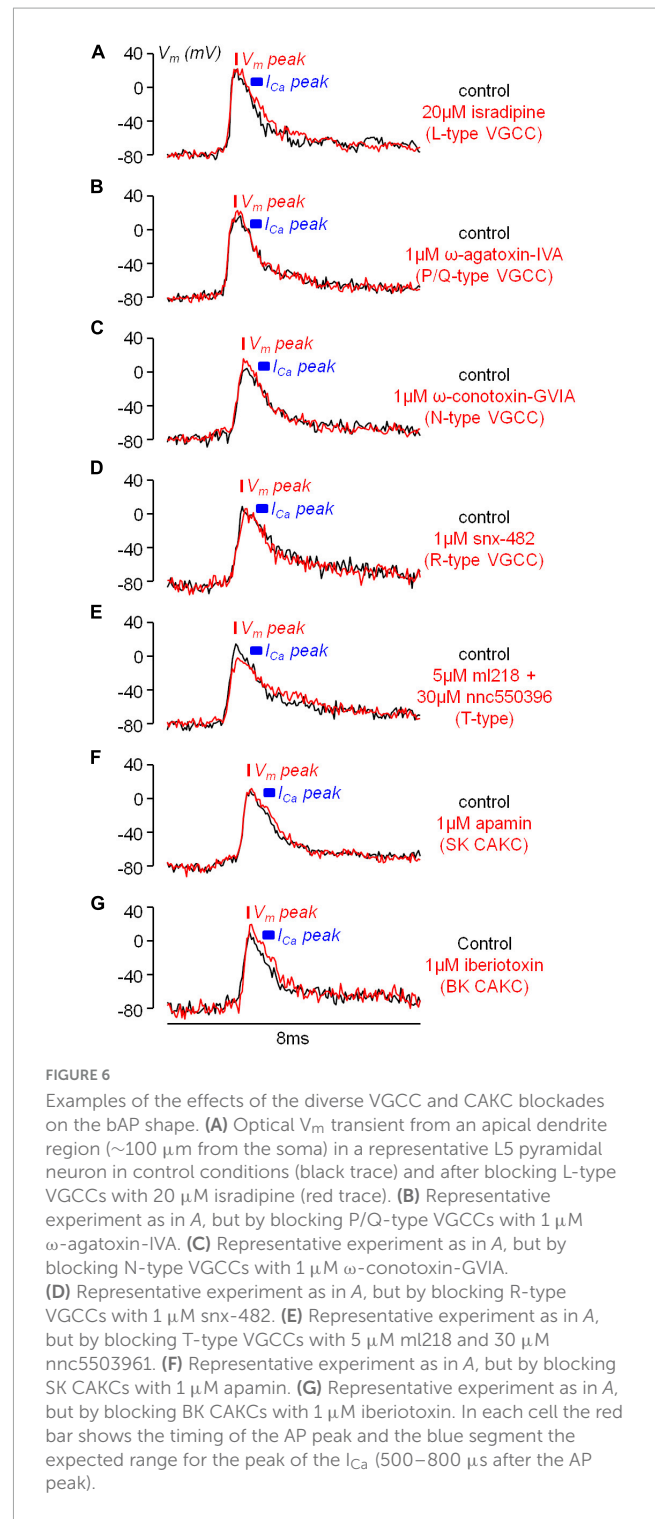
Before further analyzing the interaction between N-type VGCCs and BK CAKCs, we compared the kinetics of the dendritic



I_{Ca} with that of the eliciting bAP at $\sim 100 \mu\text{m}$ from the soma. In a previous study, we showed that in L5 pyramidal neurons the peak of the I_{Ca} occurs before the AP peak in the distal part of the axon initial segment whereas it is delayed in the proximal part (Filipis et al., 2023). In another study, using ultrafast combined V_m and Ca^{2+} imaging, we showed that the I_{Ca} was further delayed with respect to the AP peak in the proximal part of the apical dendrite of CA1 hippocampal pyramidal neurons (Jaafari and Canepari, 2016). Thus, we performed the same analysis in the proximal part of the apical dendrite in L5 pyramidal neurons using, as Ca^{2+} indicator, either Cal-520FF or Fura2FF (see Materials and Methods). A representative example of this type of measurement is reported in Figure 5A. In this particular example, the peak of the I_{Ca} corresponding to the maximal slope of the dendritic Ca^{2+} transient is delayed by 650 μs (13 samples) with respect to the dendritic AP. Consistently with the result of this experiment, a delay ranging from 500 to 800 μs was observed in $N = 8$ cells (Figure 5B). From the kinetics of the Ca^{2+} transient we concluded that VGCCs start opening during the rising phase of the AP, but they remain open during the entire falling phase of the AP increasing the cytosolic Ca^{2+} concentration over this period. As a consequence, if the activation of a Ca^{2+} -binding protein is linear with the cytosolic Ca^{2+} , the maximal effect of this activation must be observed at least 500 μs after the AP peak.

3.4 The effect of blocking N-type VGCCs or BK CAKCs occurs in the first 500 μs after the AP peak

If the inhibition of a K^+ channel occurs during an AP, then this inhibition is expected to change the shape of the AP. Thus, using ultrafast V_m imaging (Popovic et al., 2015), as shown in the examples reported in Figure 6, we investigated the kinetics of the



dendritic bAP after blocking L-type VGCCs (panel A), P/Q-type VGCCs (panel B), N-type VGCCs (panel C), R-type VGCCs (panel D), and T-type VGCCs (panel E). In addition, we investigated the kinetics of the dendritic bAP after blocking the two major types of CAKCs, namely the SK channel using 1 μM apamin (panel F) and the BK channel (panel G). Consistently with the observation that the blockade of P/Q-type and R-type VGCCs caused, on average, the smallest decrease in the I_{Ca} (Figure 2C), in the two representative cells of Figures 6B, D the blockade of

these two channels did not change the AP shape. Surprisingly, in the representative cell of **Figure 6E**, the blockade of T-type VGCCs decreased the amplitude of the bAP, suggesting an effect on dendritic excitability. We don't have an explanation for this effect that can be in principle potentially attributed to the drugs acting on a different target other than T-type VGCC, but this result suggests that the AP weakening may contribute to the relatively large inhibition of the I_{Ca} reported in **Figure 2C**. In all other representative cells, the blockade of the channels caused a visually detectable widening of the AP shape. In the case of N-type VGCCs (**Figure 6C**) and of BK CAKs (**Figure 6G**), a widening seemed occurring during the first 500 μ s following the AP peak, therefore during the phase of increasing of VGCC activation. In contrast, in the case of L-type VGCCs (**Figure 6A**) and of SK CAKs (**Figure 6F**), the widening seemed occurring mostly >500 μ s after the AP peak, therefore during or after the expected peak of the I_{Ca} . The early widening reported in the example of **Figure 6C** was also observed in the example of **Figure 7A**. In this exceptional case, where the peak-to-peak noise was <10 mV, the widening could be visually detected by plotting the difference between the samples after blocking N-type VGCCs and the samples in control conditions. Hence, whereas the 20 differences corresponding to the noise collected before the somatic current injection were either positive or negative values, the 20 differences corresponding to the signal collected after the AP were mostly positive. In the vast majority of tested cells, however, the peak-to-peak noise was >10 mV. Thus, we performed the experiment of the examples of **Figure 6** in 8–10 cells for each channel blockade and performed a statistical analysis on the ensemble of the cells tested. **Figure 7B** shows the averaged V_m signal of $N = 10$ cells where the individual traces were aligned with the AP peak. The analysis performed on the 200 sample differences corresponding to the noise indicate that their distribution is consistent with a normal behaviour, according to the large p value obtained by the Lilliefors test. In contrast, the 100 sample differences corresponding to the first 500 μ s after the AP peak and to the following 500 μ s do not follow a normal distribution. Finally, the hypothesis of deviation from the normal behaviour in the following 1.5 ms could not be rejected. The same analysis was repeated for the blockade of L-type VGCCs (**Figure 7C**), of P/Q-type VGCCs (**Figure 7D**), of R-type VGCCs (**Figure 7E**), of T-type VGCCs (**Figure 7F**), of SK CAKs (**Figure 7G**) and of BK CAKs (**Figure 7H**) and the p values obtained by the Lilliefors test are reported in **Table 2**. A significant deviation from normality in the samples collected in the first 500 μ s after the AP peak was discerned in the case of blockade of BK CAKs. Interestingly, in 4/8 cells tested for the blockade of P/Q-type VGCCs, an early widening of the AP was also observed, leading to a low value of p also in this case. Finally, the significant deviation from normality observed in the case of blockage of T-type VGCCs was due to the consistent reduction of the amplitude of the AP. In contrast to these cases, significant deviations from normality in the samples collected beyond 500 μ s after the AP peak were discerned in the case of blockade of L-type VGCCs and in the case of blockade of SK CAKs. These behaviours were due to the widening of the AP shape during or after the expected occurrence of the I_{Ca} peak. In the case of these two blockades, the time-course of the AP change is consistent with an activation of K^+ channels by cytosolic Ca^{2+} , but the hypothesis that these channels are also selectively activated by the specific Ca^{2+}

source cannot be excluded since the delayed widening can be due to a slower kinetics of the K^+ channels. Yet, in the case of the blockade of N-type VGCCs or of the blockade of BK CAKs, the results indicate that the activation of K^+ channels is not linear with the cytosolic Ca^{2+} , preceding the peak of the I_{Ca} . The fact that the AP widening occurs when only a fraction of inflowing Ca^{2+} via N-type VGCCs is detected indicates that BK channels are activated before the Ca^{2+} -dye binding reaction equilibrates. This result suggests a physical interaction between the N-type VGCC and the BK CAK at nanoscopic domain. Thus, we further explored this hypothesis using biophysical modelling in the NEURON environment.

3.5 The coupling between N-type VGCCs and BK CAKs is confirmed by a NEURON model

In order to reproduce the kinetics of the AP and of the I_{Ca} , we built a realistic NEURON model, and considered an apical dendrite at ~ 100 μ m from the soma, corresponding to the zone explored in the experiments. The model included all types of VGCCs tested, BK CAKs and SK CAKs. The coupling between a specific Ca^{2+} channel and the K^+ channel was modelled by introducing an activation factor (α) of the BK channel by the Ca^{2+} influx from that particular channel. This coupling was imposed both to L-type and N-type VGCCs, in agreement to what reported in the literature (Sun et al., 2003). We found that, to replicate the ensemble of experimental results, the coupling needed to be strong for N-type VGCCs ($\alpha = 10$) and weak for L-type VGCCs ($\alpha = 3$). **Figure 8A** shows the dendritic AP, which reproduces the experimental AP obtained in one cell, superimposed to the Ca^{2+} transient and of the I_{Ca} . Consistently with experimental observations, the model showed a delay of ~ 0.8 ms between the peak of the AP and the peak of the I_{Ca} . Next, to mimic the pharmacological blockade of channels, from the model corresponding to the control condition we removed either 90% of N-type VGCCs, 90% of BK CAKs or 90% of both channels (**Figure 8B**). The model reproduced the experimental widening of the AP preceding the peak of the I_{Ca} and the increase in the Ca^{2+} signals with all simulated pharmacological blockades. In addition, consistently with the results of the experiments reported in **Figure 4**, the blockade of both channels decreases the Ca^{2+} signals with respect to the simulation where only BK CAKs are blocked. To assess whether the selective coupling between N-type VGCCs and BK CAKs was necessary to reproduce the experimental results, we suppressed the coupling between N-type VGCCs and BK CAKs. In this modified model, the early widening of the AP disappears and the Ca^{2+} signal and current decrease when N-type VGCCs are blocked (**Figure 8C**). This result supports the experimental suggestion that a strong coupling between N-type VGCCs and BK CAKs may be in effect in these neurons.

4 Discussion

In this report we provide strong experimental evidence that BK CAKs are selectively activated by N-type VGCCs in the apical dendrite of L5 pyramidal neurons. This activation is rapid and is

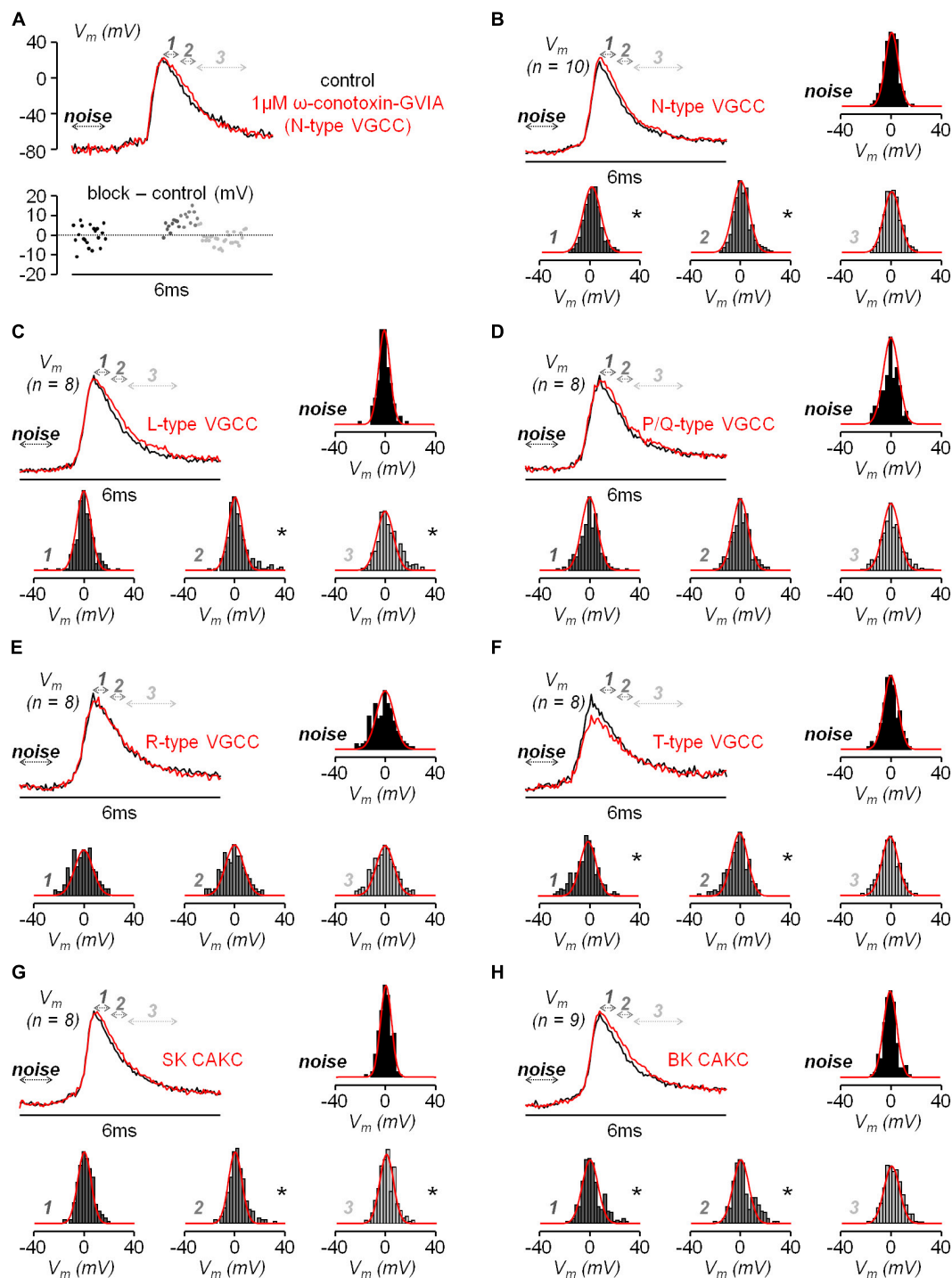


FIGURE 7

Analysis of the effect of the diverse VGCCs and CAKs on the bAP shape. (A) Top, optical V_m transient from an apical dendrite region ($\sim 100 \mu\text{m}$ from the soma) in a representative L5 pyramidal neuron in control conditions (black trace) and after blocking N-type VGCCs (red trace); arrows at different gray tones indicate the time windows corresponding to the noise (1 ms before current injection), signal "1" (first 500 μs after the AP peak), "2" (following 500 μs) and "3" (following 1.5 ms). Bottom, sample difference of the signals after the channel blockade and in control conditions in the four time windows. (B) Averaged signals aligned to the AP peak from $N = 10$ cells in control conditions (black trace) and after blocking N-type VGCCs (red trace) and histograms of the sample differences in the illustrated four time windows; normal distributions with mean and standard deviations calculated from the points are reported (red plots) for comparison with the histograms. (C) Same as in B, but in 8 cells where L-type VGCCs were blocked. (D) Same as in B, but in 8 cells where P/Q-type VGCCs were blocked. (E) Same as in B, but in 8 cells where R-type VGCCs were blocked. (F) Same as in B, but in 8 cells where T-type VGCCs were blocked. (G) Same as in B, but in 8 cells where SK CAKs were blocked. (H) Same as in B, but in 9 cells where BK CAKs were blocked. In panels B–H "*" indicates that the distribution deviates from normality ($p < 0.01$, Lilliefors test).

TABLE 2 Lilliefors test to assess whether a set of values is consistent with a normal distribution performed on sample differences in V_m imaging experiments after blocking a channel (with the name of the blocker indicated) and in control conditions.

	<i>N cells</i>	<i>p noise</i>	<i>p 1</i>	<i>p 2</i>	<i>p 3</i>
ω -conotoxin-GVIA (N-type VGCCs)	10	0.82	$8.3 \cdot 10^{-4}$	0.0082	0.29
isradipine (L-type VGCCs)	8	0.16	0.13	$0.5 \cdot 10^{-5}$	$2.5 \cdot 10^{-5}$
ω -agatoxin-IVA (P/Q-type VGCCs)	8	0.17	0.015	0.21	0.022
snx-482 (R-type VGCCs)	8	0.49	0.57	0.48	0.56
ml218 + nnc550396 (T-type VGCCs)	8	0.69	$0.1 \cdot 10^{-5}$	0.0017	0.012
apamin (SK CAKCs)	8	0.17	0.44	0.0042	$1.3 \cdot 10^{-4}$
iberiotoxin (BK CAKCs)	9	0.19	$7.1 \cdot 10^{-4}$	0.0011	0.18

The five columns report, for each channel tested, the total number of cells (*N*) and the values of *p* (*p*) for the noise (number of samples 20-*N*), for the signal 1 (first 500 μ s after the AP peak, number of samples 10-*N*), for the signal 2 (following 500 μ s after the AP peak, number of samples 10-*N*), for the signal 3 (following 1.5 ms after the AP peak, number of samples 30-*N*). Tests where *p* was < 0.01 are reported in bold characters and indicate a significant change of the AP shape caused by the blockade of the channel.

not linear with the increase of intracellular Ca^{2+} concentration associated with the transient activation of VGCCs. Thus, the blockade of N-type VGCCs widens the AP peak prolonging Ca^{2+} entry through the other VGCCs and boosting the increase of intracellular Ca^{2+} concentration associated with the AP. In contrast, the widening of the AP shape produced by inhibiting either L-type VGCCs or SK CAKCs occurs later in the AP falling phase, suggesting that SK channels might be uniformly activated by cytosolic Ca^{2+} from, possibly from low-threshold L channels (Vandael et al., 2012). The results reported here also indicate that our imaging approach can indirectly reveal functional protein-protein interactions that can be foreseen using structural imaging techniques such as Förster resonance energy transfer imaging (Masi et al., 2010). Indeed, in the case of VGCC nanodomains (Gandini and Zamponi, 2022), the close interaction with the Ca^{2+} target enables sub-millisecond activation of the target, as has been shown in synaptic terminals (Volynski and Krishnakumar, 2018). Here, we show that the combined analysis of cytosolic Ca^{2+} and of AP kinetics at 50 μ s temporal resolution can reveal VGCC-CAKC interactions at functional level in larger structures such as neuronal dendrites.

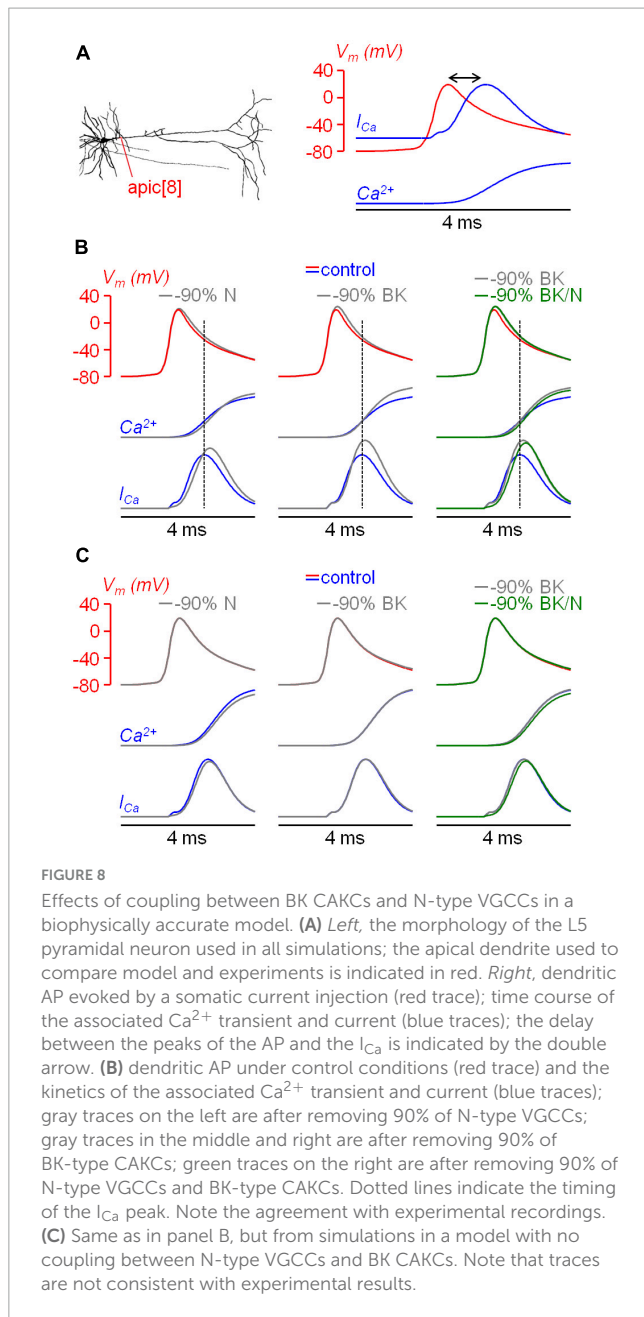
4.1 Functional consequences of the selective coupling between BK and a specific calcium source

Overall, BK channels are known to perform multiple functions in neurons (Ancatén-González et al., 2023). Compared with other CAKCs, BK channels exhibit lower affinity to Ca^{2+} and require concomitant depolarisation (Berkefeld et al., 2010). These two biophysical properties suggest that these channels must be physically close to a specific Ca^{2+} source and that they can activate at a sub-millisecond time scale when the cell is depolarised (Berkefeld et al., 2006). It is known that BK CAKCs form macromolecular complexes with L-type, P/Q-type and N-type VGCCs and, when the two proteins were co-expressed in heterologous systems, it was found that the kinetics of activation of BK CAKCs depended on the coupled VGCCs (Berkefeld and Fakler, 2008). In presynaptic terminals, BK channel activity regulate synaptic transmission (Raffaelli et al., 2004) and can modulate both the amplitude and duration of depolarization-evoked Ca^{2+} entry

as a result of the rapid repolarization and deactivation of P/Q-type and N-type VGCCs (Fakler and Adelman, 2008). In turn, reduced Ca^{2+} influx limits vesicle fusion at active zones, leading to decreased neurotransmitter release (Kyle and Braun, 2014). In the postsynaptic areas of L5 pyramidal neurons, BK channels are expressed not only in the dendritic bulk, but also in synaptic spines, and activation of BK CAKCs in small-head spines by Ca^{2+} influx through glutamate receptors reduces the size of synaptic potentials (Tazerart et al., 2022). Thus, both in synaptic terminals and spines, the selective activation of BK channels by a specific partner leads to a negative feedback of the triggering signal, which is the AP in the case of synaptic terminal and the synaptic potential in the case of spines. This negative feedback translates into a reduction of either neurotransmitter release or of neuronal excitability once the synaptic potential has reached the soma and the axon initial segment. Thus, in both cases, the functional consequences of BK CAKC activation can be tied to the underlying K^+ current that decreases the amplitude and/or the size of the triggering depolarizing event. In the apical dendrite of L5 pyramidal neurons, we found that BK CAKCs activation by N-type VGCCs anticipates the peak of the current mediated by VGCCs, providing a negative feedback to the other Ca^{2+} channels. The AP shaping produced by BK CAKCs is qualitatively similar to what we observed in the axon initial segment of L5 pyramidal neurons, where these channels are activated by the Ca^{2+} permeable voltage-gated Na^+ channel $Na_v1.2$ (Filipis et al., 2023). Compared to synaptic terminals and spines, however, the role of the regulation of the AP shape in these wider regions, produced by the K^+ current, is less straightforward.

4.2 Putative functional consequences of BK membrane conductance increase

Ion channels do not only mediate ionic currents that change the V_m , but they can also regulate the spread of V_m transient from a site to another by locally changing the membrane conductance, i.e., by “shunting” the transmission of the signal when the channels open (Blomfield, 1974). Whereas shunting inhibition is traditionally associated with localised increases of membrane conductance, the AP represents a very brief increment of membrane conductance that propagates along dendritic branches. Our realistic model shows that BK CAKCs represent a significant fraction of this



membrane conductance transient that is timely-locked to the AP. Thus, it is possible that the BK conductance transient can effectively contribute to the firing modulation produced by incoming synaptic inputs which are briefly shunted by the back-propagating AP. As for the case of localised BK channel activation in synaptic terminals and spines, the functional consequence of the spread BK channel activation is a reduction of neuronal excitability, in this case occurring at the level of dendritic integration.

4.3 Potential relevance in neurological disorders

The N-type VGCC, which is expressed in neurons predominantly at presynaptic terminals, is associated with

several neurological conditions such as anxiety, addiction, and pain in correlation with its endogenous regulator nociceptin opioid peptide receptor (Caminski et al., 2022). In contrast, dysfunction of BK CAKCs encoded by the *KCNMA1* gene, which are widely expressed in many tissues, is associated with complex combinations of disorders, including seizures, movement disorders, developmental delay and intellectual disability (Bailey et al., 2019). For instance, a *KCNMA1* knock-out mouse exhibits motor impairments and suffers from learning difficulties (Typlt et al., 2013), whereas paroxysmal non-kinesigenic dyskinesia is observed in patients with gain-of-function channelopathies of BK channels (Miller et al., 2021). Notably, BK channel dysfunction is also reported in other genetic diseases such as the fragile X syndrome (Deng and Klyachko, 2016), suggesting that BK CAKCs can be a potential general target for therapeutic intervention (Griguoli et al., 2016). In the case of the signal described in the present report, the specific coupling of BK CAKCs to N-type VGCCs in the apical dendrite might be determined by alternative splicing of the channel or through auxiliary subunits. It is known that BK CAKCs are regulated by extensive alternative splicing as well as multiple auxiliary subunits, giving BK CAKCs both cell and tissue-specific properties (Kyle and Braun, 2014; Latorre et al., 2017), even within the L5 pyramidal neuron population (Guan et al., 2015). In recent years, various β and γ auxiliary subunits of BK channels have been identified which can modulate activation and inactivation dependencies (Gonzalez-Perez and Lingle, 2019), but the specific co-expression can in principle also drive the choice of the Ca^{2+} channel partner in sub-cellular compartments. We have previously reported that in the axon initial segment, BK CAKCs interact with $\text{Na}_v1.2$ channels, but this interaction seems independent of auxiliary subunits as was indicated by heterologous expression of these channels in HEK293 cells (Filipis et al., 2023). In contrast, the BK CAKCs could be guided by auxiliary subunits to form selective nanodomains with N-type VGCCs in the dendrites of L5 pyramidal neuron. This hypothesis requiring extensive investigation might be potentially important to investigate the specific function of BK CAKC characterised in this report.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: Repository Zenodo (<https://zenodo.org/doi/10.5281/zenodo.7623897> and <https://zenodo.org/records/7623898>).

Ethics statement

The animal study was approved by the Ethics committee affiliated to the animal facility of the University of Grenoble (D3842110001). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

LB: Conceptualization, Data curation, Investigation, Methodology, Writing – review & editing. EG: Data curation, Investigation, Methodology, Software, Writing – review & editing. FA: Investigation, Writing – review & editing. LF: Investigation, Supervision, Writing – review & editing. DT: Supervision, Writing – review & editing. MM: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Software, Supervision, Writing – review & editing. MC: Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Software, Supervision, Validation, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the Agence Nationale de la Recherche through two grants (ANR-18-CE19-0024 – OptChemCom and Labex Ion Channels Science and Therapeutics: program number ANR-11-LABX-0015). EG was funded by the Regione Sicilia (CUP G79J21012770001). We are indebted to the Fédération pour la Recherche sur le Cerveau (FRC – Grant Espoir en tête, Rotary France) and the National Infrastructure France Life

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Imaging for financing part of the experimental equipment. We acknowledge a contribution from the Italian National Recovery and Resilience Plan (NRRP), M4C2, funded by the European Union – NextGenerationEU (Project IR0000011, CUP B51E22000150006, “EBRAINS-Italy”) and the EU Horizon Europe Programme under the Specific Grant Agreement No. 101147319 (EBRAINS 2.0 Project).

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