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Inhibition of dendritic Ca^{2+} spikes by $GABA_B$ receptors in cortical pyramidal neurons is mediated by a direct $G_{i/o}$-$\beta\gamma$-subunit interaction with Ca_v1 channels

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Key points

- Voltage-dependent Ca^{2+} channels mediate a large repertoire of physiological actions, including the generation of dendritic spikes in neocortical pyramidal neurons; however, the type of Ca^{2+} channels involved in their generation remains unknown.
- We found that L-type Ca^{2+} currents generate the sustained plateau potential of the Ca^{2+} spike. $GABA_B$ receptors inhibit Ca^{2+} spikes by specifically blocking dendritic L-type currents.
- This inhibition is mediated by a direct $G_{i/o}$-$\beta\gamma$-subunit interaction with the Ca_v1 channels.
- Protein kinases (protein kinase C and A) have an important influence on the generation and sustaining of dendritic Ca^{2+} spikes; however, their activity is not involved in the $GABA_B$-mediated inhibition of Ca^{2+} spikes.
- Inhibitory modulation of dendritic activity is important to understand the transformation of synaptic inputs into neuronal output activity. Our results shed light on the molecular mechanisms by which GABA acting via its $GABA_B$ receptors can exert this inhibitory action.

Abstract Voltage-dependent calcium channels (VDCCs) serve a wide range of physiological functions and their activity is modulated by different neurotransmitter systems. GABAergic inhibition of VDCCs in neurons has an important impact in controlling transmitter release, neuronal plasticity, gene expression and neuronal excitability. We investigated the molecular signalling mechanisms by which $GABA_B$ receptors inhibit calcium-mediated electrogenesis (Ca^{2+} spikes) in the distal apical dendrite of cortical layer 5 pyramidal neurons. Ca^{2+} spikes are the basis of coincidence detection and signal amplification of distal tuft synaptic inputs characteristic for the computational function of cortical pyramidal neurons. By combining dendritic whole-cell recordings with two-photon fluorescence Ca^{2+} imaging we found that all subtypes of VDCCs were present in the Ca^{2+} spike initiation zone, but that they contribute differently to the initiation and sustaining of dendritic Ca^{2+} spikes. Particularly, Ca_v1 VDCCs are the most abundant VDCC present in this dendritic compartment and they generated the sustained plateau potential characteristic for the Ca^{2+} spike. Activation of $GABA_B$ receptors specifically inhibited Ca_v1 channels. This inhibition of L-type Ca^{2+} currents was transiently relieved by strong depolarization but did not depend on protein kinase activity. Therefore, our findings suggest a novel membrane-delimited interaction of the $G_{i/o}$-$\beta\gamma$-subunit with Ca_v1 channels identifying this mechanism as the general pathway of $GABA_B$ receptor-mediated inhibition of VDCCs. Furthermore, the characterization of the contribution of the different VDCCs to the

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generation of the Ca^{2+} spike provides new insights into the molecular mechanism of dendritic computation.

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Abbreviations 4-AP, 4-aminopyridine; ACSF, artificial cerebral spinal fluid; AP, action potential; $G_{i/o}$-$\beta\gamma$, $\beta\gamma$-subunit of a $G_{i/o}$ protein; PI3K, phosphatidylinositol 3-kinase; PKA, cAMP-dependent protein kinases I and II; PKC, protein kinase C; PLC, phospholipase C; TEA, tetraethylammonium-chloride; VDCCs, voltage-dependent calcium channels.

Introduction

Voltage-dependent calcium channels (VDCCs) are a major source of calcium influx into neurons. They serve versatile roles in neuronal signaling, such as transmitter release, neuronal plasticity, gene expression and, very importantly, neuronal excitability (Tsien *et al.* 1988; Catterall, 2000; Catterall & Few, 2008). Different neurotransmitters regulate these fundamental physiological aspects by modulating the activity of VDCCs through their G-protein-coupled receptors (Catterall, 2000; Dolphin, 2003; Catterall & Few, 2008).

In particular, the neurotransmitter GABA acting through metabotropic $GABA_B$ receptors can inhibit VDCCs (Dolphin & Scott, 1987) with important implications for neuronal function. Upon activation, $GABA_B$ receptors inhibit presynaptic Ca_v 2.1 and 2.2 VDCCs (P/Q- and N-type Ca^{2+} currents, respectively), modulating synaptic release. This mechanism is exerted through a membrane-delimited pathway involving the $\beta\gamma$-subunit of a $G_{i/o}$ protein ($G_{i/o}$-$\beta\gamma$; Kavalali *et al.* 1997; Bettler *et al.* 2004; Padgett & Slesinger, 2010). $GABA_B$ receptor activation can also inhibit Ca_v1 VDCCs (L-type Ca^{2+} currents) in native neurons (Maguire *et al.* 1989; Marchetti *et al.* 1991; Scholz & Miller, 1991; Amico *et al.* 1995; Chalifoux & Carter, 2011), resulting in a reduction in somatic and dendritic Ca^{2+} influx. However, the mechanism by which this inhibition takes place is still unresolved. The possibility that this action is mediated by the $G_{i/o}$-$\beta\gamma$-subunit in a membrane-delimited fashion might *a priori* be discarded, as it has been shown that Ca_v1 VDCCs expressed in heterologous systems (Bourinet *et al.* 1996; Toth *et al.* 1996; Zhang *et al.* 1996) or in peripheral neurons (Plummer *et al.* 1989; Cox & Dunlap, 1992) can not be downregulated by neuromodulators that exert their physiological actions through this pathway. The remaining possibility would be that the $GABA_B$ modulation of the Ca_v1 is exerted by the activation of cytosolic protein kinases (Catterall, 2000; Catterall & Few, 2008).

Here, we investigated this important functional link between $GABA_B$ receptor activation and the inhibition of Ca_v1 channels. In the distal apical dendrite of neocortical pyramidal neurons, activation of $GABA_B$ receptors leads to the inhibition of calcium-mediated electrogenesis, the so-called Ca^{2+} spike (Pérez-Garci *et al.* 2006; Breton & Stuart, 2012). These events are generated in a specific region of the apical dendrite and initial tuft dendrites about 600–900 μm from the pyramidal cell soma (Amitai *et al.* 1993; Yuste *et al.* 1994; Schiller *et al.* 1997; Larkum & Zhu, 2002; Larkum *et al.* 2009), suggesting a compartmentalization of the underlying VDCCs. Ca^{2+} spikes serve important functions in dendritic computation and can transiently switch action potential (AP) output from regular to burst firing mode (Larkum *et al.* 2001). They are suggested to act as coincidence detectors for correlating feedback inputs from higher cortical areas, preferentially innervating distal tuft inputs, with feed-forward sensory information. Furthermore, Ca^{2+} spikes can amplify distal tuft synaptic inputs that are received in specific spatio-temporal sequences (Larkum *et al.* 1999*b*, 2009; Schaefer *et al.* 2003; Larkum & Nevian, 2008). Their actual influence on neuronal activity is tightly controlled by inhibitory inputs (Larkum *et al.* 1999*b*; Murayama *et al.* 2009; Gidon & Segev, 2012). $GABA_B$-mediated inhibition of Ca^{2+} spikes can occur by synaptic release of GABA from local interneurons (Pérez-Garci *et al.* 2006). However, very little is known about the composition of VDCCs in the Ca^{2+} spike initiation zone that contributes to the initiation and sustaining of the dendritic plateau potentials, as well as how $GABA_B$ receptors interact with them to inhibit the Ca^{2+} spike. Given that Ca^{2+} spikes result in large elevations of intracellular Ca^{2+} concentration in this particular region and that they are strongly modulated by $GABA_B$ receptor activation renders the apical dendrite of cortical layer 5 pyramidal neurons an ideal model system to study the interaction of $GABA_B$ receptors with VDCCs in native central neurons.

Methods

Ethical approval

All experiments were approved by the veterinary office of the canton of Berne, Switzerland; and were carried out

in compliance with *The Journal of Physiology* guidelines (Drummond, 2009).

Slice preparation

Acute cortical brain slices were obtained from Wistar rats (P28–P34). The animals were rapidly decapitated prior to brain extraction from the skull.

Parasagittal slices of the primary somatosensory cortex (300 μm thick) were cut with a vibrating microslicer on a block angled at 15 deg to the horizontal in ice-cold oxygenated artificial cerebral spinal fluid (ACSF) and then maintained at 37°C in ACSF for 15–120 min. Slices were perfused continuously with ACSF at 35 ± 2°C throughout the experiments. ACSF contained (in mM): NaCl, 125; $NaHCO_3$, 25; KCl, 2.5; NaH_2PO_4, 1.25; $MgCl_2$, 1; glucose, 25; $CaCl_2$, 2; pH 7.4.

Electrophysiology

Whole-cell recordings from somata and/or from dendrites were obtained with the aid of oblique illumination on a Nikon Eclipse E600FN or by infrared gradient contrast video microscopy on a Leica DMLFS microscope equipped with a 40× objective (Leica, HCX Apo, W40×, UVI, NA 0.8).

Somatic (4–6 MΩ) and dendritic (10–20 MΩ) recording pipettes were filled with an intracellular solution containing (in mM): potassium gluconate, 135; KCl, 7; Hepes, 10; Na_2-phosphocreatine, 10; Mg-ATP, 4; GTP, 0.3; 0.2% biocytin; pH 7.2 (with KOH); 291–293 mosmol l^{-1}. For double patch-clamp recordings, 10–20 μM Alexa 594 was added to the intracellular solution. For Ca^{2+} imaging, 300 μM Fluo-5F and 30 μM Alexa 594 were added to the internal solution. Dendritic Ca^{2+} spikes were evoked by injecting an EPSP-shaped current wave form: (double exponential shape: $f(t) = (1 - e^{-t/\tau 1})e^{-t/\tau 2}$; where $\tau_1 = 4$ ms and $\tau_2 = 10$ ms; time to peak: 5 ms) via the dendritic pipette.

Two-photon calcium imaging

Two-photon-excited fluorescence was generated with a titanium-sapphire laser (Tsunami, Spectra-Physics) with laser pulses of 100 fs at a wavelength of 800 nm. The laser was coupled to a confocal scanning unit (TCS-SP2, Leica Microsystems) attached to an upright microscope (DMLFS, Leica), equipped with a 40× objective and non-descanned detectors. Red and green fluorescence signals were separated using a dichroic mirror (560DCXR, AHF) and corresponding band-pass filters (HQ520/25 and HQ585/40, AHF; Nevian & Sakmann, 2006). Raw fluorescence $G(t)$ and $R(t)$ was derived from line scans recorded at 800 Hz by integrating the fluorescence in regions of interest enclosing the fluorescent structures. Relative fluorescence changes were calculated as $\Delta G/R(t) = (G(t) - G_0)/R_{ave}$, where G_0 is the mean resting green fluorescence during 50 ms before stimulation. The Ca^{2+}-insensitive red fluorescence R_{ave} was calculated as the mean over the entire time course of the line scan recording (Nevian & Helmchen, 2007).

Pharmacology

Drugs were freshly prepared the same day of the experiment and consisted of (in μM): RS-baclofen, 10; nimodipine, 10; Gö6850 bisindolylmaleimide I, 1; KT 5720, 0.5; wortmannin, 0.2; Rp-cAMPS triethylammonium salt, 1000 (from TOCRIS Bioscience, Switzerland); Sp-cAMPS triethylammonium salt, 30 (from SIGMA-ALDRICH); SNX-482, 0.23; ω-agatoxin TK, 0.4; ω-conotoxin GVIA, 1 (from Alomone Labs, Israel).

For isolated dendritic L-type Ca^{2+} currents, the internal solution consisted of (in mM): caesium-methanesulphonate, 108; Hepes, 9; Na_2-phosphocreatine, 14; Mg-ATP, 4; Na-GTP, 0.3; 0.2% biocytin; pH 7.3 (with CsOH). The external solution included (in mM): TTX, 0.001; tetraethylammonium-chloride (TEA), 30; 4-aminopyridine (4-AP), 5; $BaCl_2$, 0.100; $NiCl_2$, 0.05 (Sigma-Aldrich); ω-conotoxin MVIIC, 0.0005 (from Alomone Labs).

When bath-applied, a minimum wait of 15 min was allowed to assess for the effect of the drug.

Local perfusion of baclofen (50 μM) onto the dendritic recording site was achieved by means of a puffing pipette (somatic pipette) located 50–100 μm lateral from the dendritic recording electrode. The range of area covered by the bolus expelled from the pipette was estimated to have a diameter of ∼180 μm, as measured by a test application of the fluorescent indicator Alexa 594 into a brain slice.

Data analysis

For each cell tested, the sweep showing the maximal effect of baclofen on the Ca^{2+} spike was used for analysis. The effectiveness of baclofen was quantified as the area underneath the control Ca^{2+} spike sensitive to the local application of baclofen to the apical tuft. This area was estimated by subtracting the baclofen-reduced Ca^{2+} spike from the control Ca^{2+} spike. For simplification, the area underneath the Ca^{2+} spike contributed by the back-propagating somatic AP was not considered in the analysis (although this may well have led to an

underestimation of the physiological effect of activating $GABA_B$ receptors in the apical tuft). Wilcoxon signed-rank tests for repeated measurements and Mann–Whitney *U* tests for independent samples were performed for statistical comparisons.

Results

Subcellular compartmentalization of VDCCs in the Ca^{2+} spike initiation zone

We studied the contribution of the different VDCCs to the generation of dendritic Ca^{2+} spikes as a first step to study their inhibition by $GABA_B$ receptors. Dendritic whole-cell recordings were performed within the Ca^{2+} spike initiation zone of layer 5 neocortical pyramidal neurons around the main bifurcation of the apical dendrite (Fig. 1*A*). Dendrites were loaded with a Ca^{2+} indicator (Fluo-5F, 300 μM) and Alexa-594 (30 μM), and Ca^{2+} spikes were evoked by injecting an EPSP-shaped current with peak amplitudes of 800–1500 pA via the patch pipette into the dendrite. Ca^{2+} transients were measured in spines and adjacent dendritic shafts 50–150 μm distal from the recording pipette (Fig. 1*B* and *C*). Bath application of the Ca_v1 (L-type) VDCC blocker nimodipine (10 μM) resulted in an inhibition of the sustained depolarizing plateau that followed the initial depolarization of the Ca^{2+} spike and a large reduction of the dendritic Ca^{2+} transient by $55 \pm 4\%$ ($n = 10$; Fig. 1*C* and *F*). Ni^{2+} (50 μM), which blocks both Ca_v 3 (T-type) and Ca_v 2.3 (R-type) VDCCs prevented Ca^{2+} spike initiation in most cases, and abolished Ca^{2+} transients in both the spine and dendrite (Fig. 1*D*). Extra current (~400 pA) injected into the dendrite recovered the Ca^{2+} spike, albeit with a significant decrease in the dendritic Ca^{2+} transient by $31 \pm 8\%$ ($n = 7$). SNX-482 (230 nM), a selective blocker of Ca_v 2.3 (R-type) VDCCs, had no effect on the initiation of the Ca^{2+} spike, but it resulted in a similar reduction of the dendritic Ca^{2+} transient ($26 \pm 1\%$; $n = 3$), indicating a large contribution of R-type conductances within the Ni^{2+}-sensitive component. Ca_v 2.1 (P/Q-type) VDCCs, blocked by ω-agatoxin TK (400 nM), contributed $21 \pm 1\%$ ($n = 4$); and Ca_v 2.2 (N-type) VDCCs, blocked by ω-conotoxin GVIA (1 μM), contributed $20 \pm 1\%$ ($n = 8$) to the Ca^{2+} spike-evoked Ca^{2+} transients in the dendrite. The relative contribution of each VDCC subtype to the Ca^{2+} transients in spines was different (Fig. 1*G*). Ca_v1 VDCCs contributed $45 \pm 4\%$ to the Ca^{2+} transient in the spine, which was significantly less than in the parent dendrite ($P < 0.05$). $Ca_v2.3$ VDCCs contributed $34 \pm 6\%$ to the Ca^{2+} transient accounting for most of the combined R- and T-type contribution determined by application of Ni^{2+} ($41 \pm 7\%$). $Ca_v2.1$ VDCCs were found to contribute $21 \pm 2\%$ to the spine Ca^{2+} transient, whereas $Ca_v2.2$ VDCCs made no significant contribution ($9 \pm 5\%$).

$GABA_B$ receptor activation inhibits Ca_v1 VDCCs

Ca^{2+} spikes initiated in the distal main apical dendrite of layer 5 pyramidal neurons are modulated by the local activation of $GABA_B$ receptors inhibiting Ca^{2+} conductances (Pérez-Garci *et al.* 2006; Breton & Stuart, 2012). Therefore we assessed the effect of activating $GABA_B$ receptors on the Ca^{2+} transients evoked by Ca^{2+} spikes. Bath application of the $GABA_B$ agonist baclofen (10–30 μM) blocked the dendritic plateau potential that followed the upstroke of the Ca^{2+} spike and reduced the Ca^{2+} transients in dendrites by $59 \pm 4\%$ ($P < 0.01$; $n = 9$) and in spines by $52 \pm 5\%$ ($P < 0.01$; Fig. 1*E–G*). The effect of baclofen on the Ca^{2+} spike was similar to the effect of nimodipine in blocking the characteristic plateau potential and in the reduction of the Ca^{2+} transients associated to these events. These similarities suggested that $GABA_B$ receptors might exert their modulatory effect on the Ca^{2+} spike by inhibiting Ca_v1 VDCCs.

To test this hypothesis, double patch-clamp recordings were performed from the soma and the distal apical dendrite (>700 μm from soma; Fig. 2) and Ca^{2+} spikes were evoked by dendritic current injection. The $GABA_B$ agonist baclofen (50 μM) was applied by pressure ejection to the Ca^{2+} spike initiation zone using a third pipette located 50–100 μm lateral from the dendritic recording electrode to determine the effectiveness of $GABA_B$ receptor inhibition on the Ca^{2+} spike after blockade of the various VDCCs. Under control conditions, dendritic depolarization evoked Ca^{2+} spikes that propagated towards the soma, evoking somatic APs. Subsequent transient activation of $GABA_B$ receptors resulted in the inhibition of the depolarizing plateau that followed the initial upstroke of the Ca^{2+} spike and blocked somatic firing. The action of baclofen was completely reversed within 6 s after ceasing the pressure application. Block of Ca_v1 VDCCs either with nicardipine (5 μM; $n = 6$) or nimodipine (10 μM; $n = 7$) resulted in the inhibition of the plateau component of the Ca^{2+} spike leaving the fast initial depolarization intact. Under these conditions, baclofen reapplied locally to the apical dendrite did not cause further inhibition, indicating that blocking Ca_v1 VDCCs occluded the effect of baclofen (Fig. 2*A*). To test the possibility that the decrease in dendritic depolarization after blocking Ca_v1 channels affected the activation of other VDCCs that might be susceptible to modulation by $GABA_B$ receptors, extra current (additional ~400 pA) was injected in some experiments. Under these conditions somatic APs were reestablished; however, the effect of baclofen was still occluded ($n = 5$).

As shown above, Ni^{2+} (50 μM) prevented the initiation of dendritic Ca^{2+} spikes near threshold current injection, but extra current (~400 pA) injected into the dendrite reactivated the remaining VDCCs (Fig. 2*B*). Under these conditions, application of baclofen to the apical dendrite

was still effective in inhibiting the Ca^{2+} spike ($n = 5$), indicating that the action of baclofen on the sustained component of the Ca^{2+} spike was not mediated by inhibition of Ni^{2+}-sensitive (Ca_v3 and $Ca_v2.3$) VDCCs. Similarly, blocking $Ca_v2.1$, $Ca_v2.2$ and $Ca_v2.3$ VDCCs with ω-agatoxin TK (400 nM), ω-conotoxin GVIA (1 μM) and SNX-482 (230 nM), respectively, did not influence the effectiveness of baclofen to inhibit Ca^{2+} spikes (Fig. 2*C*–*E*). These results therefore showed that activation of GABA$_B$ receptors in the distal apical dendrite exerted its inhibitory effect on dendritic Ca^{2+} spikes by specifically inhibiting Ca_v1 VDCCs (Fig. 2*F*).

After having identified the predominant effect of GABA$_B$-mediated inhibition of Ca^{2+} spikes to act on Ca_v1, we tested the action of baclofen on pharmacologically isolated L-type Ca^{2+} currents recorded from the distal apical dendrite directly (Fig. 3). Because voltage-clamp recordings in dendrites of neurons with an extensive dendritic arborization are limited by an incomplete space clamp of the dendritic membrane (Williams & Mitchell, 2008), we used this method only to phenomenologically assess the interaction of GABA$_B$ receptors with L-type Ca^{2+} currents by comparing the relative effect of baclofen with control conditions. Dendritic patch-clamp

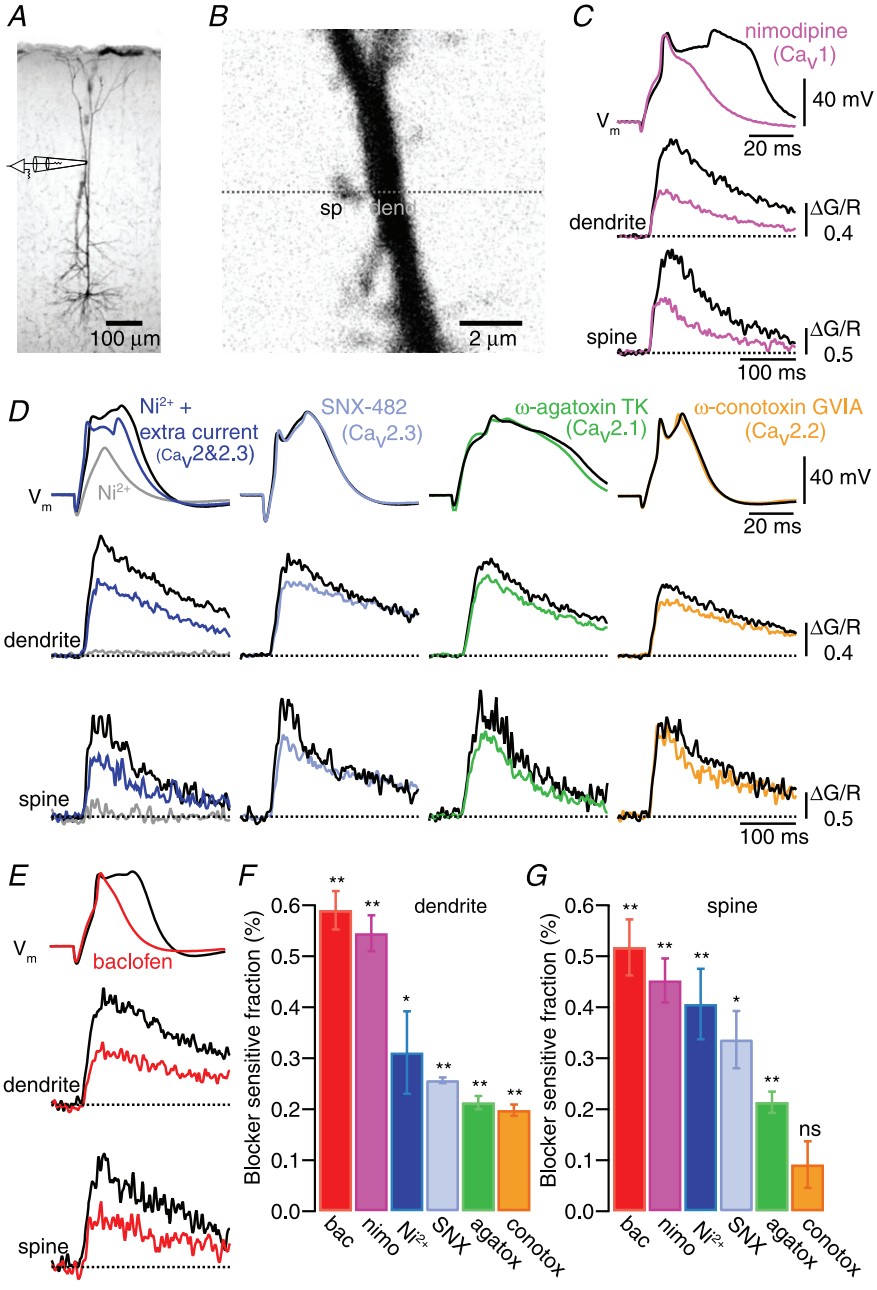

Figure 1. Subcellular compartmentalized distribution of voltage-dependent calcium channels (VDCCs) and action of GABA$_B$ receptors
A, micrograph of a biocytin-filled L5 pyramidal neuron indicating the dendritic recording site. *B*, 2-photon fluorescence image (Alexa 594) of the recording site at the distal apical dendrite and a magnified view showing a spiny region in the apical tuft. Ca^{2+} imaging (Fluo-5) was measured by establishing a line scan (dashed line) covering a spine and its adjacent dendrite. *C* and *D*, dendritic Ca^{2+} spikes (top sweep) evoked by current injection via the recording pipette elicited Ca^{2+} transients in both the dendrite (middle panel) and in the spines (bottom panel; black sweeps). Coloured sweeps show representative examples of the action of different VDCCs blockers on the Ca^{2+} spikes and the resulting inhibition of Ca^{2+} fluorescence transients in both the dendrites and in the spines: nimodipine (10 μM, magenta traces), Ni^{2+} (50 μM, grey and blue sweeps, respectively, show recordings before and after injection of additional current via the recording pipette), SNX-482 (230 nM, light blue), ω-agatoxin TK (400 nM, green sweeps) and ω-conotoxin GVIA (1 μM, orange sweeps). *E*, baclofen added to the bath (10–30 μM) partially inhibited the Ca^{2+} spikes and their associated Ca^{2+} transients (red sweeps). *F*, summary bar plot showing the relative block of Ca^{2+} transients recorded in the dendrites for each drug tested. *G*, summary bar plot showing the relative block of Ca^{2+} transients recorded in the spines for each drug tested. Error bars indicate SEM. $^*P < 0.05$, $^{**}P < 0.01$.

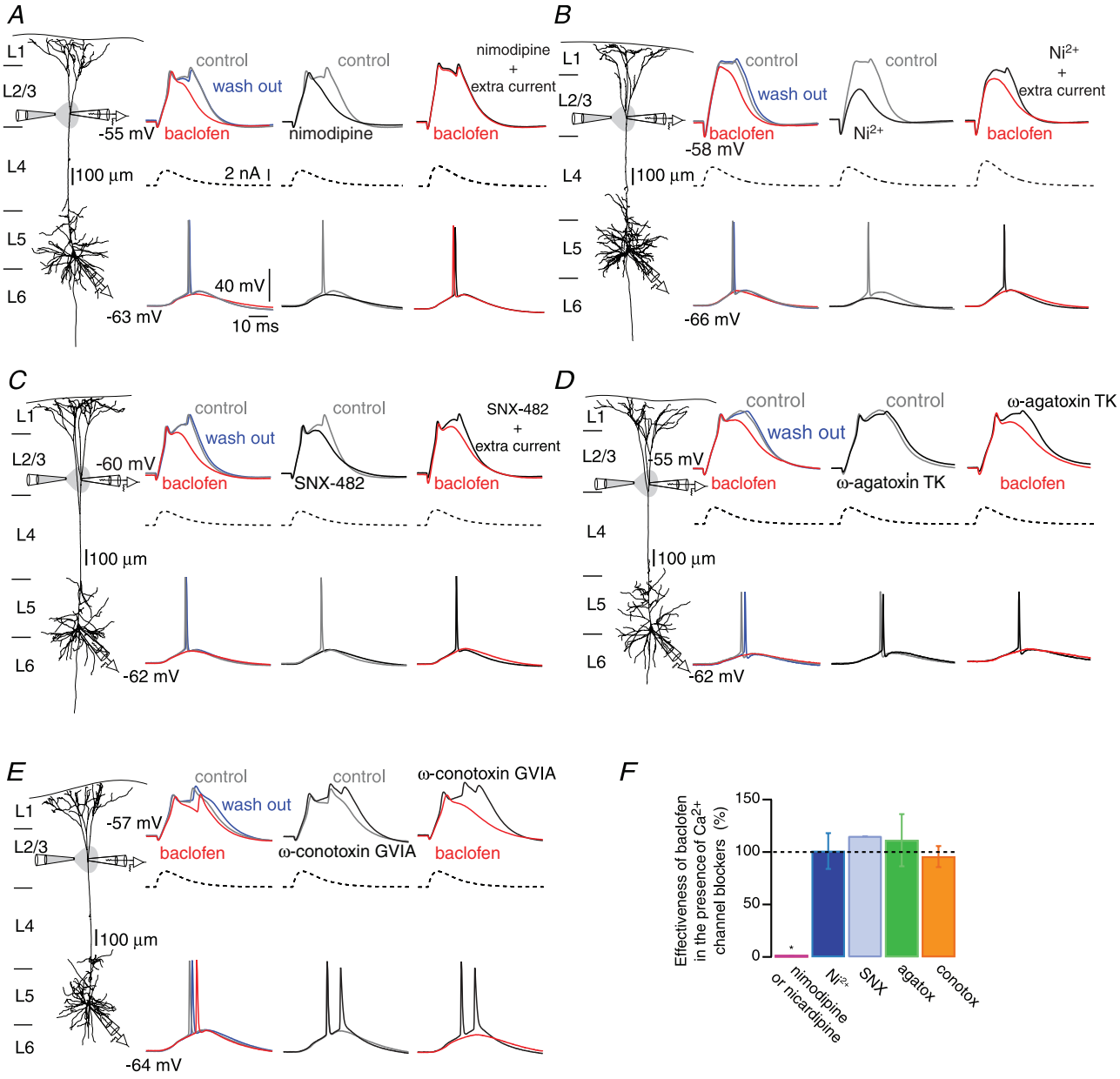

Figure 2. Activation of dendritic $GABA_B$ receptors inhibits L-type Ca^{2+} conductances involved in the generation of Ca^{2+} spikes

A–E, left columns: reconstruction of biocytin-filled L5 pyramidal neurons showing simultaneous somatic and distal (>700 μm) patch recordings, while a puff pipette expelled baclofen (50 μM) onto the apical tuft. Second columns: dendritic Ca^{2+} spikes (top, grey sweeps) were evoked by injecting an EPSP-shaped current waveform (middle, dashed sweeps) via the distal pipette. Dendritic spikes propagated towards the soma evoking APs (bottom, grey sweeps). Puffing baclofen on the dendrite shortened and reduced the amplitude of the Ca^{2+} spikes (red sweeps). The effect of baclofen was fully reversed after ceasing the local application of baclofen (blue sweeps). Third columns: control recordings and recordings obtained after bath application of different voltage-dependent calcium channels (VDCCs) blockers (black sweeps; *A*, nimodipne 10 μM; *B*, Ni^{2+} 50 μM; *C*, SNX-482, 230 nM; *D*, ω-agatoxin TK, 400 nM; *E*, ω-conotoxin GVIA, 1 μM). Fourth columns: reapplication of baclofen to the tuft in the presence of VDCCs blockers. In some cases larger current peaks were injected to the dendrite to reestablish the dendritic depolarization (see *A–C*). *F*, effectiveness of baclofen estimated as the area underneath the control Ca^{2+} spike sensitive to the local application of baclofen. Values obtained after blockade of VDCCs are normalized to those obtained before bath application of VDCCs blockers (see SI Materials and methods). Error bars indicate SEM. $^*P < 0.01$.

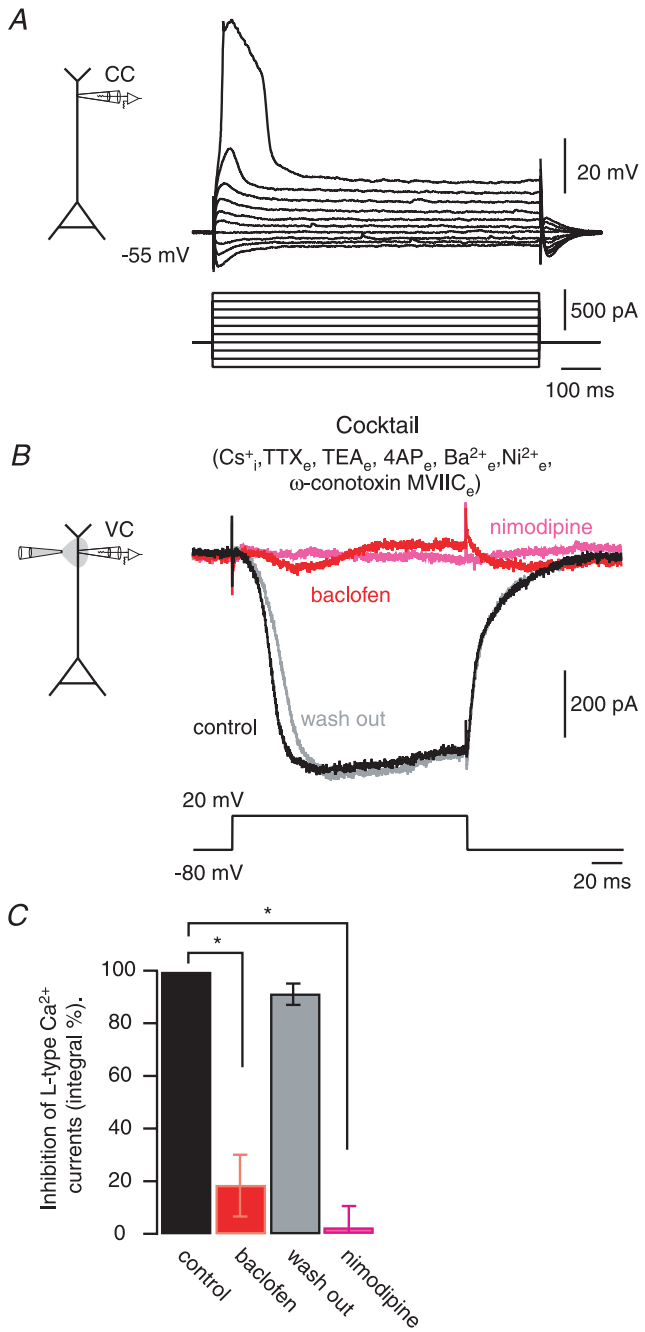

Figure 3. Activation of dendritic GABA$_B$ receptors inhibits dendritic L-type Ca^{2+} currents
A, voltage recordings performed in current-clamp mode at a distal dendritic site (700 μm) immediately after seal rupture. The internal pipette solution included 108 mM Cs^+. Dendritic Ca^{2+} spikes occurred with depolarizing current steps (lower panel). *B*, a depolarizing voltage command in voltage-clamp mode was applied to the dendrite in an external medium containing TTX (1 μM), tetraethylammounium-chloride (TEA; 30 mM), 4-aminopyridine (4-AP; 5 mM) and Ba^{2+} (100 μM). Voltage-dependent calcium channels (VDCCs) other than Ca_v1 were also blocked with Ni^{2+} (50 μM; T- and R-type) and ω-conotoxin MVIIC (500 nM; N- and P/Q-type). Under these conditions, inward L-type Ca^{2+} (after leak subtraction) currents were recorded (black sweep). Local application of the GABA$_B$ agonist baclofen (50 μM) to the apical tuft inhibited the L-type Ca^{2+} currents (red sweep). The action of baclofen was reversed after 5 s ceasing the pressure application of baclofen (grey sweep). Subsequent bath application of nimodipine (10 μM) effectively blocked the L-type Ca^{2+} current (pink sweep). *C*, summary of the inhibitory effect of baclofen and nimodipine. Error bars indicate SEM. $^*P < 0.01$.

recordings were performed with pipettes containing an internal Cs^+-methanesulphonate-based solution. We first confirmed the proximity of the recording site to the dendritic Ca^{2+} spike initiation zone using current steps in current-clamp mode to evoke Ca^{2+} spikes in normal ACSF (Fig. 3*A*). The extracellular solution was then supplemented with a 'cocktail' for isolating L-type Ca^{2+} currents by blocking all other VDCCs, K^+ channels and Na^+ channels. In addition, Cs^+ applied intracellularly effectively blocks I_h currents (Harris *et al.* 1994) and GIRK channels (Sodickson & Bean, 1996). L-type Ca^{2+} currents were then evoked in voltage-clamp mode by changing the dendritic command potential from −80 mV to 20 mV (Fig. 3*B*). Under these conditions, baclofen applied locally to the dendrite reversibly inhibited the L-type Ca^{2+} currents by 82 ± 12% ($P < 0.01$; $n = 4$; Fig. 3*C*). L-type Ca^{2+} currents subsequently recovered fully within about 3 s. Because the application of baclofen was spatially restricted (~180 μm), the remaining ~20% of the Ca^{2+} currents may be explained by L-type Ca^{2+} influx beyond the area affected. This suggested that under our experimental conditions the voltage-clamp step command still depolarized a stretch of dendrite beyond 180 μm sufficiently to activate L-type Ca^{2+} currents at even more distal sites. During the puff-application of baclofen we did not observe any change in the holding current required to clamp the dendrite at −80 mV, suggesting that baclofen did not activate or inactivate any potential leak conductances that would alter the spatial extend of the voltage-clamp. Subsequent bath application of nimodipine (10 μM) blocked the Ca^{2+} currents by 98.2 ± 8% ($P < 0.01$; $n = 4$; Fig. 3*D*), demonstrating the L-type nature of the recorded current. After having blocked L-type currents with nimodipine (and in the presence of the cocktail containing unspecific blockers for the common Na^+, K^+ and Ca^{2+} currents), a final application of baclofen was ineffective to evoke any detectable current corroborating the result that in this set of voltage-clamp experiments GABA$_B$ activation did not modulate any other currents than L-type currents under our experimental conditions (data not shown).

These results show unequivocally that activation of GABA$_B$ receptors in the distal apical dendrite directly inhibit Ca_v1 channels, which constitute the predominant conductance activated during the plateau potential of the dendritic Ca^{2+} spike.

Signalling mechanisms underlying the action of $GABA_B$ inhibition on dendritic Ca^{2+} spikes

How is the observed interaction between $GABA_B$ receptors and Ca_v1 channels mediated? $GABA_B$ receptors act via a pertussis toxin-sensitive $G_{i/o}$ protein (Bettler *et al.* 2004). They are known to inhibit Ca_v2 VDCCs (Kavalali *et al.* 1997; Bettler *et al.* 2004; Padgett & Slesinger, 2010) by a membrane-delimited pathway involving the $\beta\gamma$-subunit of the $G_{i/o}$ protein. However, this form of inhibition has not been found for Ca_v1 VDCCs when studied in heterologous expression systems (Bourinet *et al.* 1996; Toth *et al.* 1996; Zhang *et al.* 1996) or in peripheral neurons (Plummer *et al.* 1989; Cox & Dunlap, 1992) so far. Alternatively, $GABA_B$ receptors might inhibit Ca_v1 channels by cytosolic signalling pathways acting on protein kinases. Thus, we investigated the potential signalling mechanisms downstream of $GABA_B$ receptor activation that led to the inhibition of Ca_v1 channels.

We first sought to test a direct interaction of the $\beta\gamma$-subunit with Ca_v1 channels. To this end we exploited the fact that the $\beta\gamma$-subunit inhibitory interaction with VDCCs is voltage dependent and can be transiently relieved by a relatively strong depolarization, or prepulse facilitation voltage command sequence (Bourinet *et al.* 1996; Herlitze *et al.* 1996; Ikeda, 1996; Toth *et al.* 1996; Zhang *et al.* 1996; Zamponi *et al.* 1997; De Waard *et al.* 2005). Therefore, we tested whether a prepulse voltage command was able to reverse the inhibitory effect of baclofen on isolated dendritic L-type Ca^{2+} currents (Fig. 4). Bath application of baclofen (10–30 μM) abolished the L-type Ca^{2+} current evoked by a voltage command from −80 mV to 20 mV (current integral compared with control; $2.3 \pm 1\%$; $P < 0.01$; $n = 4$; Fig. 4*B* and *C*). Thereafter, a sequence of prepulse voltage commands consisting of 50 ms depolarizations from −80 mV to 70–100 mV preceding a 200 ms-long voltage step from −80 mV to 20 mV by 60 ms repeated five times every 5 s was applied to disrupt the potential $\beta\gamma$-subunit interaction with the Ca_v1 VDCCs. Indeed, after sessation of this prepulse stimulation protocol, we observed a partial recovery of the L-type Ca^{2+} current ($57.5 \pm 18\%$; Fig. 4*B* and *C*). This recovery lasted 10–30 s in the absence of the prepulse voltage commands, before baclofen exerted its inhibitory effect again ($3.4 \pm 2\%$; Fig. 4*B* and *C*). Addition of nimodipine to the bath solution maintained the complete block of the measured Ca^{2+} current as expected ($3.1 \pm 3\%$; Fig. 4*B* and *C*). However, in the presence of nimodipine, prepulse voltage commands were ineffective in rescuing the L-type Ca^{2+} current from $GABA_B$-mediated inhibition ($5.8 \pm 2.8\%$).

During the course of the experiment, neither the tonic activation of $GABA_B$ receptors by bath application of baclofen nor the prepulse voltage commands modified the holding current for a holding potential of −80 mV (Fig. 4*D*), arguing against the activation or inactivation of leak conductances that would compromise the extend of our voltage-clamp recordings and that would change the membrane length constant and therefore the number of activated Ca_v1 VDCCs. Thus, we conclude that our voltage-clamp conditions were stable and faithfully reflected the behaviour of the L-type current.

Not only was the prepulse protocol effective in recovering the L-type current, but also a 200 ms-long voltage step from −80 mV to 50–80 mV was able to reestablish the L-type Ca^{2+} currents, consistent with the voltage-dependence of the $\beta\gamma$-subunit interaction with VDCCs (Fig. S1; $n = 3$). After bath application of nimodipine the voltage steps applied did not uncover any additional currents demonstrating the L-type nature of the recorded currents. Even long (200 ms) current steps to +80 mV failed to activate any further currents (Fig. S1), arguing against the possibility that the activation of L-type currents at potentials of +20 mV resulted in a regenerative Ca^{2+}-mediated event that escaped the voltage clamp and depolarized the membrane beyond the set level of +20 mV and thereby activated additional currents that required a higher threshold for activation.

These results demonstrated that the recovery from inhibition due to strong depolarization was indeed specific to the relief of the Ca_v1 VDCC. The most parsimonious explanation for the voltage dependency of $GABA_B$ receptor-mediated inhibition of Ca_v1 channels is therefore a membrane-delimited pathway involving the $G_{i/o}$-$\beta\gamma$-subunit (Fig. 4*E*).

Next, we investigated if the $GABA_B$ receptor-mediated inhibition of Ca^{2+} spikes could additionally be mediated by cytosolic signalling cascades. These pathways involve different enzymes that have VDCCs as their main targets: protein kinase C (PKC), cAMP-dependent protein kinases I and II (PKA), phosphatidylinositol 3-kinase (PI3K; Bettler *et al.* 2004; Deng *et al.* 2009) and phospholipase C (PLC; Sickmann *et al.* 2008).

We performed six pharmacological manipulations in order to test the influence of the activity state of these enzymes on the properties of dendritic Ca^{2+} spikes directly: pre-incubation of the slices with the inhibitor of PKC, bisindolylmaleimide I (1 μM); pre-incubation of the slices with the inhibitor of PKA, KT 5720 (500 nM); inclusion in the intracellular solution of the inhibitor of PKA, Rp-cAMPS (1 mM); inclusion in the intracellular solution of the activator of PKA, Sp-cAMPS (60–100 μM); inclusion in the intracellular solution of the PI3K inhibitor wortmannin (400 nM); and inclusion in the intracellular solution of the PLC inhibitor U73122 (1 μM). Of all these manipulations, only inhibition of PKC and inhibition of PKA affected dendritic Ca^{2+} spikes (Figs S2 and S3).

We next examined if these enzymes are activated by $GABA_B$ receptors to inhibit dendritic Ca^{2+} spikes. Irreversible inhibition of PKC by bisindolylmaleimide I

(1 μM) led to prolonged dendritic Ca^{2+} spikes (Fig. S2). This indicated that activation of PKC could be a signalling cascade to inhibit Ca^{2+} spikes. If GABA$_B$ receptor activation upregulated the activity of PKC to inhibit dendritic Ca^{2+} spikes, pretreatment with bisindolylmaleimide I would occlude the effect of GABA$_B$ activation with baclofen. However, we found that local application of baclofen to the apical tuft of neurons pretreated with bisindolylmaleimide I inhibited Ca^{2+} spikes to a similar extent as in untreated cells (untreated cells, $10.5 \pm 1.6\%$; $n = 10$; bisindolylmaleimide I, $11.9 \pm 0.2\%$; $n = 3$; $P > 0.05$; Fig. 5*B* and *H*).

Irreversible inhibition of PKA with either KT 5720 or Rp-cAMPS decreased the duration or inhibited the generation of Ca^{2+} spikes (Figs S2 and S3). Under these conditions, the effect of baclofen could not be determined (Fig. 5*C*). However, the irreversible activation of PKA by adding Sp-cAMPS (60–100 μM) to the recording pipette did not occlude the effect of baclofen in inhibiting the Ca^{2+} spike ($11.2 \pm 3.4\%$, $n = 4$; $P > 0.05$; Fig. 5*D* and *H*). This shows that GABA$_B$-induced downregulation of the activity of PKA cannot explain the inhibitory action of baclofen. The inhibition of PI3K, a protein kinase known to be activated by G$_{i/o}$ proteins (Salgado *et al.* 2007), was also ineffective in modifying the inhibitory effect of baclofen ($9.4 \pm 0.4\%$; $n = 3$; $P > 0.05$; Fig. 5*E* and *H*). Inhibition of PLC by U73122 has been reported to reduce the amplitude of GABA$_B$-mediated Kir3 responses in dissociated neocortical pyramidal neurons (Sickmann *et al.* 2008); however, intracellular application of U73122

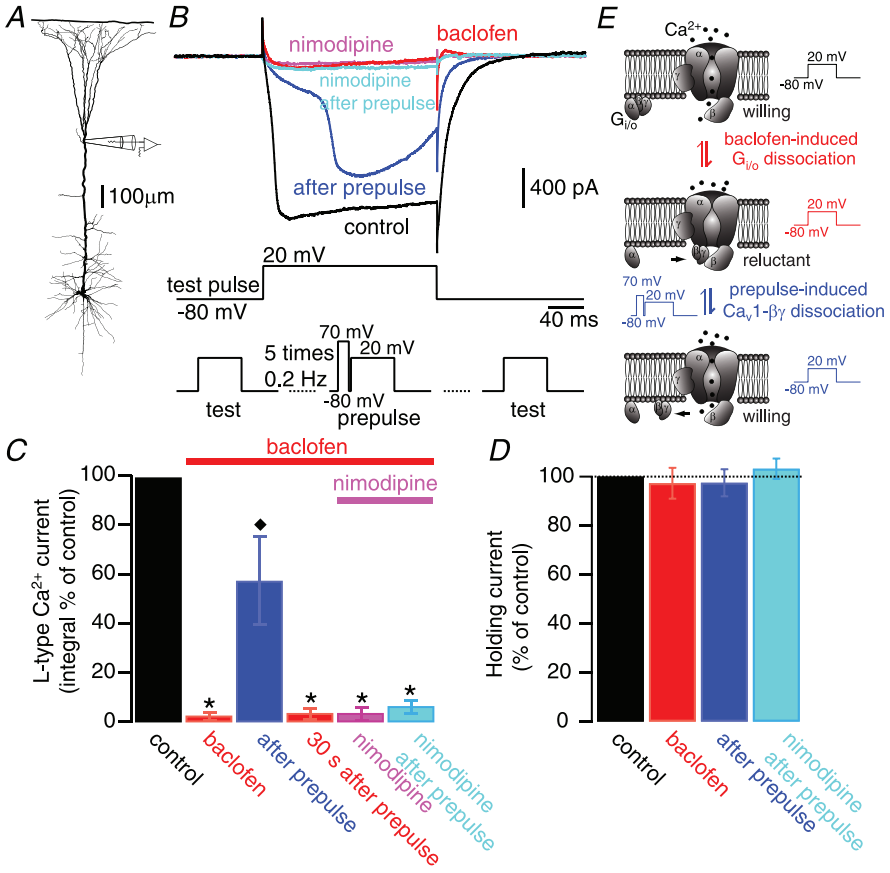

Figure 4. GABA$_B$ inhibition of dendritic L-type Ca^{2+} currents is mediated by a direct G$_{i/o}$-$\beta\gamma$-subunit interaction

A, experimental configuration of dendritic voltage-clamp experiments. *B*, pharmacologically isolated dendritic L-type Ca^{2+} currents (black trace) were evoked by injecting a test voltage command from −80 to 20 mV (test pulse; middle trace). Bath application of baclofen (10–30 μM) inhibited the L-type Ca^{2+} currents (red trace). A series of 5 prepulse voltage commands from −80 mV to 70 mV (50 ms duration) preceding a depolarization from −80 mV to 20 mV (200 ms) by 60 ms (prepulse; bottom middle trace), partially and transiently relieved the L-type Ca^{2+} current from its inhibition (blue trace). After recovery of the GABA$_B$-induced inhibition, nimodipine (10 μM) was supplemented to the bath (pink trace). In the presence of the Ca$_v$1 blocker, a new set of 5 prepulse voltage commands was ineffective to reverse the inhibition of L-type Ca^{2+} currents (cyan trace). The lower traces illustrate the sequence of test pulses and the prepulse protocol. *C*, summary of results. Error bars indicate SEM. $^*P < 0.01$, relative to control; $^\blacklozenge P < 0.01$, relative to baclofen inhibition ($n = 4$). *D*, bar graph showing the lack of variation in holding current values (at −80 mV) during the different experimental manipulations. *E*, sketch of the prepulse-induced relief of inhibition illustrating the interpretation of the current traces depicted in *B*.

did not modify the effect of baclofen on dendritic Ca^{2+} spikes, ($11.8 \pm 3.5\%$; $n = 4$; $P > 0.05$; Fig. 5*F* and *H*).

Finally, selective blockade of Kir3 K^+ channels by Ba^{2+} (Newberry & Nicoll, 1984) did not prevent the action of baclofen (control 10.5 ± 1.6 *vs.* $21.4 \pm 1.4\%$ in 200 μM Ba^{2+}; Fig. 5*G* and *H*), ruling out that the inhibition of this conductance by $GABA_B$ receptors was responsible for the inhibition of the Ca^{2+} spike. The increase in dendritic Ca^{2+} spikes under Ba^{2+} could be attributed to the increased permeability of VDCCs to Ba^{2+} *versus* Ca^{2+} ions and/or the increase in local input resistance due to blockade of K^+ channels.

Taken together our results indicate that the inhibitory effect of $GABA_B$ receptors on Ca^{2+} spikes is mediated predominantly by the downregulation of Ca_v1 VDCCs rather than the activation of Kir3 K^+ currents. Moreover, this modulation did not depend on cytosolic signalling cascades commonly associated with $GABA_B$ receptors, but is likely mediated by a direct interaction of the Ca_v1 VDCCs with the $G_{i/o}$-$\beta\gamma$-subunit.

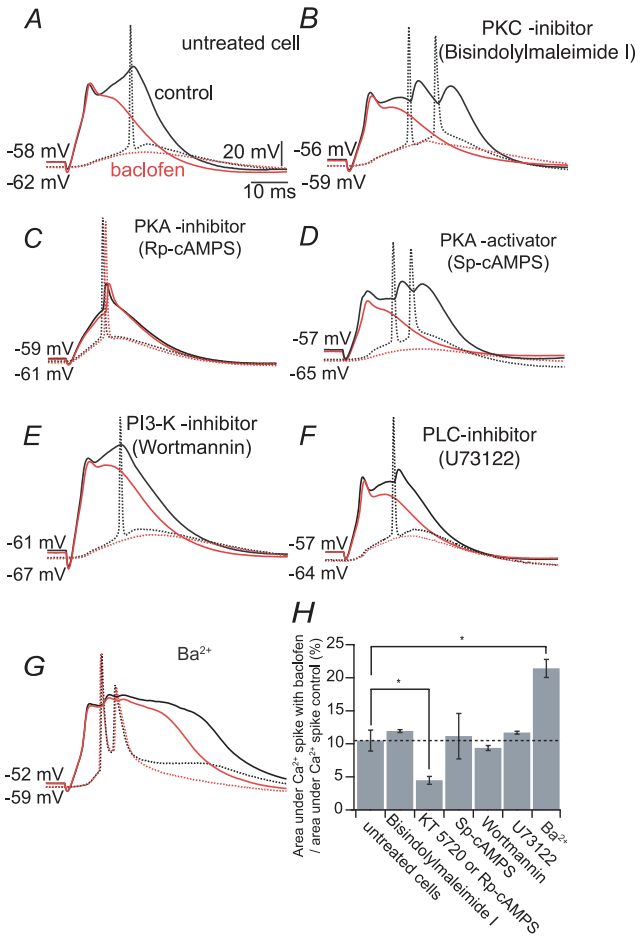

Figure 5. $GABA_B$ receptors inhibit Ca^{2+} spikes independent of protein kinases
A, dendritic Ca^{2+} spike (black solid sweep) leading to somatic AP (black dashed sweep). Baclofen (50 μM; red sweeps) applied locally to the apical tuft induced inhibition of the Ca^{2+} spike. *B–F*, simultaneous double patch-clamp recording from the apical tuft and soma as in *A*, showing the baclofen-induced inhibition of Ca^{2+} spikes in cells pretreated with bisindolylmaleimide I (1 μM) to inhibit protein kinase C (PKC; *B*); Rp-cAMPS (1 mM) to inhibit cAMP-dependent protein kinases I and II (PKA; *C*); Sp-cAMPS (60–100 μM) to overactivate PKA (*D*); wortmannin (400 nM) to inhibit phosphatidylinositol 3-kinase (PI3K; *E*); and U73122 (1 μM) to inhibit phospholipase C (PLC; *F*). *G*, simultaneous double patch-clamp recording from the apical tuft and soma as in *A*, showing the baclofen-induced inhibition of Ca^{2+} spikes in the presence of Ba^{2+} (200 μM). *H*, summary of the inhibitory effect of baclofen (normalized to the area underneath the Ca^{2+} spike control) for each pharmacological condition. Error bars indicate SEM. $^*P < 0.05$.

Discussion

Inhibitory modulation of dendritic function is gaining much attention as a key mechanism to influence dendritic computation (Murayama *et al.* 2009; Lovett-Barron *et al.* 2012; Palmer *et al.* 2012). In particular, the influence of inhibition on non-linear dendritic integration by the modulation of voltage-dependent ion channels in dendrites is important in order to understand the transformation of synaptic inputs to AP output. This study revealed a direct interaction of the $GABA_B$ receptor-activated G-protein $\beta\gamma$-subunit with Ca_v1 channels in the distal apical dendrite of cortical layer 5 pyramidal neurons. Furthermore, we demonstrated that this modulation of Ca_v1 channels is the molecular mechanism for the inhibition of Ca^{2+} spikes, which are regenerative dendritic depolarizations important for signal integration in cortical pyramidal neurons.

Differential roles of VDCCs in the generation of Ca^{2+} spikes

We found that all VDCCs are present in the distal Ca^{2+} spike initiation zone of cortical pyramidal neurons but with different relative contributions to the evoked Ca^{2+} transients. Similarly, it has been found that all VDCCs are present in the proximal stretch of the apical dendrites of various pyramidal cells and are activated by somatically evoked back-propagating APs (Jaffe *et al.* 1992; Yuste *et al.* 1994; Christie *et al.* 1995; Markram *et al.* 1995; Sabatini & Svoboda, 2000) or synaptic activity (Markram & Sakmann, 1994; Yuste *et al.* 1994; Magee *et al.* 1995; Magee & Johnston, 1995*b*; Bloodgood & Sabatini, 2007). Comparison of our results to the previous findings indicates that the VDCC subtypes are differentially distributed along the apical dendrite of layer 5 pyramidal neurons. The contribution of Ca_v1 channels to evoked Ca^{2+} transients is much larger (>50% contribution) in the distal parts of the apical dendrite,

in which Ca^{2+} spikes can be generated as compared with more proximal parts (20% contribution; Markram *et al.* 1995). This suggests that the Ca^{2+} spike initiation zone is a special compartment in terms of ion channel composition (Larkum *et al.* 2009).

The use of non-specific high-VDCC blockers such as cadmium and cobalt has been shown to block the sustained plateau potential of dendritic Ca^{2+} spikes (Kim & Connors, 1993; Schiller *et al.* 1997; Larkum *et al.* 1999*a*, 2001; Larkum & Zhu, 2002). TTX and Ni^{2+} affect the initial fast component of Ca^{2+} spikes, pointing to Na^+ and low-voltage-activated T-type Ca^{2+} currents as being responsible for the initiation of Ca^{2+} spikes (Larkum *et al.* 2001; Schaefer *et al.* 2003); however, the concentration of Ni^{2+} used in previous reports (>50 μM) is also known to block $Ca_v2.3$ channels (R-type; Soong *et al.* 1993; Randall & Tsien, 1997). Furthermore, Ca_v3 (T-type) conductances on their own can not account for the generation and duration of the Ca^{2+} spike (up to 50 ms; Larkum *et al.* 2001) given the fast inactivation kinetics (25 ms at 0 mV) and low percentage of available Ca_v3 channels at the dendritic resting membrane potential (<20% at −55 mV; Magee & Johnston, 1995*a*; Magee, 2008). We found that Ni^{2+} (50 μM) inhibited the generation of Ca^{2+} spikes to a large extent. The effect of Ni^{2+} was to increase the threshold for Ca^{2+} spike initiation because extra current injected to the distal dendrite reactivated Ca^{2+} spikes and their associated Ca^{2+} transients. Blockade of one fraction of the Ni^{2+}-sensitive conductance ($Ca_v2.3$ channels) revealed a significant contribution of R-type conductances to the Ca^{2+} spike not previously reported. Our results are consistent with the apparent lack of T-type Ca^{2+} currents observed in nucleated patch-clamp recordings from somatosensory layer 5 pyramidal neurons (Almog & Korngreen, 2009).

Here we show that Ca_v1 (L-type) channels contribute predominantly to the sustained (>30 ms) dendritic plateau potential. It is this slow and long-lasting part of the dendritic Ca^{2+} spike that determines the number and pattern of somatic APs (Larkum *et al.* 2001). In this respect, Ca^{2+} spikes and Ca_v1 VDCCs are important for the computational properties of the thick tufted pyramidal neurons, such as performing coincidence detection and non-linear integration of synaptic inputs (Larkum *et al.* 1999*b*, 2009; Schaefer *et al.* 2003). All other VDCCs contribute to the Ca^{2+} signal, but they do have only a minor effect on the electrogenesis.

GABA$_B$ modulation of VDCCs

Spines from apical dendrites are biochemically independent compartments, well isolated from their parent dendrites (Muller & Connor, 1991; Yuste & Denk, 1995; Svoboda *et al.* 1996; Sabatini & Svoboda, 2000) that contain VDCCs (Sabatini & Svoboda, 2000; Sabatini *et al.* 2001; Bloodgood & Sabatini, 2007). Evidence from hippocampal pyramidal neurons has shown a differential compartmentalization of VDCCs between shafts and spines where $Ca_v2.3$ channels form functional microdomains within the spine (Sabatini & Svoboda, 2000; Bloodgood & Sabatini, 2007). In particular, GABA$_B$ receptor activation has been shown to inhibit Ca^{2+} transients evoked by somatic back-propagating APs only in the spines of CA1 pyramidal neurons, by inhibiting $Ca_v2.3$ channels (Sabatini & Svoboda, 2000). Our findings show that activation of GABA$_B$ receptors or blockade of Ca_v1 VDCCs both strongly inhibited Ca^{2+} transients evoked by Ca^{2+} spikes in the distal apical dendritic shaft as well as the neighbouring spines. This result points to a fundamental difference between cortical and hippocampal pyramidal neurons in terms of Ca^{2+} signalling and its modulation by GABA$_B$ receptors. In agreement with our results, recent work suggests that GABA$_B$ receptor activation inhibits Ca^{2+} transients in the dendritic shafts of L2/3 cortical pyramidal neurons, evoked by back-propagating APs. This effect was only partially occluded by nimodipine, indicating a GABA$_B$-mediated inhibition of Ca_v1 VDCCs in addition to other VDCCs in these cells (Chalifoux & Carter, 2011). In the distal dendrites of L5 pyramidal neurons, only the blockade of Ca_v1 VDCCs occluded the postsynaptic actions of GABA$_B$ receptors. Furthermore, local activation of GABA$_B$ receptors inhibited isolated L-type Ca^{2+} currents recorded directly at the site of generation of Ca^{2+} spikes, showing unequivocally that GABA$_B$ receptors exert their inhibitory action on dendritic Ca^{2+} spikes by inhibiting L-type Ca^{2+} conductances. Thus, the functional coupling of GABA$_B$ receptors to VDCCs depends on cell type and/or the subcellular compartment.

GABA$_B$ receptors are localized along the apical dendrite of pyramidal neurons. At the subcellular level they are present in both dendritic spines and their parent dendritic shafts (Kulik *et al.* 2003). At the spine level, GABA$_B$ receptors form clusters together with Kir3.2 inwardly rectifying K^+ channels, whereas in the shaft both proteins are segregated (Kulik *et al.* 2006). This raises the possibility that GABA$_B$ receptors in the shaft might be co-localized to Ca_v1 channels, although this has to be determined at the electron-microscopic level. Such a degree of co-localization is consistent with a membrane-delimited coupling between these proteins via a $G_{i/o}$ protein (Karschin, 1999; Bettler *et al.* 2004; Pinard *et al.* 2010). Although this form of regulation has been shown for GABA$_B$–Kir3.2 and GABA$_B$–Ca_v2 interactions, it has never been demonstrated for GABA$_B$–Ca_v1. Previous evidence already suggested an inhibitory role of GABA$_B$ receptors on L-type Ca^{2+} conductances even though the mechanism for this interaction remained elusive (Maguire *et al.* 1989; Marchetti *et al.* 1991; Scholz & Miller, 1991; Amico *et al.* 1995; Chalifoux & Carter, 2011). Our results

now show for the first time that $GABA_B$ receptors can exert this modulatory action on dendritic Ca_v1 channels (L-type currents) via a membrane-delimited pathway. Three lines of evidence support this view. Firstly, $GABA_B$-mediated inhibition persisted after pharmacological manipulation of different cytosolic protein kinases known to be associated with the activation of $G_{i/o}$ proteins, namely PKA, PKC and PIK3 (Bettler *et al.* 2004; Salgado *et al.* 2007; Deng *et al.* 2009). Secondly, prepulse facilitation relieved the $GABA_B$-mediated inhibition of dendritic L-type Ca^{2+} currents and, thirdly, L-type Ca^{2+} currents could be recovered by strong depolarizing voltage commands. The prepulse facilitation protocol has been shown to be effective for reversing the $G_{i/o}$-$\beta\gamma$-mediated inhibition of Ca_v2, but so far not for Ca_v1 VDCCs (Bourinet *et al.* 1996; Herlitze *et al.* 1996; Ikeda, 1996; Toth *et al.* 1996; Zhang *et al.* 1996; Zamponi *et al.* 1997; De Waard *et al.* 2005). Strong depolarizing voltages have been assumed to promote the uncoupling of the $G_{i/o}$-$\beta\gamma$-dimer from the $\beta\gamma$-binding site located in the intracellular loop I–II of the pore-forming α-subunit of the channel (De Waard *et al.* 2005). Indeed, this loop has been shown to act as a voltage sensor (Sandoz *et al.* 2004), and its movement during membrane depolarization might promote the dissociation. In addition, the recovery of L-type currents at higher depolarizations is consistent with a model by which the $G_{i/o}$-$\beta\gamma$ complex shifts the activation curve of Ca_v1 VDCCs to more depolarized values, indicating their transition to a 'reluctant state' (Fig. 4*D*; De Waard *et al.* 2005).

The $G_{i/o}$-$\beta\gamma$-mediated regulation of VDCCs has mainly been tested in heterologous expression systems. This raises the possibility that native central neurons possess preserved Ca_v1 VDCCs susceptible to regulation through this pathway (Bourinet *et al.* 1996). Alternatively, $G_{i/o}$-$\beta\gamma$-mediated modulation might require additional factors like accessory proteins that were present in the specialized region of the neuron we investigated, but which were absent in the heterologous expression systems previously used. Our findings expand the repertoire of interactions between G-protein-coupled receptors and VDCCs opening new vistas in neuromodulation. Furthermore, our result implies that the $G_{i/o}$-$\beta\gamma$-subunit interaction with VDCCs is a common mechanism of action by which $GABA_B$ receptors inhibit VDCCs.

References

Almog M & Korngreen A (2009). Characterization of voltage-gated Ca^{2+} conductances in layer 5 neocortical pyramidal neurons from rats. *PLoS One* **4**, e4841.

Amico C, Marchetti C, Nobile M & Usai C (1995). Pharmacological types of calcium channels and their modulation by baclofen in cerebellar granules. *J Neurosci* **15**, 2839–2848.

Amitai Y, Friedman A, Connors BW & Gutnick MJ (1993). Regenerative activity in apical dendrites of pyramidal cells in neocortex. *Cereb Cortex* **3**, 26–38.

Bettler B, Kaupmann K, Mosbacher J & Gassmann M (2004). Molecular structure and physiological functions of $GABA_B$ receptors. *Physiol Rev* **84**, 835–867.

Bloodgood BL & Sabatini BL (2007). Nonlinear regulation of unitary synaptic signals by CaV(2.3) voltage-sensitive calcium channels located in dendritic spines. *Neuron* **53**, 249–260.

Bourinet E, Soong TW, Stea A & Snutch TP (1996). Determinants of the G protein-dependent opioid modulation of neuronal calcium channels. *Proc Natl Acad Sci U S A* **93**, 1486–1491.

Breton JD & Stuart GJ (2012). Somatic and dendritic $GABA_B$ receptors regulate neuronal excitability via different mechanisms. *J Neurophysiol* **108**, 2810–2818.

Catterall WA (2000). Structure and regulation of voltage-gated Ca^{2+} channels. *Annu Rev Cell Dev Biol* **16**, 521–555.

Catterall WA & Few AP (2008). Calcium channel regulation and presynaptic plasticity. *Neuron* **59**, 882–901.

Chalifoux JR & Carter AG (2011). $GABA_B$ receptor modulation of voltage-sensitive calcium channels in spines and dendrites. *J Neurosci* **31**, 4221–4232.

Christie BR, Eliot LS, Ito K, Miyakawa H & Johnston D (1995). Different Ca^{2+} channels in soma and dendrites of hippocampal pyramidal neurons mediate spike-induced Ca^{2+} influx. *J Neurophysiol* **73**, 2553–2557.

Cox DH & Dunlap K (1992). Pharmacological discrimination of N-type from L-type calcium current and its selective modulation by transmitters. *J Neurosci* **12**, 906–914.

De Waard M, Hering J, Weiss N & Feltz A (2005). How do G proteins directly control neuronal Ca^{2+} channel function? *Trends Pharmacol Sci* **26**, 427–436.

Deng PY, Xiao Z, Yang C, Rojanathammanee L, Grisanti L, Watt J, Geiger JD, Liu R, Porter JE & Lei S (2009). $GABA_B$ receptor activation inhibits neuronal excitability and spatial learning in the entorhinal cortex by activating TREK-2 K^+ channels. *Neuron* **63**, 230–243.

Dolphin AC (2003). G protein modulation of voltage-gated calcium channels. *Pharmacol Rev* **55**, 607–627.

Dolphin AC & Scott RH (1987). Calcium channel currents and their inhibition by (−)-baclofen in rat sensory neurones: modulation by guanine nucleotides. *J Physiol* **386**, 1–17.

Drummond GB (2009). Reporting ethical matters in the *Journal of Physiology*: standards and advice. *J Physiol* **587**, 713–719.

Gidon A & Segev I (2012). Principles governing the operation of synaptic inhibition in dendrites. *Neuron* **75**, 330–341.

Harris NC, Libri V & Constanti A (1994). Selective blockade of the hyperpolarization-activated cationic current (I_h) in guinea pig substantia nigra pars compacta neurones by a novel bradycardic agent, Zeneca ZM 227189. *Neurosci Lett* **176**, 221–225.

Herlitze S, Garcia DE, Mackie K, Hille B, Scheuer T & Catterall WA (1996). Modulation of Ca^{2+} channels by G-protein $\beta\gamma$ subunits. *Nature* **380**, 258–262.

Ikeda SR (1996). Voltage-dependent modulation of N-type calcium channels by G-protein $\beta\gamma$ subunits. *Nature* **380**, 255–258.

Jaffe DB, Johnston D, Lasser-Ross N, Lisman JE, Miyakawa H & Ross WN (1992). The spread of Na^+ spikes determines the pattern of dendritic Ca^{2+} entry into hippocampal neurons. *Nature* **357**, 244–246.

Karschin A (1999). G protein regulation of inwardly rectifying K^+ channels. *News Physiol Sci* **14**, 215–220.

Kavalali ET, Zhuo M, Bito H & Tsien RW (1997). Dendritic Ca^{2+} channels characterized by recordings from isolated hippocampal dendritic segments. *Neuron* **18**, 651–663.

Kim HG & Connors BW (1993). Apical dendrites of the neocortex: correlation between sodium- and calcium-dependent spiking and pyramidal cell morphology. *J Neurosci* **13**, 5301–5311.

Kulik A, Vida I, Fukazawa Y, Guetg N, Kasugai Y, Marker CL, Rigato F, Bettler B, Wickman K, Frotscher M & Shigemoto R (2006). Compartment-dependent colocalization of Kir3.2-containing K^+ channels and $GABA_B$ receptors in hippocampal pyramidal cells. *J Neurosci* **26**, 4289–4297.

Kulik A, Vida I, Lujan R, Haas CA, Lopez-Bendito G, Shigemoto R & Frotscher M (2003). Subcellular localization of metabotropic $GABA_B$ receptor subunits $GABA_{B1a/b}$ and $GABA_{B2}$ in the rat hippocampus. *J Neurosci* **23**, 11026–11035.

Larkum ME, Kaiser KM & Sakmann B (1999*a*). Calcium electrogenesis in distal apical dendrites of layer 5 pyramidal cells at a critical frequency of back-propagating action potentials. *Proc Natl Acad Sci U S A* **96**, 14600–14604.

Larkum ME & Nevian T (2008). Synaptic clustering by dendritic signalling mechanisms. *Curr Opin Neurobiol* **18**, 321–331.

Larkum ME, Nevian T, Sandler M, Polsky A & Schiller J (2009). Synaptic integration in tuft dendrites of layer 5 pyramidal neurons: a new unifying principle. *Science* **325**, 756–760.

Larkum ME & Zhu JJ (2002). Signaling of layer 1 and whisker-evoked Ca^{2+} and Na^+ action potentials in distal and terminal dendrites of rat neocortical pyramidal neurons in vitro and in vivo. *J Neurosci* **22**, 6991–7005.

Larkum ME, Zhu JJ & Sakmann B (1999*b*). A new cellular mechanism for coupling inputs arriving at different cortical layers. *Nature* **398**, 338–341.

Larkum ME, Zhu JJ & Sakmann B (2001). Dendritic mechanisms underlying the coupling of the dendritic with the axonal action potential initiation zone of adult rat layer 5 pyramidal neurons. *J Physiol* **533**, 447–466.

Lovett-Barron M, Turi GF, Kaifosh P, Lee PH, Bolze F, Sun XH, Nicoud JF, Zemelman BV, Sternson SM & Losonczy A (2012). Regulation of neuronal input transformations by tunable dendritic inhibition. *Nature Neurosci* **15**, 423–430, S421–S423.

Magee JC (2008). Dendritic voltage-gated ion channels. In *Dendrites*, ed. Stuart G, Spruston N & Häusser M, pp. 225–250. Oxford University Press, Oxford.

Magee JC, Christofi G, Miyakawa H, Christie B, Lasser-Ross N & Johnston D (1995). Subthreshold synaptic activation of voltage-gated Ca^{2+} channels mediates a localized Ca^{2+} influx into the dendrites of hippocampal pyramidal neurons. *J Neurophysiol* **74**, 1335–1342.

Magee JC & Johnston D (1995*a*). Characterization of single voltage-gated Na^+ and Ca^{2+} channels in apical dendrites of rat CA1 pyramidal neurons. *J Physiol* **487**, 67–90.

Magee JC & Johnston D (1995*b*). Synaptic activation of voltage-gated channels in the dendrites of hippocampal pyramidal neurons. *Science* **268**, 301–304.

Maguire G, Maple B, Lukasiewicz P & Werblin F (1989). Gamma-aminobutyrate type B receptor modulation of L-type calcium channel current at bipolar cell terminals in the retina of the tiger salamander. *Proc Natl Acad Sci U S A* **86**, 10144–10147.

Marchetti C, Carignani C & Robello M (1991). Voltage-dependent calcium currents in dissociated granule cells from rat cerebellum. *Neuroscience* **43**, 121–133.

Markram H, Helm PJ & Sakmann B (1995). Dendritic calcium transients evoked by single back-propagating action potentials in rat neocortical pyramidal neurons. *J Physiol* **485**(Pt 1), 1–20.

Markram H & Sakmann B (1994). Calcium transients in dendrites of neocortical neurons evoked by single subthreshold excitatory postsynaptic potentials via low-voltage-activated calcium channels. *Proc Natl Acad Sci U S A* **91**, 5207–5211.

Muller W & Connor JA (1991). Dendritic spines as individual neuronal compartments for synaptic Ca^{2+} responses. *Nature* **354**, 73–76.

Murayama M, Pérez-Garci E, Nevian T, Bock T, Senn W & Larkum ME (2009). Dendritic encoding of sensory stimuli controlled by deep cortical interneurons. *Nature* **457**, 1137–1141.

Nevian T & Helmchen F (2007). Calcium indicator loading of neurons using single-cell electroporation. *Pflugers Arch* **454**, 675–688.

Nevian T & Sakmann B (2006). Spine Ca^{2+} signaling in spike-timing-dependent plasticity. *J Neurosci* **26**, 11001–11013.

Newberry NR & Nicoll RA (1984). Direct hyperpolarizing action of baclofen on hippocampal pyramidal cells. *Nature* **308**, 450–452.

Padgett CL & Slesinger PA (2010). $GABA_B$ receptor coupling to G-proteins and ion channels. *Adv Pharmacol* **58**, 123–147.

Palmer LM, Schulz JM, Murphy SC, Ledergerber D, Murayama M & Larkum ME (2012). The cellular basis of $GABA_B$-mediated interhemispheric inhibition. *Science* **335**, 989–993.

Pérez-Garci E, Gassmann M, Bettler B & Larkum ME (2006). The $GABA_{B1b}$ isoform mediates long-lasting inhibition of dendritic Ca^{2+} spikes in layer 5 somatosensory pyramidal neurons. *Neuron* **50**, 603–616.

Pinard A, Seddik R & Bettler B (2010). $GABA_B$ receptors: physiological functions and mechanisms of diversity. *Adv Pharmacol* **58**, 231–255.

Plummer MR, Logothetis DE & Hess P (1989). Elementary properties and pharmacological sensitivities of calcium channels in mammalian peripheral neurons. *Neuron* **2**, 1453–1463.

Randall AD & Tsien RW (1997). Contrasting biophysical and pharmacological properties of T-type and R-type calcium channels. *Neuropharmacology* **36**, 879–893.

Sabatini BL, Maravall M & Svoboda K (2001). Ca^{2+} signaling in dendritic spines. *Curr Opin Neurobiol* **11**, 349–356.

Sabatini BL & Svoboda K (2000). Analysis of calcium channels in single spines using optical fluctuation analysis. *Nature* **408**, 589–593.

Salgado H, Bellay T, Nichols JA, Bose M, Martinolich L, Perrotti L & Atzori M (2007). Muscarinic M2 and M1 receptors reduce GABA release by Ca^{2+} channel modulation through activation of PI3K/Ca^{2+}-independent and PLC/Ca^{2+}-dependent PKC. *J Neurophysiol* **98**, 952–965.

Sandoz G, Lopez-Gonzalez I, Stamboulian S, Weiss N, Arnoult C & De Waard M (2004). Repositioning of charged I-II loop amino acid residues within the electric field by β subunit as a novel working hypothesis for the control of fast P/Q calcium channel inactivation. *Eur J Neurosci* **19**, 1759–1772.

Schaefer AT, Larkum ME, Sakmann B & Roth A (2003). Coincidence detection in pyramidal neurons is tuned by their dendritic branching pattern. *J Neurophysiol* **89**, 3143–3154.

Schiller J, Schiller Y, Stuart G & Sakmann B (1997). Calcium action potentials restricted to distal apical dendrites of rat neocortical pyramidal neurons. *J Physiol* **505**, 605–616.

Scholz KP & Miller RJ (1991). $GABA_B$ receptor-mediated inhibition of Ca^{2+} currents and synaptic transmission in cultured rat hippocampal neurones. *J Physiol* **444**, 669–686.

Sickmann T, Klose A, Huth T & Alzheimer C (2008). Unexpected suppression of neuronal G protein-activated, inwardly rectifying K^+ current by common phospholipase C inhibitor. *Neurosci Lett* **436**, 102–106.

Sodickson DL & Bean BP (1996). $GABA_B$ receptor-activated inwardly rectifying potassium current in dissociated hippocampal CA3 neurons. *J Neurosci* **16**, 6374–6385.

Soong TW, Stea A, Hodson CD, Dubel SJ, Vincent SR & Snutch TP (1993). Structure and functional expression of a member of the low voltage-activated calcium channel family. *Science* **260**, 1133–1136.

Svoboda K, Tank DW & Denk W (1996). Direct measurement of coupling between dendritic spines and shafts. *Science* **272**, 716–719.

Toth PT, Shekter LR, Ma GH, Philipson LH & Miller RJ (1996). Selective G-protein regulation of neuronal calcium channels. *J Neurosci* **16**, 4617–4624.

Tsien RW, Lipscombe D, Madison DV, Bley KR & Fox AP (1988). Multiple types of neuronal calcium channels and their selective modulation. *Trends Neurosci* **11**, 431–438.

Williams SR & Mitchell SJ (2008). Direct measurement of somatic voltage clamp errors in central neurons. *Nat Neurosci* **11**, 790–798.

Yuste R & Denk W (1995). Dendritic spines as basic functional units of neuronal integration. *Nature* **375**, 682–684.

Yuste R, Gutnick MJ, Saar D, Delaney KR & Tank DW (1994). Ca^{2+} accumulations in dendrites of neocortical pyramidal neurons: an apical band and evidence for two functional compartments. *Neuron* **13**, 23–43.

Zamponi GW, Bourinet E, Nelson D, Nargeot J & Snutch TP (1997). Crosstalk between G proteins and protein kinase C mediated by the calcium channel α_1 subunit. *Nature* **385**, 442–446.

Zhang JF, Ellinor PT, Aldrich RW & Tsien RW (1996). Multiple structural elements in voltage-dependent Ca^{2+} channels support their inhibition by G proteins. *Neuron* **17**, 991–1003.

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E.P.-G., M.E.L. and T.N.: designed the experiments; E.P.-G. and T.N.: performed and analysed the experiments; E.P.-G., M.E.L. and T.N. wrote the manuscript. All authors approved the final version for publication.

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