Nuclear BK channels regulate gene expression via the control of nuclear calcium signaling

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Ion channels are essential for the regulation of neuronal functions. The significance of plasma membrane, mitochondrial, endoplasmic reticulum and lysosomal ion channels in the regulation of Ca\textsuperscript{2+} is well established. In contrast, surprisingly little is known about the function of ion channels on the nuclear envelope (NE). Here we demonstrate the presence of functional large-conductance, calcium-activated potassium channels (BK channels) on the NE of rodent hippocampal neurons. Functionally, blockade of nuclear BK channels (nBK channels) induces NE-derived Ca\textsuperscript{2+} release, nucleoplasmic Ca\textsuperscript{2+} elevation and cyclic AMP response element binding protein (CREB)-dependent transcription. More importantly, blockade of nBK channels regulates nuclear Ca\textsuperscript{2+}-sensitive gene expression and promotes dendritic arborization in a nuclear Ca\textsuperscript{2+}-dependent manner. These results suggest that the nBK channel functions as a molecular link between neuronal activity and nuclear Ca\textsuperscript{2+} to convey signals from synapse to nucleus and is a new modulator, operating at the NE, of synaptic activity–dependent neuronal functions.

As transmembrane proteins, ion channels are present not only on the plasma membrane but also on intracellular membrane-bound organelles. Much is known regarding the control of action potential firing, synaptic transmission and gene expression by plasma membrane ion channels through the regulation of membrane potential and Ca\textsuperscript{2+} influx\textsuperscript{1}. In addition, the significance of mitochondrial\textsuperscript{3}, lysosomal\textsuperscript{3,4} and endoplasmic reticulum (ER)\textsuperscript{5} ion channels in regulating ATP production, stress response and apoptosis via the control of Ca\textsuperscript{2+} signals has been well established. In contrast, despite the obvious structural and functional importance of the nucleus in gene expression and regulation, the functions of ion channels on the NE (nuclear ion channels) remain largely unknown, especially in neurons.

Recent studies have shown that the NE is a key source of nucleoplasmic Ca\textsuperscript{2+}: Ca\textsuperscript{2+} in the perinuclear space, the lumen between the outer and inner nuclear membrane of the NE, can be released into the nucleoplasm via nuclear inositol-1,4,5-trisphosphate receptors (IP\textsubscript{3}R) and ryanodine receptors (RyR)\textsuperscript{6–10}. Therefore, the NE is a functional perinuclear Ca\textsuperscript{2+} store and acts independently of the ER and cytoplasm in regulating the nucleoplasmic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{nu})\textsuperscript{6–10}. Meanwhile, numerous studies have demonstrated that nucleoplasmic Ca\textsuperscript{2+} is one of the most important signals in mediating neuronal activity–dependent gene expression\textsuperscript{11,12} and is critical for several long-lasting adaptive responses, including modulation of synapse formation and dendritic complexity\textsuperscript{13}, regulation of synaptic plasticity and memory consolidation\textsuperscript{13,14}, and activity-dependent acquired neuroprotection\textsuperscript{12,15}.

As a key signal in neuronal functions, numerous fundamental questions remain concerning this NE-derived Ca\textsuperscript{2+} release, such as how it is regulated in neurons; whether it can be regulated by nuclear ion channels through changing the NE transmembrane potential, similarly to the regulation of Ca\textsuperscript{2+} fluxes in the plasma membrane and mitochondria; which type of nuclear ion channel governs this transmembrane potential change; and whether this nuclear ion channel also regulates transcription factor activation and gene expression induced by neuronal activity. To address these questions, we applied a combination of biochemical and molecular biological methods, together with calcium imaging, voltage imaging and patch clamp techniques in wild-type and gene knockout mice, to study the function of the nuclear ion channel. We found that functional BK channels were present on the NE of hippocampal neurons. Pharmacological or genetic blockade of nBK channels directly regulated the NE-derived release of Ca\textsuperscript{2+}; CREB phosphorylation, gene expression and dendritic arborization, suggesting that nBK channels play a key role in neuronal activity–dependent functions and serve as a new molecular link, operating at the NE, between neuronal activities, nuclear Ca\textsuperscript{2+} signaling and gene expression.

RESULTS
BK channels are expressed on the NE
To investigate the subcellular localization of BK channels in intact hippocampal neurons, we performed immunocytochemistry using a monoclonal antibody to the α subunit of the BK channel\textsuperscript{16}. The confocal optical sections demonstrated an intracellular ring-like labeling

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around the nucleus (Fig. 1a). Analysis of intensity profiles indicated that the labeling colocalized with the NE marker lamin B, suggesting that the BK channels are localized to the NE (Fig. 1a). To confirm that this observation did not result from nonspecific binding of the BK antibody, we conducted the same experiment in the neurons from BK channel α subunit–null mice (Kcnma1−/−) mice. The BK channel antibody failed to detect any signal in the neurons from Kcnma1−/− mice (Fig. 1a), validating its specificity. Furthermore, we used immunoelectron microscopy to investigate BK channel localization at high spatial resolution. Gold particles signaling BK channels were located on the NE (Fig. 1b), further confirming the NE localization of the BK channels. Consistent with previous findings17,18, we also observed mitochondrial localization of the BK channels (Fig. 1b). The negative labeling in Kcnma1−/− mice once again validated the specificity of the BK antibody (Fig. 1b).

To further verify the nuclear localization of the BK channels, we isolated nuclei from cultured hippocampal neurons. We observed a ring-like labeling of BK channel immunofluorescence in isolated

**Figure 1** BK channels expression on the NE of hippocampal neurons. (a) Confocal immunofluorescence of BK channels and lamin B in cultured hippocampal neurons. Intensity profile analysis along a vertical line across the center of the nucleus is shown at right. Over 85% of neurons (>100 neurons) show BK channel staining on the NE. (b) Top, immunoelectron microscopy images of BK channel localization in cultured hippocampal neurons. Gold particles marking BK channels were located on the NE (arrows) and mitochondria (M) (arrowhead) in wild-type mice. Forty-six immunoelectron microscopy images were taken in total. NE-localized gold particles were observed in all the images from the wild-type mice, with no signal in Kcnma1−/− mice. Scale bar represents 1 μm. Middle, schematic of top images; N, nucleus. Bottom, enlargements of the rectangular regions indicated in the top images. (c) Left, immunofluorescence of BK channels and lamin B in isolated nuclei. Right, intensity profile analysis of a horizontal line across the center of the isolated nuclei. (d) Immunofluorescence of BK-GFP and lamin B. (e) Western blot of the subcellular localization of BK channels in the membrane fraction (M), whole cell lysate (W) and nuclear fraction (N). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; COX IV, cytochrome c oxidase subunit IV. (f) Western blot of BK channel expression in intact isolated nuclei (1), denuded nuclei (2) and NE (3). (g) Single-channel activities of the BK channel in isolated nuclei with 10 μM Ca2+ at different voltage in the dimethylsulfoxide (DMSO) control group (left), with paxilline treatment (10 μM, middle) and in nuclei from Kcnma1−/− mice (right) (n = 6 nuclei). (h) Left, single-channel activities recorded in solutions with 10 μM or 5 μM Ca2+ at 40 mV. Right, BK channel open probability (p0) recorded in solutions with 10 μM (n = 10 nuclei for each group, unpaired t-test, P = 2.30 × 10−14, t18 = 21.71 between control and paxilline) or 5 μM Ca2+ (n = 10 nuclei for each group, unpaired t-test, P = 1.80 × 10−8, t18 = 9.55, between control and paxilline) at 40 mV; error bars are mean ± s.d. (i) I–V curve of channel activity. For single-channel recording, BK channel activity was recorded from 15 of the 20 successful patching events on nuclei from wild-type mice. All data are from 3 independent cultures from at least 3 litters. All experiments in a–f were successfully performed at least 3 times. WT, wild-type. Scale bars in a,c,d represent 10 μm. Full-length blots for e,f are presented in Supplementary Figure 7.
nuclei from wild-type mice (Fig. 1c). Analysis of intensity profiles of the isolated nuclei indicated that BK channel colocalized with lamin B (Fig. 1c). The BK channel antibody failed to detect any signal in the nucleus from Kcnma1−/− mice (Fig. 1c). Additionally, we transfected neurons with a GFP-tagged BK channel construct. Green fluorescence was detected around the nucleus and colocalized with lamin B (Fig. 1d), once again suggesting the NE localization of the BK channels. Subcellular fractionation and immunoblot analysis further supported this analysis: we detected BK channels in the nuclear fraction, as well as the plasma membrane fraction and the whole cell lysate (Fig. 1e). The purity of the nuclear fraction was validated by the presence of lamin B and the absence of the cytosolic marker glyceraldehyde-3-phosphate dehydrogenase, ER marker calcin and mitochondrial marker cytochrome c oxidase subunit IV (Fig. 1e). The presence of immunoreactivity in the NE fraction, but not in the denuded (NE-depleted) nuclear fraction, further verified the localization of the BK channel on the NE (Fig. 1f). The absence of the immunobands in Kcnma1−/− mice confirmed that the immunoreactivity arose from authentic BK channels (Fig. 1f,g).

Besides the pore-forming α-subunit, BK channels also contain auxiliary β-subunits. The brain-specific auxiliary subunits β4 were also observed and colocalized with lamin B (Supplementary Fig. 1), suggesting that a fully assembled BK channel complex is located on the NE. To further determine whether nBK channels are functional and exhibit similar electrophysiological properties to those of plasma membrane BK (pmBK) channels, we performed single-channel recordings with patch clamps on isolated nuclei. We recorded a voltage-dependent and Ca2+-dependent K+ channel activity with a conductance of approximately 220 pS, similar to that of the neuronal pmBK channel19–22 (Fig. 1g–i). Blockade of channel activity with paxilline, a BK channel-specific blocker, and the absence of the channel activity in Kcnma1−/− mice further confirmed that the channel activity arose from BK channels (Fig. 1g,h). Taken together, these observations demonstrate that BK channels are present and functional on the NE of hippocampal neurons.

**nBK channels regulate nuclear Ca2+ concentrations**

pmBK channels can affect the plasma membrane potential and accordingly regulate voltage-dependent calcium influx19–22, nBK channels, with similar electrophysiological properties to those of pmBK channels, may also regulate the nuclear transmembrane potential (∆Ψn), thereby regulating nuclear calcium concentration. To address this hypothesis, we first examined whether nBK channels could regulate ∆Ψn. We loaded isolated nuclei with the carbocyanine dye DiOC6(3), which has been validated as a potentiometric probe in NE21,24. Paxilline elicited a consistent increase in DiOC6(3) fluorescence (0.28 ± 0.02 ΔF/F0 (mean ± s.e.m.), n = 20 nuclei), indicating that the perinuclear lumen became more negative23,24 (Fig. 2a). In accord, the paxilline-induced ΔΨn was completely absent in the nuclei from Kcnma1−/− mice (0.02 ± 0.01 ΔF/F0 (mean ± s.e.m.), n = 31 nuclei), unpaired t-test, P = 2.81 × 10−13, t0.9 = 9.90, compared with wild-type group; Fig. 2a). These results suggest that nBK channels can regulate ΔΨn.

Next we investigated whether nBK channels can regulate [Ca2+]nu in hippocampal neurons. Paxilline is membrane permeant and inhibits BK channels on both the plasma membrane and intracellular organelles. Iberiotoxin (IbTx), a membrane-impermeant BK channel blocker, only inhibits pmBK channels on the plasma membrane. Calcium imaging of the confocal mid-nuclear section demonstrated that paxilline induced [Ca2+]nu elevation in intact neurons (Fig. 2b), whereas IbTx lacked an influence on [Ca2+]nu (Fig. 2c). Moreover, paxilline elicited [Ca2+]nu elevation even after complete blockade of the pmBK channels with a high concentration of IbTx (100 μM) (Fig. 2c), which suggests that the effect of paxilline was mainly mediated by intracellular BK channels and not pmBK channels. This idea was further supported by the finding that paxilline induced [Ca2+]nu elevation in digitonin-permeabilized neurons with a Ca2+-buffered intracellular medium (Fig. 2d). Paxilline-induced [Ca2+]nu elevation was largely suppressed by prior depletion of intracellular Ca2+ stores with thapsigargin, but not by chelation of extracellular Ca2+ with EGTA (Fig. 2c), further suggesting that the nucleoplasmic Ca2+ transient is likely to be due to direct Ca2+ release from the NE29. Notably, neurons from Kcnma1−/− mice were completely devoid of paxilline-induced [Ca2+]nu elevation (Fig. 2e). Taken together, these results strongly support the idea that the intracellular BK channels and perinuclear Ca2+ stores are responsible for eliciting nuclear Ca2+ signals.

To further verify that the mediators of the paxilline-induced [Ca2+]nu elevation are the nBK channels and perinuclear Ca2+ stores, we loaded isolated nuclei from cultured hippocampal neurons with either the Ca2+ probe Fluo-4 AM or Fluo-4 dextran. Fluo-4 AM, a membrane-permeant probe, preferentially accumulated in the NE (Fig. 2f), whereas Fluo-4 dextran, a membrane-impermeant probe, was distributed uniformly in the nucleoplasm20,24 (Fig. 2g). Confocal microscopy revealed that paxilline produced opposite Ca2+ changes in the NE and the nucleoplasm. Application of paxilline led to a decrease of NE Ca2+ (Fig. 2f), which paralleled a transient Ca2+ increase in the nucleoplasm (Fig. 2g). The paxilline-induced Ca2+ transient was diminished by knockdown of BK channel with lentiviral short hairpin RNAs (Supplementary Fig. 2) and was completely abolished in neurons from Kcnma1−/− mice (Fig. 2f,g). Fluo-4 AM intensity, 0.29 ± 0.03 ΔF/F0 in wild-type group, 0.01 ± 0.02 ΔF/F0 in Kcnma1−/− group, n = 25 nuclei for each group, unpaired t-test, P = 1.16 × 10−10, t0.9 = 8.19; Fluo-4 dextran intensity, 0.33 ± 0.04 ΔF/F0 in wild-type group, 0.03 ± 0.02 ΔF/F0 for Kcnma1−/− group, n = 22 nuclei for each group, unpaired t-test, P = 2.12 × 10−6, t0.9 = 6.89; mean ± s.e.m.). Depletion of perinuclear Ca2+ stores with thapsigargin completely blocked the paxilline-induced Ca2+ transient (Fig. 2h), suggesting that the perinuclear Ca2+ stores are responsible for eliciting the paxilline-induced nuclear Ca2+ signals.

Numerous studies have demonstrated that IP3Rs and RyRs, located on the inner membrane of the NE, mediate the release of Ca2+ from the perinuclear space into the nucleoplasm6,10. Therefore, we used specific IP3R inhibitors (heparin and 2-aminoethoxydiphenyl borate (2-APB)) and a RyR inhibitor (ruthenium red) to test which receptor is responsible for the nBK channel–regulated NE Ca2+ release. The paxilline-induced Ca2+ release was attenuated by 10 μM ruthenium red and was completely blocked by 100 μM ruthenium red (Fig. 2h). Conversely, results with the IP3R blockers indicated that neither heparin (50 μM) nor 2-APB (100 μM) affected paxilline-induced Ca2+ release (Fig. 2h). To further confirm that RyRs mediate paxilline-induced Ca2+ release, we designed two independent shRNAs to knock down RyR expression in neurons23. Using the shRNAs individually reduced RyR expression to 56.4% and 51.5% of control (non-silencing shRNA group), respectively, and using the two together reduced it to 25.5% of control (Supplementary Fig. 3). Knockdown of RyRs compromised paxilline-induced [Ca2+]nu elevation in intact neurons (Fig. 2i), providing direct evidence that RyRs mediate the paxilline-induced Ca2+ release. Collectively, these results indicate that blockade of nBK channels induces NE-derived Ca2+ release and [Ca2+]nu elevation via RyRs.
nBK channels regulate nuclear Ca\(^{2+}\) signaling

Activity-dependent activation of CREB is heavily implicated in neuromodulation of transmembrane potential and nuclear Ca\(^{2+}\) concentration in intact neurons and isolated nuclei. (a) Fluorescence intensity changes of DiOC\(_6(3)\)-loaded isolated nuclei (inset) upon addition of paxilline. (b) Confocal nuclear calcium imaging with Fluo-4 AM of the midnuclear region in hippocampal neurons. (c) Left, changes in fluorescence intensity in nuclear calcium imaging of intact neurons after indicated treatments. Right, summary data. One-way ANOVA (n = 15 cells for each group, P = 4.70 × 10\(^{-15}\), F\(_{14,37} = 11.15\) and post hoc test. **P < 0.01 as compared with paxilline group. (d) Fluorescence intensity changes in the nuclei of digitonin-permeabilized neurons after paxilline treatment. (e) Paxilline-induced fluorescence intensity changes in intact neurons from wild-type and Kcnma1\(^{−/−}\) mice (n = 8 cells for each group, unpaired t-test, P = 2.56 × 10\(^{-5}\), t\(_{4,3} = 6.14\)). (f,g) Paxilline-induced changes in Fluo-4 AM (f) or Fluo-4 dextran (g) fluorescence intensity in isolated nuclei. (h) Left, changes in fluorescence intensity in isolated nuclei after knockdown of RyRs. Right, summary data. One-way ANOVA (n = 5, 6, 6, 5 cells, respectively; P = 0.0016, F\(_{3,18} = 7.72\) and post hoc test. **P < 0.01 as compared with N.S. group. Fisher’s least significant difference test was used for post hoc testing in one-way ANOVAs. Error bars represent mean ± s.e.m. All data are from 3 independent cultures from at least 3 litters. All experiments were successfully performed at least 3 times. WT, wild-type; Pax, paxilline; RR, ruthenium red; TG, thapsigargin; shRNA, RyR-specific shRNAs; N.S., non-silencing shRNAs; EGTA, chelation of extracellular Ca\(^{2+}\) with EGTA. RR concentrations indicated in μM. X-Rhod-1 AM is a Ca\(^{2+}\) indicator compatible with GFP. Scale bars in a,b,f,g represent 5 μm.

nBK channels regulate activity-evoked gene expression

Synaptic excitation on the dendritic spines of pyramidal neurons induces transcription factor activation and gene expression in the nucleus. This excitation–transcription coupling is critically important...
for a broad range of processes and behaviors, including neuronal development, learning and memory, and cell survival.\(^{12,29,31}\) To identify the role of nBK channels in excitation–transcription coupling, we investigate the effects of the nBK channels on the regulation of synaptic activity–evoked CREB phosphorylation and gene expression.

Hippocampal neurons were preincubated with IbTx to exclude the involvement of pmBK channels (Supplementary Fig. 4) and stimulated with the GABA\(_A\) receptor antagonist bicusculine (50 μM). GABAergic interneurons exert a tonic inhibition on the network. Removal of GABAergic inhibition with bicusculine leads to enhancement of neuronal activity and induces robust [Ca\(^{2+}\)]\(_{nu}\) elevation and CREB phosphorylation\(^{12,21}\) (Fig. 4a). Bicusculine-induced [Ca\(^{2+}\)]\(_{nu}\) elevation and CREB phosphorylation are highly dependent on the filling of perinuclear Ca\(^{2+}\) stores, and a reduction of the Ca\(^{2+}\) store will significantly compromise the bicusculine-induced effects\(^{31}\). Paxilline pretreatment attenuated both bicusculine-induced [Ca\(^{2+}\)]\(_{nu}\) elevation and CREB phosphorylation in wild-type mice because it depleted the perinuclear Ca\(^{2+}\) store content (Fig. 4a,b and Fig. 2). In contrast, paxilline failed to compromise bicusculine-induced [Ca\(^{2+}\)]\(_{nu}\) elevation and CREB phosphorylation in the neurons from Kcnma1\(^{-/-}\) mice (Fig. 4a,b).

To determine whether the effects of nBK channels blockade extended to differences in activity-evoked gene transcription, we performed real-time quantitative reverse transcription PCR (qRT-PCR) to analyze the expression of activity-dependent and nuclear Ca\(^{2+}\)-sensitive genes\(^{12,33,34}\) after IbTx preincubation. Paxilline indeed increased Fos, Npas4, Atf3, Btg2, Bcl6 and Ifi202 expression (Fig. 4c–h), but not Gadd45b and Gadd45g expression (data not shown), during basal neuronal activity. Paxilline induction of gene expression was blocked by STO-609 and was completely abolished in the neurons from Kcnma1\(^{-/-}\) mice (Fig. 4c–h). Similarly to the effects on

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**Figure 3** Blockade of nBK channels induces CREB phosphorylation in isolated nuclei and intact neurons. (a) Immunoblots of isolated functional nuclei after stimulation as indicated; p, phospho.

One-way ANOVA (\(F_{5,9} = 28.7, P < 0.0001\); \(F_{3,16} = 47.4, P < 0.0001\) and \(F_{2,9} = 15.2, P < 0.001\)) and post hoc test. \(*P < 0.05, **P < 0.01, ***P < 0.001\) as compared with 60 nM Ca\(^{2+}\) group. (b) Immunofluorescence for CREB phosphorylation. (c) Statistics for b. One-way ANOVA (\(F_{5,9} = 15.2, P < 0.001\); \(F_{3,16} = 6.7, P < 0.01\) and \(F_{2,9} = 15.2, P < 0.001\)) and post hoc test. \(*P < 0.05, **P < 0.01, ***P < 0.001\) as compared with 60 nM Ca\(^{2+}\) group. (d) Immunohistochemistry for CREB phosphorylation in isolated functional nuclei (d) and intact neurons (f) after paxilline treatment. (e,g) Immunofluorescence intensity of CREB phosphorylation in isolated functional nuclei (e) and intact neurons (g) after treatments as indicated. One-way ANOVA (\(F_{5,9} = 20.5, P < 0.001\); \(F_{5,9} = 15.2, P < 0.001\)) and post hoc test. Fisher’s least significant difference test was used for post hoc testing in one-way ANOVAs. Error bars represent mean \(\pm\) s.e.m. All data in a are from 3 independent cultures from at least 3 litters. All other data are from 5 coverslips of cells from 3 independent cultures from at least 3 litters. All experiments were successfully repeated at least 3 times. **\(P < 0.01\); n.s., no significant difference: \(P = 0.65\) for e; \(P = 0.71\) for g; \(P = 0.97\) for i; \(P = 0.63\) for j. STO, STO-609; WT, wild-type; Scale bars in d,f,h represent 10 μm. Full-length blots for a,d,f,h are presented in Supplementary Figure 7.
bicuculline-induced \([Ca^{2+}]_{\text{in}}\) and CREB phosphorylation, paxilline pretreatment significantly attenuated bicuculline-induced elevation of gene expression (Fig. 4c–h). Taken together, these results indicate that the nBK channels are important to synaptic activity–evoked CREB phosphorylation and gene expression during excitation–transcription coupling.

**nBK channels regulate dendritic arborization**

Dendritic arborization is critically important for neuronal development and function, and the regulation of dendritic geometry is highly dependent on excitation–transcription coupling\(^{13}\). Given that the nBK channel is important to the control of nuclear Ca\(^{2+}\) signaling (Figs. 2 and 3) and gene expression in excitation–transcription coupling (Fig. 4), we further explored the roles of nuclear Ca\(^{2+}\) signaling and the nBK channels in the regulation of dendritic arborization.

To first determine the role of nuclear Ca\(^{2+}\) signaling in the regulation of dendritic arborization in the basal condition, we used CaMBP4, which contains four repeats of the M13 CaM binding peptide and can effectively inactivate the nuclear Ca\(^{2+}/\text{CaM}\) complex and block genomic responses induced by nuclear Ca\(^{2+}\) signaling\(^{12,13,15}\). We expressed CaMBP4 or dnCaMKIV in hippocampal neurons. As is consistent with a previous study\(^{13}\), morphological analyses showed that the expression of CaMBP4 or dnCaMKIV could reduce dendritic arborization under basal conditions.

To further investigate the role of nBK channels in the regulation of dendritic arborization, hippocampal neurons were pretreated with IbTx to exclude the involvement of pmBK channel (Fig. 5 and Supplementary Fig. 4). Morphometric analyses revealed that neurons treated with paxilline showed an increase both in the complexity of the dendrites (Fig. 5a) and in the total dendritic length (Fig. 5b). The expression of CaMBP4 or dnCaMKIV reversed this paxilline-induced increase (Fig. 5a,b), suggesting that the increase also depended on nuclear Ca\(^{2+}\) signaling. Similarly, neurons transfected with BK channel–specific shRNAs showed an increase both in the dendritic...
Figure 5 nBK channels regulate dendritic arborization via nuclear Ca\textsuperscript{2+}–CaMKIV signaling. (a) Representative micrographs of hippocampal neurons transfected with an expression vector for GFP or cotransfected with expression vectors for GFP and CaMBP4 or for GFP and CaMKIVK75E (dnCaMKIV), with DMSO or paxilline treatment. (b) Sholl analysis (left) and quantification of total dendritic length (right) in hippocampal neurons treated as indicated. Right, one-way ANOVA (P = 0.0070, \(F_{2,57} = 5.42\) for DMSO group; \(P = 6.22 \times 10^{-6}, F_{2,57} = 22.51\) for paxilline group) and post hoc test; unpaired \(t\)-test \(P = 0.101, t_{38} = -2.70\) for comparison between vector groups after DMSO or paxilline treatment. (c) Representative micrographs of hippocampal neurons transfected with GFP-tagged plasmids containing BK channel-specific shRNAs or non-silencing shRNAs, with DMSO or STO-609 treatment. (d) Sholl analysis (left) and quantification of total dendritic length (right) in hippocampal neurons treated as indicated. Right, one-way ANOVA \(P = 3.00 \times 10^{-6}, F_{6,133} = 6.74\) and post hoc test. All experiments were successfully performed at least 3 times. \(n = 20\) cells from 4 independent cultures from at least 4 litters for each group. Fisher’s least significant difference test was used for post hoc testing in one-way ANOVAs. Error bars represent mean \(\pm\) s.e.m. Scale bars, 30 \(\mu\)m. * \(P < 0.05\), ** \(P < 0.01\). STO, STO-609; Pax, paxilline; N.S., non-silencing shRNAs.

complexity and in the total dendritic length (Fig. 5c,d). STO-609 suppressed basal dendritic arborization and reversed the shRNA-induced increase, once again suggesting that both basal and paxilline-induced dendritic arborizations depended on nuclear Ca\textsuperscript{2+} signaling.

To determine the specific genes mediating paxilline-induced morphological changes, we used shRNAs to knock down the expression of nBK channel target genes (Supplementary Fig. 5). Only shRNA against Npas4 reduced dendritic arborization and reversed paxilline-induced morphological changes (Fig. 5e,f and Supplementary Fig. 6), suggesting the involvement of Npas4 in both basal and paxilline-induced dendritic arborization. Taken together, these results indicate that nBK channels can regulate dendritic arborization in a nuclear Ca\textsuperscript{2+}/CaMKIV signaling–dependent manner.

DISCUSSION

In this study, we demonstrate for the first time, to our knowledge, that functional BK channels are expressed on the NE of hippocampal neurons and that the activities of nBK channels regulate synaptic activity–triggered nuclear Ca\textsuperscript{2+} signaling, CREB activation, gene expression and dendritic arborization. These findings reveal the importance of the nBK channel as a key regulator and molecular link, operating at the NE, between neuronal activities, nuclear Ca\textsuperscript{2+} signaling and gene expression.

The expression of BK channels in the nuclear envelope was established on the basis of immunofluorescence, immunoelectron microscopy and immunoblot data from both intact cells and isolated nuclei. The use of Kcnn4a\textsuperscript{−/−} mice further confirmed the specificity of the labeling. Most importantly, excised patches of nuclear membrane confirmed the presence of nBK channels that exhibited similar voltage dependence, Ca\textsuperscript{2+} dependence, single-channel conductance and pharmacological properties to those of BK channels found in the plasma membrane. Thus, several independent lines of evidence indicate the
presence of functional BK channels in the NE, supporting the notion that the BK channels have multiple sites of function, including the plasma membrane, mitochondria and as shown here, the NE.

Nuclear Ca\(^{2+}\) is a potent signal regulating neuronal gene expression and represents a key player in the dialog between synapse and nucleus. However, the source of nuclear Ca\(^{2+}\) has been debated for many years, and the mechanisms by which nuclear Ca\(^{2+}\) is regulated by synaptic activity are incompletely understood. It has been proposed that the nuclear pore complex (NPC) is large enough to provide a route for passive diffusion of ions across the NE, which would imply that nuclear Ca\(^{2+}\) signaling depends on cytosolic Ca\(^{2+}\) changes. However, much evidence suggests that the nucleus has an autonomous Ca\(^{2+}\) signaling system that can generate its own, independent Ca\(^{2+}\) transients. (i) NPC size is regulated by the filling state of the NE lumen. Depletion of perinuclear Ca\(^{2+}\) stores has been shown to induce a conformational change in the NPC and reduce NPC permeability. (ii) Patch clamping in the nucleus-attached mode puts many NPCs in the tip of a patch pipette. The high-resistance seal between the pipette and NE suggests that NPCs can shift to a closed state that restricts ion flux. (iii) Ions do not seem to move freely across the NPC, as there are differences in ion concentration between cytoplasm and nucleoplasm. (iv) When human liver hepatocellular carcinoma HepG2 cells are stimulated with extracellular ATP, the nucleoplasmic Ca\(^{2+}\) signal precedes the cytosolic Ca\(^{2+}\) signal by ~450 ms, suggesting the independence of the nucleoplasmic Ca\(^{2+}\) signal. (v) In the present study, application of paxilline to intact neurons induced nucleoplasmic Ca\(^{2+}\) elevation without raising cytosolic Ca\(^{2+}\) concentration, suggesting that the nucleoplasmic Ca\(^{2+}\) is independent of cytoplasmic Ca\(^{2+}\) and NPC-mediated Ca\(^{2+}\) transport. Nonetheless, the existence of this autonomous nuclear Ca\(^{2+}\) signaling system does not preclude the presence of the NPC-mediated Ca\(^{2+}\) transport.

Partly owing to a lack of suitable experimental methods, the ionic gradient for K\(^+\) across the NE remains unknown in intact cells. Recent studies in isolated nuclei loaded with K\(^+\)-sensitive fluorescent indicators showed that the K\(^+\) concentration in the perinuclear space is much lower than the cytoplasm and nucleoplasm. Therefore, the changes in the nuclear K\(^+\) channel activity would alter K\(^+\) flux across the NE and ultimately lead to alteration of \(\Delta \Psi \text{n}\). Indeed, our results showed that nBK channels, with their similar electrophysiological properties to those of pmBK channels, regulated \(\Delta \Psi \text{n}\), supporting the existence of a K\(^+\) gradient across the NE. Nevertheless, all these results were obtained from isolated nuclei and need further confirmation in intact cells. It is noteworthy that the open probability of RyRs is elevated upon changes in voltage. Thus, it is highly probable that RyRs may be affected by paxilline-induced \(\Delta \Psi \text{n}\) as revealed by the voltage-sensor DiOC\(_6\)(3). Similar voltage-induced RyR-mediated Ca\(^{2+}\) release has been observed in pancreatic beta cells. Nonetheless, we do not exclude the existence of other voltage-sensitive pathways that may directly or indirectly trigger the release of nuclear calcium.

Coupling between synaptic excitation and gene expression is critical for a broad range of long-term changes in nervous system function. Although many intracellular molecules, such as calmodulin, MAP kinases and CaMKs, have been shown to participate in synapse-to-nucleus signaling, faster modulators are also required to convey fast information-laden synaptic electrical signals. Via rapid ion channel kinetics, the nBK channel may function as a fast molecular mediator between neuronal activity and nuclear Ca\(^{2+}\) to convey signals from synapse to nucleus. The NE is inaccessible to analysis with patch clamp pipettes and voltage-sensitive dyes in intact neurons, which makes it difficult to measure the activity of nBK channels synchronously during neuronal activity. Therefore, the effects of neuronal activity on the activity of the nBK channel require further exploration. It is noteworthy that neuronal activity can modulate pmBK channels in a bidirectional manner: activity-induced intracellular Ca\(^{2+}\) elevation and CaMKII activation activate pmBK channels, whereas activity-induced calcineurin activation has the opposite effect. If the same regulation also applies to nBK channels, the regulation of nBK channel activity by neuronal activity may be bidirectional as well. The development of suitable experimental systems will help to elucidate this matter.

The long-term maintenance of activity-induced, functional adaptations requires nuclear Ca\(^{2+}\) signaling—dependent, CREB-mediated transcription to activate gene expression. Using immunofluorescence, immunoblot and qRT-PCR, we found that blockade of the nBK channel increased CREB phosphorylation and neuronal activity—dependent gene expression during basal neuronal activity, but compromised those during bicuculline-induced action potential bursting. Bicuculline-induced CREB phosphorylation and gene expression need sustained, Ca\(^{2+}\) store-derived nuclear Ca\(^{2+}\) elevation, and depletion of the Ca\(^{2+}\) store will significantly compromise these effects. In our studies, paxilline pretreatment induced the release of nuclear Ca\(^{2+}\) and the reduction of the Ca\(^{2+}\) store content, and, consequently, compromised the bicuculline-induced nuclear Ca\(^{2+}\) elevation needed for CREB phosphorylation and gene expression. These results are consistent with studies proposing that filling of the nuclear Ca\(^{2+}\) store is required for synaptically evoked nuclear Ca\(^{2+}\) signaling and CREB-mediated gene expression. nBK channel–modulated CREB phosphorylation and gene expression depend on nuclear Ca\(^{2+}\) signaling, as specific blockade of nuclear Ca\(^{2+}\) signaling via dnCaMKIV, PV-NLs and STO-609, but not via the ERK inhibitor U0126, abolished the effects of nBK channel blockade. In the intact cell, we cannot exclude the possibility that Ca\(^{2+}\) release from other organelles may drive gene expression in our conditions. However, the NE would be responsible for an important portion of these intracellular Ca\(^{2+}\) signals.

The dendritic tree determines the neuronal connectivity and has a strong influence on synaptic transmission and integration of synaptic signals. Moreover, dendritic geometry itself is regulated by neuronal activity. Our results show here that nBK channel blockade caused an increase both in the total dendritic length and in the complexity of the dendrites, further confirming the importance of nuclear Ca\(^{2+}\) in the regulation of dendritic arborization. Knockdown of expression of one of the nBK channel-target gene, Npas4, completely blocked paxilline-induced morphological changes, indicating that Npas4 is critical to this regulation. Npas4 also functions as a transcriptional regulator, with a key role in activity-dependent regulation of inhibitory synapse development, contextual memory formation and neuronal protection. Knockdown of Npas4 significantly inhibits brain-derived neurotrophic factor (BDNF) expression, implicating a mechanism by which Npas4 knockdown may block paxilline-induced morphological changes.

By controlling nuclear Ca\(^{2+}\) signaling, the nBK channel links neuronal activity to the regulation of CREB activation and gene expression, the control of total dendritic length and branching patterns. Blockade of the nBK channels represents a new strategy for the development of effective therapies for the reduction of dendrite complexity in aging, Alzheimer’s disease and autism. These findings may also help explain the various phenotypes, such as Purkinje cell dysfunction, cerebellar ataxia and abnormal circadian behavioral rhythms, found in BK channel global knockout mice.
METHODS

Methods and any associated references are available in the online version of the paper.

Note. Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

B.L. and T.-M.G. designed the study; B.L., WJ, and L.H. conducted biochemistry. Ca2+ imaging and immunocytochemistry experiments; B.L., P.W. and Z.L. conducted electrophysiological experiments; B.L. and S.L. cultured neurons; A.K.E., A.L.M., M.H.-H. and X.H.Z. contributed new reagents or analytic tools; B.L., WJ, P.W., Z.L. and L.H. collected and analyzed data; B.L., L.H. and T.-M.G. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Cell culture and transfection and virus infection. Wild-type or Kcnma1−/− mice (C57BL/6 strain) and Sprague-Dawley rats were used for cell culture. All the experiments were conducted in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (China) and were approved by the Southern Medical University Animal Ethics Committee. Three to four animals were housed per cage on a 12 h light, 12 h dark cycle, as reported in previous publications. Pure primary hippocampal neurons from postnatal day 1 wild-type or Kcnma1−/− mice (C57BL/6J/129 strain)36 were cultured in Neurobasal medium with 2% B27 supplement, 0.5 mM glutamine and 100 U/ml penicillin/streptomycin (Invitrogen, San Diego, CA). Cells were grown at 37 °C in a humidified atmosphere containing 5% CO2 and 95% air. The data in Figures 1d, 2b, and 3b were obtained from intact neurons or isolated nuclei of primary cultured hippocampal neurons from postnatal day 1 Sprague-Dawley rats. All experiments were performed on cells cultured for 14 d. DNA transfection was done on DIV 8 by using Lipofectamine 2000 (Invitrogen, San Diego, CA) as described39. BK-GFP plasmid was a gift from X. Tang40,41. PV-NLS plasmid was a gift from M. Nathanson42. Plasmids for the kinase-inactive form of CaMKIV (dnCaMKIV, CaMKIVK75E) and CaMP84 were gifts from H. Bading43. For shRNA, different sequences were obtained from Shanghai GeneChem (shBK1, shBK2 and shBK3), cloned and tested for silencing efficiency. shBK (1) and (2) (GGATGTCGCAAGAAGATGTA and GCACCTACGTACTGGAGATGTTA) were chosen. The targeting sequences for all RyRs or RyR2 are ACATGGAGACCAAGTGCTT and GGAAAGAAGTCGATGGCAT, respectively25. As control, a scrambled version of this sequence was used. For the studies on dendrite morphology, neurons were chilled on ice, washed twice with ice-cold PBS containing 1 mM CaCl2, and then incubated with peroxidase- or IRDye-conjugated secondary antibody. Membranes were incubated in Odyssey Blocking Buffer (LiCor) for 2 h at room temperature. High-resistance seals were formed (5–7 GΩ), indicating minimal ER contamination–free circumstances, it has been proposed that nuclear pores would be occluded or nonconducting because of experimental conditions or lack of cytosolic factors6,39,53. PCLAMP software was used to measure the patch-clamp amplifier (Axopatch 200B; Axon Instruments, Foster City, CA) at a sampling rate of 1–5 kHz and leak-subtracted online. Series resistance never exceeded 25 MΩ and was compensated. Data were acquired and analyzed with pClamp9 (Axon Instruments). Single-channel currents were recorded from nuclei positively labeled with ethidium homodimer-1 by using standard patch-clamp recording procedures24. Currents were recorded in symmetrical 133 mM K+ using a patch-clamp amplifier (Axopatch 200B; Axon Instruments) at a sampling rate of 5 kHz and filtered at 1 kHz filter. Experiments were performed at room temperature. High-resistance seals were formed (5–7 GΩ), indicating minimal contamination by the endoplasmic reticulum (ER) membrane24. Under these ER contamination–free circumstances, it has been proposed that nuclear pores would be occluded or nonconducting because of experimental conditions or lack of cytosolic factors6,39,53. PCLAMP software was used to measure the current magnitude and duration.

Patch-clamp experiments. For whole-cell recordings, electrodes had a final resistance of 5–10 MΩ when filled with pipette solution (in mM): 120 potassium gluconate, 15 HEPES, 2.2 CaCl2, 1.0 MgCl2, 4.0 EGTA, and 4.0 HEDTA, pH 7.35, 305 mOsm. Currents were recorded using an Axopatch 200B (Axon Instruments, Foster City, CA) amplifier at a sampling rate of 1–5 kHz. Currents were filtered at 5 kHz and leak-subtracted online. Series resistance never exceeded 25 MΩ and was compensated. Data were acquired and analyzed with pClamp9 (Axon Instruments). Single-channel currents were recorded from nuclei positively labeled with ethidium homodimer-1 by using standard patch-clamp recording procedures24. Currents were recorded in symmetrical 133 mM K+ using a patch-clamp amplifier (Axopatch 200B; Axon Instruments) at a sampling rate of 5 kHz and filtered at 1 kHz filter. Experiments were performed at room temperature. High-resistance seals were formed (5–7 GΩ), indicating minimal contamination by the endoplasmic reticulum (ER) membrane24. Under these ER contamination–free circumstances, it has been proposed that nuclear pores would be occluded or nonconducting because of experimental conditions or lack of cytosolic factors6,39,53. PCLAMP software was used to measure the current magnitude and duration.

Ca2+ imaging. Intact neurons were loaded with Fluo-4 AM or X-Rhod-1 a.m. (Life Technology) in transfected neurons, in HEPPS buffer for 30 min, and washed three times before imaging. Single isolated nuclei were loaded as reported24. The membrane-impermeant Ca2+ probe Fluo-4 dextran (30 µg/ml; 30 min at 4 °C; Life Technologies) loaded the nucleoplasm while the membrane permeant Ca2+ probe Fluo-4 AM (20 µM; 60 min at 4 °C) loaded the nuclear envelope. After loading, the nuclei were washed twice with the intracellular medium (125 mM KCl, 2 mM KH2PO4, 0.1 mM MgCl2, 40 mM HEPES, pH 7.2, 100 mM Ca2+, with 10.2 mM EGTA and 1.65 mM CaCl2) and then were equilibrated in the same medium supplemented with 1 µM of ATP and 300 nM Ca2+ for a few minutes to load nuclei with Ca2+24. After that, they were washed twice again with the intracellular buffer (without ATP and Ca2+). Experiments were done at room temperature. No probe leakage was detected during the experiment. Ca2+ imaging in single isolated nuclei was performed by using an Olympus FV1000 confocal microscope. Images were collected at 1-s intervals, and fluorescence was measured by using the Olympus FV1000
software package. The difference (ΔF) between the mean fluorescence measured in a given region of interest (ROI) and the corresponding control value for each ROI (F₀) was expressed as fraction of the control (ΔF/F₀) and plotted as a function of time.

Nuclear transmembrane potential (ΔΨₙ) measurements. Isolated nuclei were equilibrated in intracellular medium with DiOOC(3) (200 nM, room temperature, Life Technologies), which is a fluorescent cationic lipophilic dye whose incorporation into the lumen is proportional to ΔΨₙ (ref. 24). After 10 min, the nuclear envelope became stained. The nuclei were washed three times before imaging with the Olympus FV1000 confocal microscope. Images were collected at 1-s intervals, and fluorescence was measured by using the Olympus FV1000 software package. The difference (ΔF) between the mean fluorescence measured in a given ROI and the corresponding control value for each ROI (F₀) was expressed as fraction of the control (ΔF/F₀) and plotted as a function of time.

Immunocytochemistry. Cells were washed with PBS and fixed for 20 min with 4% paraformaldehyde at room temperature. To permeabilize cells, they were treated with 0.3% Triton X-100 in PBS. Nonspecific binding was blocked for 1 h at room temperature using 5% bovine serum. Primary antibodies were then added and incubated overnight at 4 °C. Cells were then washed and incubated for 1 h with the fluorescently labeled secondary antibodies at room temperature and washed three times to remove unbound antibodies.

Phospho-CREB immunofluorescence. CREB phosphorylation was induced in isolated nuclei by increasing Ca²⁺ in an EGTA-buffered intracellular medium from 60 nM to 200 nM Ca²⁺ or by addition of parvalbumin for 3 min. Nuclei were fixed with 4% paraformaldehyde (w/v) and permeabilized with 1% Triton X-100 for 10 min. Nuclei were preincubated with blocking buffer, then anti–phospho-CREB antibodies were applied for 16 h at 4 °C. Phospho-CREB was visualized with TRITC-conjugated secondary antibodies (1 h, room temperature). High Ca²⁺ gave the maximum response.

Morphometric analyses. For three-dimensional Sholl analysis, total dendritic length and spine morphology were calculated using ImageJ software. Briefly, a z-stack was imported, calibrated in ImageJ, and manually traced. Total dendritic length was then computed. For Sholl analysis, the shell interval was set to 10 µm. All analyses were performed blind.

RNA extraction and cDNA synthesis. Total RNA was isolated from hippocampal primary neuron cultures with RNeasy Mini Kit (Takara) including an optional DNase I treatment at room temperature for 15 min according to manufacturer’s instructions. 1 µg of extracted RNA was reverse transcribed into first strand cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA).

Real-time quantitative PCR. Quantitative reverse transcriptase PCR (qRT-PCR) was done on a Stratagene Mx3000P thermal cycler using Universal qRT-PCR master mix for the indicated genes (Takara). The primers were designed and synthesized as follows:

| Sense            | Antisense          |
|------------------|--------------------|
| 5′-GGCGGAGTTGGAAGACATC-3′ | 3′-ATGATGCTTCAACACCCAGGC-3′ |
| 5′-ATGATGCTTCAACACCCAGGC-3′ | 3′-GGCGGAGTTGGAAGACATC-3′ |

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Corrigendum: Nuclear BK channels regulate gene expression via the control of nuclear calcium signaling

Boxing Li, Wei Jie, Lianyan Huang, Peng Wei, Shuji Li, Zhengyi Luo, Allyson K Friedman, Andrea L Meredith, Ming-Hu Han, Xin-Hong Zhu & Tian-Ming Gao
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In the version of this article initially published, item (ii) in the third paragraph of the Discussion read, "The ability to obtain high-resistance gigaohm seals during patch clamping in the nucleus-attached mode, when dozens of NPCs may be encompassed by the patch pipette, suggests that NPCs can exist in a closed state that restricts ion flow\textsuperscript{39}." This sentence has been amended to cite its source, which the authors inadvertently eliminated while reducing the reference count for publication. That work has been added to the reference list as ref. 72: Matzke, A.J., Weiger, T.M. & Matzke, M. Ion channels at the nucleus: electrophysiology meets the genome. Mol. Plant 3, 642–652 (2010). In addition, the text has been reworded to avoid directly quoting from the source without attribution; the revised text reads, "Patch clamping in the nucleus-attached mode puts many NPCs in the tip of a patch pipette. The high-resistance seal between the pipette and NE suggests that NPCs can shift to a closed state that restricts ion flux\textsuperscript{39,72}." The error has been corrected in the HTML and PDF versions of the article.