Tonic PKA activity regulates SK channel nanoclustering and somatodendritic distribution

Abbreviated Title: PKA controls SK channel location and clustering

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Abstract

Small-conductance calcium-activated potassium (SK) channels mediate a potassium conductance in the brain and are involved in synaptic plasticity, learning, and memory. SK channels show a distinct subcellular localization that is crucial for their neuronal functions. However, the mechanisms that control this spatial distribution are unknown. We imaged SK channels labeled with fluorophore-tagged apamin, and monitored SK channel nanoclustering at the single molecule level by combining atomic force microscopy and toxin (i.e. apamin) pharmacology. Using these two complementary approaches, we found that native SK channel distribution in pyramidal neurons, across the somatodendritic domain, depends on ongoing cyclic adenosine monophosphate (cAMP)-protein kinase A (PKA) levels, strongly limiting SK channel expression at the pyramidal neuron soma. Furthermore, tonic cAMP-PKA levels also controlled whether SK channels were expressed in nanodomains as single entities or as a group of multiple channels. Our study reveals a new level of regulation of SK channels by cAMP-PKA and suggests that ion channel topography and nanoclustering might be under the control of second messenger cascades.

Keywords: single-molecule atomic force microscopy; dual-color fluorescence; potassium channels; second messengers;

Introduction

Calcium (Ca^{2+})-activated potassium channels, in particular, small-conductance Ca^{2+} -activated potassium (SK) channels, play an important role in regulating the excitability and synaptic plasticity of neurons [1]. Multiple SK channel subtypes are expressed in cortical pyramidal neurons. However, only SK2 channels are required for SK channel-mediated currents [2], as blocking SK channels using apamin, a neurotoxic polypeptide from bee venom, has no effect on currents in SK2-null (but not SK1- or SK3-null) cortical neurons [2]. As a result, recent work in these neurons has focused primarily on SK2 channels.

SK2 channels are found in two isoforms, a short (SK2-S) and long (SK2-L) form [3]. The two isoforms differ structurally in the length of their N-terminus [3] and also in their propensity to cluster into groups of multiple channels [4]. Recent work also suggests that the two SK2 isoforms might serve different functions in neurons. Specifically, SK2-L is primarily localized in spines and controls excitatory postsynaptic potential (EPSP) amplitudes, whereas SK2-S predominantly mediates the apamin-sensitive current following strong somatic depolarization [5]. However, the mechanisms that underlie the differences in subcellular compartmentalization of SK channels are largely unknown.

The cell-surface expression of SK2 channels is regulated by protein kinase A (PKA) activity [6-8]. In a previous study, we used atomic-force microscopy (AFM) to show that native SK channels predominantly reside along neuronal dendrites as both single entities and in pairs [4]. We also found that stimulation of neurons with forskolin, a strong PKA activator, altered the density of SK channels at the cell surface, establishing proof-of-principle that this approach is

highly quantitative and amenable to live studies of receptor trafficking at superior (i.e., nanometer) resolution. However, it remains unknown whether PKA phosphorylation also regulates the somatodendritic distribution of SK channels and SK channel nanoclustering at the cell surface, and if so, what is the source of PKA required for these effects? This is particularly important as SK channel somatodendritic distribution and gradient is crucial for their neuronal functions [9, 10].

In the present study, we sought to answer these questions using confocal microscopy, single molecule atomic force microscopy (AFM) and toxin pharmacology. Specifically, we tested whether cyclic adenosine monophosphate (cAMP)-activated PKA regulates SK2 channel nanoclustering at a single molecule level. We primarily focused on the cAMP-PKA pathway because it regulates SK2 channel surface expression and endosome recycling [7], and is a key determinant of SK2 channel activity following the induction of long-term potentiation [7]. We demonstrate that (i) the somatodendritic distribution of SK channels in neurons is dictated by basal PKA activity and (ii) SK channel nanoclustering in heterologous cells and in neurons is under the control of cAMP and PKA activity. Thus, our work reveals a new level of regulation of SK channels by PKA and also demonstrates, for the first time, that SK channel somatodendritic distribution is dynamic and depends on the levels and regulation of intracellular signal-transduction pathways.

Results

We and others have shown that SK channels are enriched on dendrites compared to the soma [4, 11, 12]. However, the mechanism for this somatodendritic distribution in neurons is currently

unknown. To address this, we first used confocal microscopy to image neurons that were incubated with 500 pM apamin conjugated to biotin. Apamin is a specific SK channel blocker that binds to the outer vestibule of SK channels with high affinity ($K_d=10$ pM) in a 1:1 ratio [13]. We visualized apamin distribution with Alexa-488 fluorophore conjugated to streptavidin, a high affinity binding partner of biotin, thus enabling the localization of SK channels. Neurons were also stained against the dendritic marker MAP2 and the synaptic marker MAGUK.

Consistent with our previously published work [4], apamin labeling was strong on dendrites (Figure 1A) and appeared as puncta co-localized with MAGUK (Figure 1B) in spines. Indeed, the overlap between MAGUK and SK channels exhibited a Pearson's correlation coefficient of 0.34 ± 0.06 ($n=37$). Surprisingly, we also saw robust somatic labeling of SK channels, but it was unclear whether the staining was on the surface or primarily intracellular (Figure 1D(i) and 1D(ii)).

To determine and quantify the distribution of cell surface versus internalized SK channels, we employed a dual-color fluorescence approach [14]. We first incubated live neurons with apamin-biotin followed by fixation and incubation with Alexa-488 fluorophore conjugated with streptavidin to label SK channels at the cell surface, as described in the methods. Then, after thoroughly washing the cells, we permeabilized the neurons and incubated the neurons with Alexa-546 fluorophore conjugated with streptavidin to label internalized SK channels and with antibodies against the dendritic marker MAP2. The ratio between the Alexa-488 (green) and Alexa-546 (red) fluorescence signal reflected the distribution between surface and internalized SK channels. We found that the majority of both dendritic and somatic apamin-bound SK

channels were intracellular (dendrites: mean corrected total fluorescence (CTF) ratio of Alexa-488 to Alexa-546 = 0.450 ± 0.069 , n= 27; soma: CTF ratio of Alexa-488 to Alexa-546 = 0.603 ± 0.068 , n= 18; Figure 2A, top panel, 2B and 2C). Control experiments in which the cells were not permeabilized showed essentially no staining with Alexa-546 (data not shown), indicating that this stain indeed measured internal apamin-bound SK channels. The simplest explanation for these results is that SK channels internalize to intracellular compartments within a short time frame upon binding apamin. Thus, we repeated our experiments at 4°C to arrest or significantly retard the rate of internalization. Consistent with our hypothesis, apamin labeling became more prominent at the cell surface (dendrites: CTF ratio of Alexa-488 to Alexa-546 = 1.606 ± 0.105 , n=27; soma; mean CTF ratio of Alexa-488 to Alexa-546 = 2.754 ± 0.370 , n=18; Figure 2A, middle panel, 2B and 2C) when neurons were incubated at 4°C. This suggests that SK channels are found on the soma and dendrites but can be rapidly internalized.

We next wondered what could regulate the surface expression of SK channels. Previous work has shown that PKA can trigger the removal of SK channels from spines in an activity-dependent manner [7]. Therefore, it is possible that SK channels are continuously retrieved from the cell surface due to elevated cAMP-PKA levels in their vicinity, independent of strong synaptic activity. To examine this mechanism, we incubated neurons with KT5720, a PKA inhibitor that previous work has shown to inhibit activity dependent internalization of SK channels in neurons [7]. We found that neurons incubated for 30 minutes with 1μM KT5720 exhibited a strong surface localization (Figure 2A, bottom panel). The strongest effect was in the soma, suggesting that the sparse surface labeling of SK channels on the soma when the neurons were not treated with KT5720, might be due to very strong PKA activity (soma: CTF ratio of Alexa-488 to

Alexa-546 = 3.965 ± 0.208 , $n=18$; dendrites: CTF ratio of Alexa-488 to Alexa-546 = 1.705 ± 0.105 , $n=27$; Figure 2B and 2C). Our data, therefore, suggests that PKA activity might continuously promote the retrieval of SK channels from the cell surface.

To obtain a more quantitative assessment of this finding and to use an experimental approach that did not rely on extended apamin incubation, we turned to single-molecule AFM. Apamin was functionalized on an AFM cantilever, allowing us to detect surface SK channels by scanning a $1 \mu\text{m} \times 1 \mu\text{m}$ area using 30 nm lateral steps. We thereby obtained the unbinding forces between apamin molecules and SK channels (Figure 3A) [4] by measuring the deflection of the AFM cantilever in the z direction (Figure 3B).

To determine the dynamic range of single-molecule AFM in detecting the unbinding forces between apamin and SK2 channels, a prerequisite for studying clustering, we took advantage of the previous finding that the rat homologue of SK1 (rSK1) does not bind to apamin [15]. However, rSK1 readily forms heteromers with the apamin-sensitive short SK2 channel splice isoform (SK2-S) [15], and therefore, the rSK1/SK2-S heteromer will presumably have lower apamin unbinding forces. Indeed, co-transfecting rSK1 channels with SK2-S channels in HEK293T cells led to clearly distinguishable unbinding forces with average strength of $\mu=20 \pm 3$ pN (surface density = 2.6 ± 1.3 %, $n=6$; Figure 3C, red). The values of the unbinding forces between apamin and rSK1/SK2-S heteromers were lower than the forces obtained from cells expressing SK2-S alone ($\mu=28 \pm 5$ pN; $P=0.0013$, Mann Whitney test; Figure 3C, green) and higher than forces obtained with non-functionalized AFM tips ($\mu=11 \pm 1$ pN, surface density = 0.7 ± 0.6 %, $n=4$; $P<0.0001$, Mann Whitney test; Figure 3D, dark green) or forces from

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4 cells transfected with rSK1 alone ($\mu=13\pm1$ pN, surface density = 1.0 ± 0.6 %, $n=6$; $P<0.0001$,
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6 Mann Whitney test, Figure 3C, blue). The data are consistent with the presence of rSK1/SK2-S
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8 heteromers on HEK293T cell surfaces having lower apamin affinity than SK2-S homomers.
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10 Together, our data clearly establish the dynamic range of single-molecule AFM to identify SK
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12 channels in living cells.
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18 Similar to our imaging data with confocal microscopy in neurons, when we employed single
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20 molecule AFM in living neurons under control conditions, we found that SK channels are
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22 enriched on dendritic membranes (Figure 4C(i) and 4C(ii), -bath apamin). Preincubating neurons
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24 with apamin drastically lowered the detection of apamin unbinding events (Figure 4A(i)-C(i) and
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26 4A(ii)-C(ii), +bath apamin) indicating that the unbinding forces observed were indeed between
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28 apamin and SK channels in neurons. However, neurons incubated with KT5720, a PKA blocker,
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30 showed a large increase in the surface expression of SK channels in both soma and dendrites
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32 (Figure 5A(i)-C(i) and 5A(ii)-C(ii), +KT5720), consistent with our findings using confocal
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34 microscopy. Additionally, the unbinding force histograms of neurons treated with KT5720
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36 shifted to significantly larger values than untreated neurons, indicating an association between
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38 multiple SK channels and apamin molecules attached to the cantilever tip. This suggested that (i)
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40 SK channels do not accumulate in the soma due to ongoing PKA activity and (ii) SK channels
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42 might cluster in groups in the absence of PKA activity. Since PKA inhibition promotes the
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44 expression of multiple SK channels in nanodomains on the cell surface, we refer to this as
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46 nanoclustering.
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To further probe the possibility that PKA controls SK channel nanoclustering, we compared the properties of SK2 channels expressed in HEK293T cells. This experiment was necessary as neurons express multiple SK channel isoforms including two SK2 splice variants. Consistent with earlier studies [4, 8], the application of forskolin (FSK) (30 μ M), an adenylate cyclase activator that strongly stimulates PKA, led to a pronounced loss of SK2-S channels from the surface of HEK293T cells (μ =13 $\pm$ 1 pN, surface density = 0.3 $\pm$ 0.2%, n=6; Figure 6B). In contrast, in cells pretreated with the PKA inhibitor KT5720 (1 μ M), FSK application did not cause any loss of SK2-S channel surface expression. Rather, the frequency of unbinding events increased three-fold (surface density = 12.1 $\pm$ 0.37%, n=6; Figure 6C). Along with the higher frequency of unbinding events, we also found that in the presence of KT5720+FSK, the recorded unbinding forces were best fit with two Gaussian distributions rather than one (74% μ =36 $\pm$ 6 pN and 26% μ =53 $\pm$ 13 pN; Figure 6C).

To determine whether the increase in SK2-S cell surface expression was mainly due to ongoing basal PKA activity and not forskolin-induced signaling independent of PKA (i.e., via activation of Epac), we also measured the unbinding forces of SK2-S-transfected cells pre-treated only with KT5720. Again, we found a biphasic force distribution and an increased density of detected unbinding events in the force maps (64% μ =35 $\pm$ 6 pN and 36% μ =55 $\pm$ 15 pN, surface density = 14.1 $\pm$ 2.7%, n=6; Figure 6D). The emergence of the second peak indicates that PKA inhibition promotes the expression of pairs of SK2 channels in nanodomains on the cell surface.

To further test this possibility, we functionalized AFM cantilevers with apamin via an acetal-PEG-NHS linker. In this method, the probability that the tip is functionalized with only one

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4 molecule is greater than 50% [16]. In comparison, in the glutaraldehyde-based functionalization
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6 method used in the experiments above, the number of apamin molecules attached to the probe
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8 cannot be controlled. We performed experiments on HEK293T cells transfected with SK2-S and
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10 found that at low surface expression of SK2-S channels, the unbinding forces obtained when
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12 using an acetal-PEG-NHS-apamin tip were similar to those obtained when a glutaraldehyde-
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14 apamin tip was used (acetal-PEG-NHS-apamin: $\mu=25\pm4$ pN, surface density= 3.6%, n=4, Figure
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16 7C, blue; glutaraldehyde-apamin: $\mu=29\pm5$ pN, surface density= 4.4%, n=12; Figure 7C, red).
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18 However, when the cells were pre-incubated with KT5720, which significantly increased their
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20 cell-surface expression, and then probed with an acetal-PEG-NHS-apamin functionalized tip, we
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22 observed only a single peak with a mean unbinding force similar to that obtained at baseline
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24 ($\mu=30\pm5.5$ pN, surface density= 8.2%, n=4; Figure 7D, blue), as opposed to the biphasic force
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26 distribution histogram observed when KT5720-treated cells were probed with a glutaraldehyde-
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28 apamin tip (Figure 7D, red). These results suggest that the presence of more than one channel
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30 was the reason for the observed second peak in the experiments in which PKA was inhibited and
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32 a glutaraldehyde-apamin functionalized tip was used. As the acetal-PEG-NHS functionalization
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34 method cannot detect possible channel clustering, we performed the rest of the experiments using
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36 the glutaraldehyde-based functionalization method.
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48 We next tested whether the ongoing PKA activity is due to increased basal cAMP levels, using a
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50 PKA inhibitor. We determined the unbinding force map distributions in cells incubated with (R)-
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52 adenosine-3',5'-cyclic triethylammonium salt (Rp-cAMPS), a cAMP analog that prevents
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54 activation of PKA by cAMP. Application of Rp-cAMPS (100 μ M) led to similar results as those
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56 obtained with the PKA inhibitor KT5720. In particular, the unbinding force histogram required
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two Gaussian distributions to fit the data (77% $\mu=36\pm7$ pN and 23% $\mu=57\pm15$ pN, surface density = $10.7\pm2.32\%$, n=6; Figure 8A). This suggests that tonic cAMP activity was responsible for the observed basal activation of PKA.

Multiple studies have shown that PKA is anchored to the plasma membrane through its association with A-kinase anchor protein (AKAP) scaffolding molecules [17, 18]. In turn, this complex can give rise to cAMP-PKA microenvironments that promote basal cAMP signaling [19]. HEK293T cells express multiple AKAPs [20, 21], raising the possibility that tonic PKA activity in the proximity of SK2 channels also requires AKAPs. To directly test this possibility, we incubated SK2-S-transfected cells with St-Ht31, a peptide that interferes with regulatory PKA subunit binding to AKAPs and thus, disperses PKA from the SK channel microenvironment. Similar to the above experiments using cAMP-PKA blockers, treatment with St-Ht31 (5 μ M) led to increased unbinding event frequency and force distributions that required fitting with two Gaussian distributions (62% $\mu=36\pm7$ pN and 38% $\mu=56\pm14$ pN, surface density = $12.8\pm4.0\%$, n=6; Figure 8B). Importantly, cells treated with St-Ht31P (5 μ M), a control peptide for St-Ht31 that does not interfere with the association of PKA with AKAPs, had force distributions no different than in control conditions ($\mu=30\pm5$ pN, surface density = $4.3\pm0.9\%$, P=0.86, ns, Mann Whitney test, n=6; Figure 8C).

Lastly, we tested whether the tonic cAMP activity was due to basal activation of beta-adrenergic receptors that reside in HEK293T cells. Beta-adrenergic receptors are G-protein-coupled receptors that signal by elevating cAMP levels through stimulation of adenylate cyclase. Since beta adrenergic receptors are endogenously expressed in HEK293T cells [22], the observed tonic

PKA activity could be due to their basal activation. However, we found that application of the inverse agonist propranolol (10 μ M), a compound that shifts the equilibrium of beta-adrenergic receptors towards the inactive state, led to force histograms no different from non-treated cells (μ =27 $\pm$ 4 pN, surface density = 4.0 $\pm$ 0.9%, P=0.42, ns, Mann Whitney test, n=6; Figure 8D). This suggests that the PKA activity seen in HEK293T cells is not due to basal activation of beta-adrenergic receptors, but rather tonic PKA activity and its association with AKAPs.

Pyramidal neurons also express the SK2-L isoform, a more recently described SK2 channel found in dendrites [5]. Therefore, we also investigated whether PKA activity regulates the spatial distribution of SK2-L. In previous work, we showed that SK2-L has a higher propensity for nanoclustering than SK2-S [4]; thus, SK2-L might be more amenable to PKA modulation than SK2-S. Indeed, unlike SK2-S-expressing cells, HEK293T cells transfected with 3 μ g of SK2-L cDNA showed a prominent biphasic unbinding force distribution under control conditions (SK2-L 3 μ g: 77% μ =31 $\pm$ 6 pN, 23% μ =53 $\pm$ 15 pN, surface density = 13.4 $\pm$ 2.2%, n=9; Figure 9A(i)) indicating that some SK2-L are clustered in groups under basal conditions. In cells pretreated with KT5720, the SK2-L force distribution histogram retained its biphasic nature but readily shifted to much higher values consistent with the majority of SK2-L channels found in groups of multiple channels (90% μ =48 $\pm$ 9 pN, 10% μ =76 $\pm$ 7 pN, surface density = 10.9 $\pm$ 2.7%, n=5; Figure 9A(ii)) and almost devoid of single channels. Similar results were also obtained in cells treated with the AKAP inhibitor St-Ht31 (80% μ =42 $\pm$ 7pN and 20% μ =62 $\pm$ 13pN, surface density = 11.7 $\pm$ 0.7 %, n=5; Supplementary figure 1), indicating that, as with SK2-S, SK2-L nanoclustering critically depends on AKAP activity.

To confirm that the rightward shift of the biphasic curves to higher values when the cells are treated with KT5720 and St-Ht31 corresponds to nanoclustering of SK2-L channels, we performed experiments using the acetal-PEG-NHS-apamin functionalization method on cells treated with KT5720. We found that the mean unbinding force shifted to values similar to those obtained from SK2-L cells probed with glutaraldehyde-apamin under basal conditions ($\mu=35\pm8.5$ pN, surface density= 10.9%, n=5; blue, supplementary figure 2).

To better quantify the extent to which PKA can promote SK2-L nanoclustering, we repeated our experiments in cells transfected with a low concentration of SK2-L cDNA plasmid (0.25 μ g) (Figure 9B). The use of a low plasmid concentration was to ensure that a greater number of SK2-L channels were initially expressed as single entities on the cell surface, thereby increasing the resolution for detecting the emergence of nanoclustering following inhibition of PKA. Indeed, cells transfected with 0.25 μ g SK2-L had a force distribution histogram that required one Gaussian distribution to fit the data, with unbinding forces tending towards smaller values, closer to those obtained previously with SK2-S (SK2-L 0.25 μ g: $\mu=28\pm 6$ pN, surface density = 4.4 $\pm$ 0.7%, n=5; Figure 9B(i)). However, following incubation with the PKA inhibitor KT5720, we observed two major changes: (i) SK2-L expression increased by two-fold (surface density = 8.8 $\pm$ 2.6%), and (ii) the force histograms required three Gaussian curves to describe the data with almost equal likelihood of finding SK2-L channels as single entities or as groups of multiple channels (44% $\mu=29\pm4$ pN; 49% $\mu=44 \pm9$ pN, 6% $\mu=73\pm 7$ pN,n=5; Figure 9B(ii)). To corroborate our SK2-L experiments, we repeated them using the dual-color fluorescence method and confocal microscopy as described previously. Indeed, we found that SK2-L channels shifted from an intracellular to cell surface pool, with CTF ratio of 0.422 $\pm$ 0.051 (n=17) to 1.395 $\pm$ 0.092

(n=10) after a 30 minute incubation with KT5720 (Figure 10A and 10B). We did not see any changes to this ratio when KT5720 was incubated for less than 10 minutes (Figure 10B). In the vast majority of cells (78.5%), we noticed that treatment of cells with KT5720 for 30 minutes also led to the appearance of large SK channel clusters on the cell surface. This further demonstrates that PKA limits both SK2-L surface expression and clustering.

Discussion

Our study provides insight into the role of cAMP-PKA signaling in the regulation of the spatial distribution of SK channels. Our data demonstrates that tonic PKA activity exerts a powerful regulation of SK channel surface expression, clustering, and subcompartment distribution (soma versus dendrites) in heterologous cells and neurons. We present two main findings: first, we show that basal cAMP-PKA activity dictates the cell surface expression and nanoclustering of SK channels. High PKA levels lead to lower surface expression of SK channels in both HEK293T and primary hippocampal neurons, whereas low PKA activity leads to robust and elevated cell surface expression and clustering. Second, we show that the somatodendritic distribution of apamin-sensitive SK channels is determined by tonic neuronal PKA activity. To our knowledge, this is the first study demonstrating that the topographic distribution of a neuronal Ca^{2+} -activated potassium channel is plastic and under the control of a second messenger cascade. This observation is significant in the context of neuronal excitability, as SK channel activity can have profound effects on controlling neuronal output [23].

The role of cAMP and PKA in regulating the properties of SK channels has been largely unexplored until recently. In 2006, Ren et al. showed that PKA directly phosphorylates SK2

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4 channels at multiple sites, leading to their retention in the endoplasmic reticulum and removal
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6 from the cell surface [6]. Similarly, Lin et al. (2008) demonstrated that elevated cAMP and PKA
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8 activity following the induction of long-term potentiation promotes the internalization of SK2
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10 channels from spines, leading to larger EPSPs [7]. Recent work also showed that intrinsic
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12 plasticity in Purkinje cells following robust activation of parallel fibers also depends on PKA
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14 activity and downregulation of SK2 channels [24, 25]. The present study extends these previous
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16 observations by demonstrating that PKA activity also controls the spatial distribution and
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18 nanoclustering of SK channels. We show that blocking basal PKA activity determines whether
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20 multiple SK channels versus single entities are expressed in nanodomains. Importantly, we found
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22 that the propensity of SK2 channels to cluster following inhibition by PKA is isoform specific,
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24 with SK2-L being more susceptible to PKA-regulated nanoclustering. SK2-L has an extended N-
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26 terminus compared to the more widely studied SK2-S isoform [3]. The extended N-terminus
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28 contains a series of cysteine residues and additional PKA phosphorylation sites. Previous work
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30 suggested that the N-terminus of SK2-L might promote clustering of neighboring SK2 channels
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32 [3], although additional studies are needed to establish that assertion.
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43 One possible explanation for the differential nanoclustering of SK2-L over SK2-S is their
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45 different locations. Unlike SK2-S, SK2-L is preferentially found in dendritic spines, where its
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47 primary function is to limit the amplitude of synaptically evoked EPSPs [5]. SK2-L channels are
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49 gated by voltage-gated calcium channels that become activated during spine depolarization by
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51 glutamate-gated AMPA and NMDA receptors [26]. Recent work has shown that induction of
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53 long-term potentiation also leads to removal of SK2 channels from the cell surface through
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55 endocytosis [7]. This is primarily driven by elevated PKA activity and occurs in parallel with the
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4 insertion of AMPA receptors at synaptic sites [7, 27]. We suggest that, concomitant with SK2
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6 endocytosis, increased PKA activity will also disperse SK2-L nanoclusters, thereby decreasing
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8 their density in the vicinity of the spine calcium nanodomains necessary for their gating. Such an
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10 effect might lead to either a rapid boost of EPSPs, as fewer SK2-L channels might be available
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12 for activation, or an increase in the SK2-L membrane diffusion coefficient, allowing SK2-L to
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14 reach the endocytosis zones quicker. These findings therefore lay a foundation for future studies
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16 aimed at identifying the precise effects of SK2-L nanoclustering in synaptic transmission and
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18 plasticity.
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26 SK2 channels exhibit a distinct somatodendritic distribution in cultured neurons and brain tissue
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28 [4, 11, 12], being primarily located in dendrites with limited expression on somatic membranes.
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30 Consistent with this concept, blocking SK channel activity in dendrites enhances dendritic
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32 excitability and synaptic transmission, but has only minimal effects on spike frequency
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34 adaptation in pyramidal neurons (a measure of somatic excitability) [23, 28] but see [29]. Recent
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36 work using freeze-fracture immuno-EM also demonstrated that less than 10% of the SK2
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38 channels found in the soma of pyramidal neurons are expressed on the cell surface [12]. Instead,
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40 the vast majority of SK2 channels are retained on the rough endoplasmic reticulum [12] (see also
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42 [30] for SK1). Our work suggests that the modest SK channel somatic expression might be due
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44 to elevated PKA activity in the vicinity of membrane SK channels promoting their retrieval from
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46 the surface. Such internalization forms the basis for the distribution of SK channels in pyramidal
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48 neurons. This assertion is supported by our finding that pharmacological block of PKA led to a
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50 large increase in surface expression of SK channels and complete redistribution from primarily
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4 intracellular to predominantly on the surface. Future work is needed to test whether PKA
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11 In summary, our study indicates an expanded role of PKA in controlling SK channel properties
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13 and suggests that ion channel distribution in neurons is highly dynamic and under the control of
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15 second messenger cascades.
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21 **Materials and Methods**

22 **Reagents**

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24 Forskolin, KT5720, Rp-Adenosine 3',5'-cyclic monophosphorothioate triethylammonium salt
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26 (Rp-cAMPS) and propranolol were purchased from Sigma Aldrich (St. Louis, MO). St-Ht31
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28 inhibitor peptide and St-Ht31P control peptide were obtained from Promega (Madison, WI).
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30 Apamin conjugated to biotin was obtained from Alamone Labs (Jerusalem, Israel).
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38 **Primary culture of rat hippocampal neurons**

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40 For confocal experiments, E18 rat hippocampal tissue obtained from embryonic day 18 rats were
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42 prepared as previously described [31]. Briefly, dissected hippocampi were treated with trypsin
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44 and plated on coverslips coated with poly-L-lysine and laminin (Sigma Aldrich, St. Louis, MO)
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46 in primary neuron basal medium (Lonza, Allendale, NJ) supplemented with neuron survival
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48 factor-1 (Lonza, Allendale, NJ), 500μM glutamine (Lonza, Allendale, NJ) and 25μM β-
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50 mercaptoethanol. The cells were grown at 37°C in a humidified incubator with 5% CO₂. The
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52 cells were fed five days after plating and weekly thereafter with media without added β-
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7 For AFM experiments, E18 hippocampal tissue obtained from Brainbits was treated with trypsin
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9 and plated onto poly-D-lysine (Sigma Aldrich, St. Louis, MO)-coated glass bottom petri dishes
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11 (Ted Pella, Redding, CA) in neurobasal media (Thermo Scientific, Waltham, MA) supplemented
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13 with B27 (Thermo Scientific, Waltham, MA), penicillin streptomycin (Thermo Scientific,
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15 Waltham, MA) and glutamax (Thermo Scientific, Waltham, MA). . The cells were grown at
16
17 37°C in a humidified incubator with 5% CO₂.
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23 **Immunocytochemistry and dual-color fluorescence method**

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26 1μM KT5720 or 1μl DMSO carrier was added to the assigned wells for 30 minutes at 37°C in a
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28 humidified CO₂ incubator. 500 pM of apamin-biotin (Alamone Labs, Jerusalem, Israel) was
29
30 added to each well and plates were returned to the incubator for 15 minutes. Following
31
32 incubation, wells were washed with Dulbecco's PBS (dPBS, Thermo Scientific, Waltham, MA)
33
34 and neurons were fixed with 2% Paraformaldehyde/4% Sucrose/PBS for 5 minutes at room
35
36 temperature. After washing with dPBS and blocking with pre-block buffer (20mM phosphate
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38 buffer, pH 7.4, 5% normal goat serum, 450mM NaCl) for 1 hour at 4°C, neurons were incubated
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40 with Streptavidin-Alexa Fluor 488 conjugate (1:500, Thermo Fisher Scientific Cat# S11223,
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42 RRID:AB_2336881) for 2 hours at room temperature in the dark. After washing with dPBS,
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44 neurons were permeabilized with preblock buffer+0.05% Triton X-100 for 1 hour at 4°C.
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53 Neurons were then incubated with Streptavidin-Alexa Fluor 546 conjugate (1:500, Thermo
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55 Fisher Scientific Cat# S-11225, RRID:AB_2532130) and the primary antibody chicken anti-
56
57 MAP2 (1:1000, Abcam Cat# ab5392, RRID:AB_2138153) in preblock buffer+0.05% Triton X-
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4 100 overnight at 4°C. The following day, neurons were washed with preblock buffer+0.05%
5
6 Triton X-100 and incubated with the secondary antibody anti-chicken Alexa Fluor 647 (1:750,
7
8 Thermo Fisher Scientific Cat# A-21449, RRID:AB_2535866) in preblock buffer+0.05% Triton
9
10 X-100 for 1 hour at RT in the dark.
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16 Following incubation and washing with preblock buffer, dPBS and 0.1M NaCO₃, coverslips
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18 were mounted on glass slides using Vectashield with DAPI (Vector laboratories, CA) mounting
19
20 media. For the experiments shown in Figure 1, we used the primary antibody mouse anti-pan-
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22 MAGUK (1:500, UC Davis/NIH NeuroMab Facility Cat# 75-029, RRID:AB_10673115) and
23
24 anti-mouse Alexa Fluor 546 (1:750, Thermo Fisher Scientific Cat# A-11003,
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26 RRID:AB_2534071) as secondary. HEK293T cells were stained similarly to neurons using the
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28 dual color fluorescence method.
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36 **Image acquisition**

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38 All images were taken on a laser-scanning Leica SP8 (Leica Microsystems, Wetzlar,
39
40 Germany) confocal microscope. Z-stacks were taken with a step size of 0.25µm with 11 to 15
41
42 slices for each cell. Imaging conditions for all the paraformaldehyde-fixed cells were as follows:
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44 40X oil and 63X oil immersion lens were used to view the cells; white-light laser excitation was
45
46 set at 25% laser intensity; 405 Diode, DPSS 561, HeNe 633 excitation wavelengths were used to
47
48 excite the fluorophores at 5% laser intensity; the Z-stack images were acquired using sequential
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50 acquisition settings of the different channels to avoid cross-talk and bleed-through between the
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52 different fluorophores; the pinhole size for all the channels was set at 1 airy unit; the raw images
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54 were exported as .tiff files and viewed on Adobe Photoshop and quantified on NIH Image J
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software. For all the acquired images, only brightness and contrast for the entire frame was adjusted on Adobe Photoshop and no singular section of the frame was modified in any way.

Image quantitation

Z-stacks from the same cell were projected using the Z-project function of Image J software. The channels from the processed Z-stacks were then split into individual red, green, blue channels representing 546-streptavidin, 488- streptavidin and 647-anti mouse MAP2, respectively. To quantify the fluorescence intensity of the red (SK2 channels stained after permeabilization) and green (SK2 channels stained before permeabilization) channels, the same region of interest (ROI) was chosen. Both area and XY coordinates were kept the same when comparing fluorescence intensities between the two channels for the ROI. The measurement analysis was set to yield the area, min and max pixel strength, integrated and raw density and mean grayscale value.

The raw values were exported to Excel. The corrected total fluorescence (CTF) was calculated for the chosen ROIs for the soma and dendrites for the different conditions. CTF is the resultant output of subtracting the mean background intensity over the chosen ROI from the integrated density for each ROI. The ratio of CTF of green channel (488 streptavidin) over red channel (546 streptavidin) represents surface/internalized SK2 channels. Cells were chosen at random for each condition to quantify fluorescence in the soma, and sections of MAP2-labeled areas were chosen at random to quantify fluorescence in the dendrites. All analysis was done blind. CTF ratios are reported as mean $\pm$ s.e.m.

To quantify colocalization of SK and MAGUK labeling, ten dendritic segments derived from 4 different images were analyzed. Pixel intensity profiles for each labeled pair on each image were measured with Fiji software. The pixel intensity were imported into Microsoft Excel and Pearson's correlation coefficient values were calculated on a scales from -1.0 (absolute negative colocalization) to 1.0 (absolute colocalization) and are reported as the mean $\pm$ s.e.m.

Transfection of SK2 channels in HEK293T cells

Cultured HEK293T were transfected with recombinant DNA using Lipofectamine 2000 according to manufacturer directions (Thermo Scientific, Waltham, MA). All measurements took place two days following transfection. SK2-S subcloned into a pEGFP-N1 vector (Clontech) was a gift from Chul-Seung Park, PhD (Gwangju Institute of Science and Technology, Gwangju, Korea). SK2-L was a gift from John P. Adelman, PhD (Vollum Institute, OHSU) and SK1 was a gift from Rodrigo Andrade, PhD (Wayne State University).

Preparation of AFM cantilever probes

Acetal-PEG-NHS-apamin functionalization: Silicon nitride probes were functionalized with apamin via acetal-PEG-NHS (Institute of Biophysics, Johannes Kepler University, Linz, Austria) according to manufacturer instructions (<http://www.jku.at/biophysics/content>).

Glutaraldehyde-apamin functionalization: Silicon nitride probes were silanized with 2% v/v 3-aminopropyltriethoxysilane (Sigma Aldrich, St. Louis, MO) in acetone (Sigma Aldrich, St. Louis, MO) for 10 min at room temperature. The probes were then rinsed in deionized water and immersed in 0.5% glutaraldehyde for 30 min, after which they were washed and then incubated

with 100 nM apamin for an additional 30 min. Lastly, the probes were treated with 100 μ g/mL bovine serum albumin in tris-buffered saline to block the remaining aldehyde groups on the cantilever surface.

AFM measurements

Force-distance curves were obtained from Asylum MFP 3D-BIO AFM (Asylum Research, Santa Barbara, CA). HEK293T cell measurements were performed in a solution containing (mM): 2.5 KCl, 144 NaCl, 10 HEPES, 22 D-glucose and 2.2 CaCl₂, pH 7.2. Neuronal experiments were carried out in neurobasal media supplemented with 2% B27, 1% penicillin-streptomycin, 0.25% glutamax, and HEPES to maintain constant pH. All measurements and drug incubations were carried out at 37°C. Cells were identified using differential interference contrast microscopy, and a scan area of 1 μ m*1 μ m with a lateral resolution of 31.25 nm was chosen. All force measurements were carried out at a nominal loading rate of 24,000 pN/s, calculated by multiplying the tip retraction velocity (nm/s) by the cantilever spring constant (pN/nm). The cantilevers used had a nominal spring constant of 30 pN/nm as provided by the manufacturer, the exact values of which are obtained via a thermal noise-based method implemented by the manufacturer. The approach and retraction velocities are held constant at 800 nm/s. Probes had nominal tip radii of 20 nm and nominal angle of 20°, as provided by the manufacturer.

AFM data processing and analysis

The distribution of SK channels on the cell surface was detected by recording spatially resolved unbinding force maps over 1 μ m*1 μ m areas using an apamin functionalized probe. Unbinding forces were extracted from each of the 1024 force curves generated from each test (Figure 3B).

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4 A MATLAB (Mathworks) program developed by the Lykotrafitis lab was then used to create
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6 unbinding force maps where each pixel represents an independent rupture force obtained from
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8 the retraction portion of the force-distance curve. Unbinding forces are reported as mean $\pm$ S.D.
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11 12 13 14 **Statistical Analysis**

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16 Statistical analysis was performed using version 3.0.3 of the R programming language [32]. The
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18 maximum likelihood method was used to estimate the parameters for single univariate Gaussian
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20 distributions or for mixed univariate Gaussian distributions. For the single Gaussian
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22 distributions, we used the function fitdistr() available in MASS package. For the mixed Gaussian
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24 distributions, we used the function fitdistr() available in MASS package. For the mixed Gaussian
25
26 distributions we used the function normalmixEM(), available in “mixtools” package [33] and
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28 based on the expectation-maximization algorithm. The number of mixtures was determined by
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30 employing the function “boot.comp” from the “mixtools” package that uses difference in log-
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32 likelihoods to determine if the increase in the number of Gaussian distributions is significant at
33
34 the predefined level of 1%. Mann Whitney U tests and one-way ANOVA were performed using
35
36 GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA,
37
38 www.graphpad.com).

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41
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54
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56
57 interpretation of data, drafting and revising the article. KA, Acquisition of AFM data and
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analysis, confocal specimen preparation, acquisition of confocal data and analysis of HEK293T cells. MS, confocal specimen preparation, acquisition of confocal microscopy data and analysis of neurons. RSW, Analysis and interpretation of data, revising the article.

Ethics

All experiments were carried out according to the guidelines of the University of Connecticut–Storrs Institutional Animal Care and Use Committee.

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Figure Captions

Figure 1. Apamin labels SK channels in both the soma and dendrites of cultured hippocampal neurons. Dissociated hippocampal neurons at 16-19 DIV were fixed and labeled with apamin-biotin followed by streptavidin conjugated Alexa-488 fluorophore to stain biotin-conjugated apamin (green), and with antibodies against the dendritic marker MAP2 (blue) and the synaptic marker MAGUK (red). Confocal microscopy images showing labeling in a segment of dendrite for (A) apamin and MAP2, (B) apamin and MAGUK, and (C) the overlay of all three labels. Boxed areas are shown enlarged in insets (right panel). (Di,ii) Permeabilized neurons fixed and doubled-labeled with an antibody against the dendritic marker MAP2 (blue) and Alexa-488-conjugated avidin (green) to stain biotin-conjugated apamin. The image in D(ii) is the amplified region of the box shown in D(i).

Figure 2. Surface and internalized SK channel localization in hippocampal neuronal cultures visualized using a dual- fluorescence labeling approach. A) Representative images of hippocampal neuronal cultures at 18 DIV incubated with 500pM apamin conjugated biotin at 37°C, 4°C or at 37°C after preincubation with 1µM PKA inhibitor KT5720 for 30 min. Surface SK (green) panels show surface SK channels labeled with streptavidin conjugated Alexa 488 fluorophore prior to permeabilization, internal SK (red) panels show internalized SK channels labeled with streptavidin conjugated Alexa 546 fluorophore after permeabilization of neurons, MAP2 (blue) shows dendrites labeled with MAP-2 antibody and visualized using Alexa 647 secondary antibody. The dual-fluorescence labeling shows a significant increase in ratio of surface SK channels (green) to internal SK (red) in the 37°C+KT5720 conditions compared to the 37°C condition in both the soma and the dendrites. In the soma, the ratio of surface to

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4 internal labeling of SK channels at 4° C was intermediate between 37°C and 37°C +KT5720. **B**,
5
6 **C**) Bar graphs showing the corrected total fluorescence (CTF) ratios between the 488nm and
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8 546nm channels which represents the ratio of surface to internalized SK channels in the soma
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10 and dendrites respectively. The data are represented as mean $\pm$ s.e.m and are normalized against
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12 the 37°C conditions for both the soma and the dendrites. n=18 for the soma, n=27 for the
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14 dendrites from 2 independent experiments. **, $P<0.01$, *** $P<0.001$, N.S., no significant
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16 difference (one-way ANOVA).
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24 **Figure 3. Apamin unbinding forces depend on SK channel apamin affinity.**

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26 **(A)** (i) Illustration of an AFM probe (black) functionalized with apamin (green) approaching a
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28 cell membrane (blue) expressing SK channels (yellow); (ii) binding of functionalized probe to
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30 SK channel; (iii) retraction of probe from the surface of the cell; (iv) further retraction and
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32 rupture of protein-protein bond between functionalized probe and SK channel. **(B)** Force-
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34 displacement curve. The red trace represents the approach curve whereas the blue trace
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36 represents the retraction curve. i-iv represents AFM phases described in panel A. **(C)** Unbinding
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38 force frequency histograms showing detected SK channels in living HEK293T cells expressing
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40 SK1 (blue), SK1/SK2-S (red) and SK2-S alone (green) using apamin functionalized AFM probe.
41
42 **(D)** Cumulative distributions of unbinding forces from living HEK293T cells expressing SK2-S
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44 probed with non-functionalized AFM tip (dark green), living HEK293T cells expressing SK1
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46 (blue), SK1/SK2-S (red) and only SK2S cells (green) and probed with apamin functionalized
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48 AFM tip. Cyan shows cumulative distribution of unbinding forces from living untransfected
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50 HEK293T cells probed with an apamin functionalized AFM tip.
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Figure 4. Apamin unbinding forces reveal a somatodendritic distribution of SK channels in cultured hippocampal neurons. Unbinding force maps and corresponding histogram showing detected SK channels in living cultured hippocampal neurons 16-19 DIV using apamin functionalized AFM probe. The unbinding force maps represent $1\mu\text{m} \times 1\mu\text{m}$ scan area on the cell surface. For each unbinding force map, 1024 force-distance curves were obtained with a lateral resolution of 31.25nm. Each pixel represents one force-distance curve. Left panels, unbinding force maps showing detected SK channels on the **A(i)** soma, **B(i)** dendrite proximal, and **C(i)** dendrite distal in the presence or absence of bath apamin. Right panel, unbinding force frequency histograms showing the distribution of unbinding forces at the **A(ii)** soma (-bath apamin: $\mu = 13 \pm 4$ pN, surface density = $0.4 \pm 0.05\%$, $n=7$, purple; + bath apamin: surface density = $0.09 \pm 0.01\%$, $n=4$, red), **B(ii)** dendrite proximal (-bath apamin: $\mu = 17 \pm 4$ pN, surface density = $0.8 \pm 0.09\%$, $n=6$, purple; + bath apamin: surface density = $0.7 \pm 0.12\%$, $n=4$, red), and **C(ii)** dendrite distal (-bath apamin: 58% $\mu = 23 \pm 3$ pN and 42% $\mu = 37 \pm 10$ pN, surface density = $3.2 \pm 0.7\%$, $n=9$, purple; + bath apamin: surface density = $0.81 \pm 0.1\%$, $n=6$, red) in either the presence or absence of bath apamin. Scale bars, 250 nm. Color scale as shown. N represents number of cells probed.

Figure 5. PKA activity regulates the somatodendritic distribution and nanoclustering of SK channels in hippocampal neurons. Unbinding force maps and corresponding histogram showing detected SK channels in living cultured hippocampal neurons 16-19 DIV using apamin functionalized AFM probe. The unbinding force maps represent $1\mu\text{m} \times 1\mu\text{m}$ scan area on the cell surface. For each unbinding force map, 1024 force-distance curves were obtained with a lateral resolution of 31.25nm. Each pixel represents one force-distance curve. Left panels, unbinding force maps showing detected SK channels on the **A(i)** soma, **B(i)** dendrite proximal, and **C(i)**

dendrite distal in the presence or absence of PKA inhibitor KT5720 (1 μ M for 30 min). Right panel, unbinding force frequency histograms showing the distribution of unbinding forces at the **A(ii)** soma (-KT5720: μ = 13 $\pm$ 4 pN, surface density= 0.4 $\pm$ 0.05%, n=7, purple; +KT5720: 41% μ =40 $\pm$ 6pN, 19% μ =55 $\pm$ 7pN, 40% μ =69 $\pm$ 16pN, surface density= 14.1 $\pm$ 5.7%, n=5, red), **B(ii)** dendrite proximal (-KT5720: μ = 17 $\pm$ 4 pN, surface density= 0.8 $\pm$ 0.09%, n=6, purple; +KT5720: 71% μ =44 $\pm$ 7pN, 13% μ =65 $\pm$ 5pN, 16% μ =77 $\pm$ 12pN, surface density= 12.2 $\pm$ 6.2%, n=6, red), and **C(ii)** dendrite distal (-KT5720: 58% μ =23 $\pm$ 3pN and 42% μ =37 $\pm$ 10pN, surface density= 3.2 $\pm$ 0.7 %, n=9, purple; +KT5720: 28% μ =35 $\pm$ 5pN, 52% μ =48 $\pm$ 8pN and 20% μ =69 $\pm$ 16pN, surface density= 13.7 $\pm$ 2.1%, n=8, red) in either the presence or absence of KT5720. Scale bars, 250 nm. Color scale as shown. N represents number of cells probed.

Figure 6. Tonic PKA activity regulates SK2-S channel nanoclustering. Unbinding force maps and corresponding histogram showing detected SK2 channels in living HEK293T cells expressing SK2-S using apamin functionalized AFM probe. The unbinding force maps represent 1 μ m*1 μ m scan area on the cell surface. For each unbinding force map, 1024 force-distance curves were obtained with a lateral resolution of 31.25nm. Each pixel represents one force-distance curve. Unbinding force maps (left panel) and histogram (right panel) of **(A)** untreated SK2-S-transfected cells (μ =28 $\pm$ 5pN, surface density = 4.2 $\pm$ 2.2%, n=12) and SK2-S-transfected cells preincubated with **(B)** 30 μ M Forskolin for 15 min (μ =13 $\pm$ 1 pN, surface density = 0.3 $\pm$ 0.2%, n=6), **(C)** 1 μ M KT5720 and 30 μ M Forskolin for 30 min each (74% μ =36 $\pm$ 6 pN and 26% μ =53 $\pm$ 13 pN, surface density = 12.1 $\pm$ 0.37%, n=6) and **(D)** 1 μ M KT5720 for 30 min (64% μ =35 $\pm$ 6pN and 36% μ =55 $\pm$ 15 pN, surface density = 14.1 $\pm$ 2.7 %, n=6). Scale bars, 250 nm. Color scale as shown. N represents number of cells probed.

Figure 7. PKA inhibition leads to increased SK2-S nanoclustering. Schematic of acetal-PEG-NHS-apamin functionalized tip probing **A(i)** low density and **B(i)** high density of channels and glutaraldehyde-apamin functionalized tip probing **A(ii)** low and **B(ii)** high density of channels. Histogram showing unbinding forces detected between SK2 channels in living HEK293T cells expressing SK2-S and apamin functionalized AFM probe. **(C)** SK2-S transfected HEK293T cells probed with acetal-PEG-NHS-apamin functionalized tip ($\mu=25\pm4$ pN, surface density= 3.6%, n=4, blue) and glutaraldehyde-apamin functionalized tip ($\mu=29\pm5$ pN, surface density= 4.4%, n=12, red), **(D)** SK2-S transfected HEK293T cells preincubated with 1 μ M KT5720 for 30 min probed with acetal-PEG-NHS-apamin tip ($\mu=30\pm5.5$ pN, surface density= 8.2%, n=4, blue) and glutaraldehyde-apamin tip (72% $\mu=35\pm6$ pN and 27% $\mu=55\pm15$ pN, surface density = 14.1 $\pm$ 2.7%, n=6, red). N represents number of cells probed.

Figure 8. Tonic cAMP and AKAP activity regulates SK2-S channel nanoclustering. Unbinding force maps and corresponding histogram showing detected SK channels in living HEK293T cells expressing SK2-S using apamin functionalized AFM probe. The unbinding force maps represent 1 μ m*1 μ m scan area on the cell surface. For each unbinding force map, 1024 force-distance curves were obtained with a lateral resolution of 31.25nm. Each pixel represents one force-distance curve. Unbinding force maps (left panel) and histogram (right panel) of cells preincubated with **(A)** 100 μ M Rp-cAMPS for 30 min (77% $\mu=36\pm7$ pN and 23% $\mu=57\pm15$ pN, surface density = 10.7 $\pm$ 2.32 %, n=6), **(B)** 5 μ M St-Ht31 for 2 h (62% $\mu=36\pm7$ pN and 38% $\mu=56\pm14$ pN, surface density = 12.8 $\pm$ 4.0 %, n=6), **(C)** 5 μ M St-Ht31P for 2 h ($\mu=30\pm5$ pN, surface density = 4.3 $\pm$ 0.9 %, P=0.86, ns, Mann Whitney test, n=6), and **(D)** 10 μ M propranolol

for 30 min ($\mu=27\pm4$ pN, surface density = 4.0 ± 0.9 %, $P=0.42$, ns, Mann Whitney test, $n=6$). Scale bars, 250 nm. Color scale as shown. N represents number of cells probed.

Figure 9. Tonic PKA activity regulates SK2-L channel nanoclustering. Histogram showing unbinding forces between detected SK2 channels in living HEK293T cells expressing SK2-L and apamin functionalized AFM probe. Histograms of **A(i)** Untreated HEK293T cells expressing SK2-L (3 μ g) transfected cells (77% $\mu=31 \pm 6$ pN and 23% $\mu=53 \pm 15$ pN, surface density = 13.4 ± 2.2 %, $n=9$), **A(ii)**, SK2-L (3 μ g) transfected cells preincubated with 1 μ M KT5720 for 30 min (90% $\mu=48 \pm 9$ pN and 10% $\mu=76 \pm 7$ pN, surface density = 10.9 ± 2.7 %, $n=5$) and **B(i)**, untreated SK2-L (0.25 μ g) transfected cells ($\mu=28\pm 6$ pN, surface density = 4.4 ± 0.7 %, $n=5$), **B(ii)**, SK2-L (0.25 μ g) transfected cells preincubated with 1 μ M KT5720 for 30 min (44% $\mu=29\pm4$ pN, 49% $\mu=44 \pm 9$ pN and 6% $\mu=73\pm 7$ pN, surface density = 8.8 ± 2.6 %, $n=5$). N represents number of cells probed.

Figure 10. Apamin labeling of HEK293T cells expressing SK2-L channels shows increased surface expression and clustering of SK2-L upon PKA inhibition. HEK293T cells expressing SK2-L were pretreated with 1 μ l DMSO or 1 μ M KT5720 for 5, 10, 15 or 30 minutes and live labeled with 500pM apamin-biotin. Then, the cells were fixed and labeled with streptavidin conjugated Alexa 488 to stain surface SK2-L (green), cells were then permeabilized and labeled with streptavidin conjugated Alexa 546 (red) to visualize internalized SK2-L pool. DAPI was used to stain the nucleus (cyan). Confocal microscopic images of **(A)** (left), SK2-L (middle), SK2-L treated with 1 μ M KT5720 for 15 min and (right), SK2-L treated with 1 μ M KT5720 for 30 min. **(B)** Bar graphs showing total corrected fluorescence ratio between 488nm and 546nm

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4 channels which represents the ratio of surface to internalized SK2-L channels in HEK293T cells
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6 expressing SK2-L (n=17) and HEK293T cells expressing SK2-L pretreated with 1 μ M KT5720
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8 for 5, 10, 15 and 30 min (n=10 for cells pretreated with KT5720 for varying times). ****
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10 ,p<0.0001 , N.S., no significant difference (one-way ANOVA).
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