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cAMP At Perinuclear mAKAPα Signalosomes

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Abstract

The second messenger cyclic adenosine monophosphate (cAMP) is important for the regulation of neuronal structure and function, including neurite extension. A perinuclear cAMP compartment organized by the scaffold protein muscle A-Kinase Anchoring Protein α (mAKAPα/AKAP6α) is sufficient and necessary for axon growth by rat hippocampal neurons *in vitro*. Here, we report that cAMP at mAKAPα signalosomes is regulated by local Ca^{2+} signaling that mediates activity-dependent cAMP elevation within that compartment. Simultaneous Forster resonance energy transfer (FRET) imaging using the PKA activity reporter AKAR4 and intensiometric imaging using the RCaMP1h fluorescent Ca^{2+} sensor revealed that membrane depolarization by KCl selectively induced activation of perinuclear PKA activity. Activity-dependent perinuclear PKA activity was dependent upon expression of the mAKAPα scaffold, while both perinuclear Ca^{2+} elevation and PKA activation were dependent upon voltage-dependent L-type Ca^{2+} channel activity. Importantly, chelation of Ca^{2+} by a nuclear envelope-localized parvalbumin fusion protein inhibited both activity-induced perinuclear PKA activity and axon elongation. Together, this study provides evidence for a model in which a neuronal perinuclear cAMP compartment is locally regulated by activity-dependent Ca^{2+} influx, providing local control for the enhancement of neurite extension.

Significance statement

cAMP-dependent signaling has been implicated as a positive regulator of neurite outgrowth and axon regeneration. However, the mechanisms regulating cAMP signaling relevant to these processes remain largely unknown. Live cell imaging techniques are used to study the regulation by local Ca^{2+} signals of an mAKAPα-associated cAMP compartment at the neuronal nuclear envelope, providing new mechanistic insight into CNS neuronal signaling transduction conferring axon outgrowth.

Introduction

Central nervous system (CNS) neurons responsible for higher order functions fail to survive or regenerate their axons after injury, resulting in permanent disability in common diseases such as stroke, Alzheimer's disease, Parkinson's disease and glaucoma. To combat this disability, strategies are being sought to promote CNS neuron survival and axon regeneration after injury, including the identification of intracellular signaling pathways whose activation might be beneficial in disease. Enhanced cyclic adenosine monophosphate (cAMP) signaling has been shown to potentiate neurotrophic signaling and to promote neuron survival and axon regeneration (Wang et al., 2015; Wild and Dell'Acqua, 2018). cAMP associated with these processes can be activity dependent (Goldberg and Barres, 2000; Goldberg et al., 2002; Corredor et al., 2012), but the mechanisms conferring this regulation remain largely unknown.

Although cAMP is in theory a freely diffusible second messenger present throughout the cell, it is now established that the specific effects of cAMP signaling in response to different stimuli often occur in discrete intracellular compartments organized by scaffold proteins that form multimolecular signaling complexes or "signalosomes" (Wild and Dell'Acqua, 2018). Scaffolds that bind the cAMP effector protein kinase A (PKA) are called "A-kinase anchoring proteins (AKAPs)." Diverse neuronal functions, including synaptic plasticity, neuronal excitability and transduction of sensory information, have been shown to be associated with AKAP-mediated compartmentation (Wild and Dell'Acqua, 2018). Recent studies have implicated muscle A-kinase anchoring protein α (mAKAPα) in pro-survival and pro-growth neurotropic and cAMP signal transduction, including in the extension of neurites by hippocampal and retinal neurons *in vitro* (Wang et al., 2015; Boczek et al., 2019).

Expressed in neurons and striated myocytes, the large 250 kDa mAKAP (AKAP6) scaffold (α-isoform in neurons, β-isoform in myocytes) is localized to the nuclear envelope via binding to the

Klarsicht/ANC-1/Syne-1 homology (KASH) domain, transmembrane protein nesprin-1α (Pare et al., 2005a; Boczek et al., 2019). mAKAP binds >20 different signaling enzymes and gene regulatory proteins, thereby regulating stress-induced gene expression in these excitable cells (Dodge-Kafka et al., 2019). mAKAP was the first AKAP to be shown to be capable of binding an adenylyl cyclase (AC), a phosphodiesterase (PDE) and a cAMP effector, thus having the potential to orchestrate completely compartmentalized cAMP signaling (Dodge et al., 2001; Dodge-Kafka et al., 2005; Kapiloff et al., 2009). By expression of nesprin-1α-localized constitutive active AC and PDE fusion proteins, cAMP at mAKAPα signalosomes has been shown to be sufficient and necessary for neurite extension by e18 rat hippocampal neurons *in vitro* (Boczek et al., 2019). Inhibition of local cAMP signaling by the PDE-nesprin-1α fusion protein both suppressed forskolin-induced PKA activity detected by a nuclear envelope-localized FRET PKA reporter (PN-AKAR4) and blocked activity-dependent neurite extension. In contrast, expression of the AC-nesprin-1α fusion protein that increased cAMP levels at mAKAPα signalosomes promoted neurite outgrowth. In addition, anchoring disruptor peptide-mediated displacement of endogenous type 4D3 PDE from mAKAPα signalosomes similarly elevated perinuclear cAMP levels and potentiated neurite extension. mAKAPα signalosomes have been implicated not only in the regulation of neurite extension, but also pro-survival signaling. PDE displacement enhanced retinal ganglion survival *in vivo* after optic nerve crush, consistent with prior findings that the mAKAPα scaffold is required for the neuroprotective effects of exogenous cAMP after crush injury (Wang et al., 2015; Boczek et al., 2019). Using live cell imaging, we now consider the activity-dependent regulation of cAMP levels at mAKAPα signalosomes in neurons, demonstrating local production of cAMP in that compartment and local regulation of neurite extension.

Materials and Methods

Plasmids and adenovirus: The cerulean-cpVE172 FRET-based PKA activity sensor AKAR4 was a gift from Dr. Jin Zhang, the University of California, San Diego (Depry et al., 2011). To assay PKA activity at the nuclear envelope with spatiotemporal resolution, AKAR4 was expressed in fusion to the N-terminus of nesprin-1α (PN-AKAR4). pCAG cyto-RCaMP1h was a gift from Franck Polleux (Addgene plasmid #105014; RRID:Addgene_105014) (Hirabayashi et al., 2017). To assay Ca^{2+} at the nuclear envelope using "PN-RCaMP1h," pS-RCaMP1h-nesprin-1α was constructed by fusing the RcAMP1h cDNA to the 5' end of a myc-tagged nesprin-1α cDNA. pS-mCherry-Parv-Nesprin and pS-Parv-EGFP-Nesprin plasmids express mCherry- and GFP-tagged cyprinus carpio β-parvalbumin – nesprin-1α fusion proteins, respectively, under the control of the CMV immediate early promoter. pS-mCherry-nesprin and pS-EGFP-nesprin plasmids express nesprin-1α fusion protein controls. New plasmids were constructed by GENEWIZ (South Plainfield, NJ, USA). Additional details and plasmid sequences will be provided upon request.

Hippocampal neuron isolation and culture: All procedures for animal handling were approved by the Institutional Animal Care and Use Committee at [Author University]. Primary hippocampal neurons were isolated from embryonic day 18 (E18) Sprague-Dawley rat embryos of either sex. Briefly, the hippocampal CA1-CA3 region was dissected in PBS medium with 10 mM D-glucose and digested with 0.05% trypsin-EDTA in PBS with 11 mM D-glucose for 30 min at 37°C. The dissociated tissues were centrifuged at 250*g* for 2 min and then triturated with fire polished glass pipet in Hank's balanced salt solution (HBSS) with calcium and magnesium in plating medium (10% v/v horse serum in DMEM). Dissociated neurons were plated on nitric acid-treated 25-mm cover glass coated with poly-L-lysine in plating medium. Four hours after plating, the medium was replaced with maintenance Neurobasal defined medium supplemented with 1% N2, 2% B27 (Invitrogen, Carlsbad, California, USA), 5 mM D-glucose, 1 mM sodium pyruvate. On day 4-5 in culture, 4 μM arabinosyl cytosine was added to inhibit glial proliferation,

and the neurons were plasmid transfected with Lipofectamine 3000 and/or infected with adenovirus.

Live cell FRET imaging: Imaging was performed using an automated, inverted Zeiss Axio Observer 7 Marianas™ Microscope equipped with a X-Cite 120LED Boost White Light LED System and a high-resolution Prime™ Scientific CMOS digital camera. The workstation was controlled by SlideBook imaging and microscope control software (Intelligent Imaging Innovations, Inc.). The filters used were as follows (Semrock): Dichroics - FF459/526/596-Di01 (CFP/YFP/mCherry) and FF409/493/596-Di02 (DAPI/GFP/mCherry); CFP: Exciter - FF02-438/24, Emitter - FF01-482/25; YFP: Exciter - FF01-509/22, Emitter - FF01-544/24; mCherry and RcAMP1h: Exciter - FF01-578/21, Emitter - FF02-641/75; GFP: Exciter - FF01-474/27, Emitter: FF01-525/45. Cells were washed twice before imaging in PBS with 11 mM D-glucose and perfused during imaging with Tyrode solution (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.2 mM Na_2HPO4, 12 mM $NaHCO_3$, 11 mM D-glucose, 25 mM HEPES, 1% BSA) at room temperature (23-25°C) in a perfusion chamber (Warner Instruments). A peristaltic pump (Harvard Apparatus) was used to perfuse the imaging chamber with different drugs in Tyrode solution; delay attributable to perfusion rates was similar across experiments. 100 ms images were acquired every 5-15 s, depending upon the tracing. Baseline images were acquired for 2-5 min, with analysis using Slidebooks software. Net AKAR4 FRET for regions of interest was calculated by subtracting bleedthrough for both the donor and acceptor channels after background subtraction. FRET ratio “R” is defined as net FRET ÷ background-subtracted donor signal, with R_0 being the ratio for time = 0. RCaMP1h intensiometric data are normalized to the intensity (I) at time = 0.

Neurite extension assays: For neurite extension assays, the cells were cultured for 3 days in maintenance Neurobasal defined medium supplemented with 1% N2, 2% B27 (Invitrogen, Carlsbad, California, USA), 1 mM sodium pyruvate. On day 3-4 in culture, 4 μM arabinosyl

cytosine was added to inhibit glial proliferation, and on day 4 the neurons were co-transfected with pmCherry-C1 and Parv-GFP-nesprin or control GFP-nesprin expression plasmids using Lipofectamine LTX with Plus Reagent (ThermoFisher Scientific, *cat:* 15338030). KCl (40 mM) was added to the medium after transfection. Two days later, the neurons were fixed and counterstained with Hoechst (Invitrogen, *cat:* 33342). Images were acquired with a Zeiss 880 confocal microscope by 20x objective tile scan and processed with Fiji Image J. The length of the longest neurite for 20-40 neurons per condition was measured for each experiment using ImageJ with the Simple Neurite Tracer plugin.

Statistical analysis: Statistical analyses were performed using GraphPad Prism. Data are presented as mean ± SEM. Normally distributed datasets by D'Agostino-Pearson omnibus (K2) test were compared by unpaired *t*-tests (for two groups) or one-way ANOVA (for three groups) with subsequent Tukey's post-hoc testing. Other datasets were analyzed by Mann-Whitney *U* test (for two groups) or Kruskal-Wallis *H* test (for three groups), followed by Dunn's post-hoc testing. Repeated symbols are used as follows: single - $p \leq 0.05$; double - $p \leq 0.01$; triple - $p \leq 0.001$.

Results

KCl depolarization induces PKA and Ca^{2+} transients at the nuclear envelope

cAMP-dependent PKA signaling is highly compartmentalized in cells by AKAPs that organize localized signalosomes regulating specific cellular processes (Wild and Dell'Acqua, 2018). Given that perinuclear cAMP signaling has been shown to be required for activity-dependent neurite extension (Boczek et al., 2019), we now considered whether KCl-mediated depolarization can regulate cAMP at mAKAPα signalosomes. Cultured primary e18 rat hippocampal neurons were transfected on day 4-5 in culture with expression plasmids for perinuclear-localized PN-AKAR4 or diffusely localized parent AKAR4 PKA activity FRET biosensors (Figure 1A). At the same time, the neurons were co-transfected with plasmids expressing perinuclear-localized PN-RCaMP1h or diffusely localized parent RCaMP1h intensiometric Ca^{2+} sensors (Figure 1A). The neurons were imaged 36-72 hours after transfection. As AKAR4 emits cyan and yellow light, while RCaMP1h is red, we were able to image simultaneously similarly localized PKA and Ca^{2+} sensors.

Membrane depolarization with 40 mM KCl for 60 sec induced a pronounced increase in perinuclear PKA activity, but had no significant effect on PKA detected with the diffusely localized PKA parent sensor (Figure 1B-D). In addition, whereas 40 mM KCl resulted in robust perinuclear PKA activation, 10 mM KCl did not induce activation of perinuclear PKA, and 30 mM KCl inconsistently resulted in PN-AKAR4 signals (Figure 1E-F). This was in contrast to the similarly robust response by AKAR4 and PN-AKAR4 to the transmembrane adenylyl cyclase activator forskolin previously observed in these neurons (Boczek et al., 2019). KCl induced Ca^{2+} transients in both compartments, notably with less increase in RcAMP1h signal at the nuclear envelope (Figure 1C-D). These results imply KCl depolarization can selectively activate PKA at the nuclear envelope, despite elevating [Ca^{2+}] more generally in the cell.

mAKAPα is required for activity-induced perinuclear PKA signaling

As PKA is recruited to the nesprin-1α perinuclear compartment by the scaffold mAKAPα, it was likely that KCl-induced PN-AKAR4 signal would be due to activation of mAKAPα-bound PKA. Expression of a shRNA that has been used previously to deplete cells of mAKAP (Pare et al., 2005b; Boczek et al., 2019) inhibited KCl-induced perinuclear PKA activity (Figure 2A-B). In contrast, mAKAPα expression was not required for KCl-induced Ca^{2+} transients at the nuclear envelope, such that mAKAP depletion had no effect upon Ca^{2+} transients detected with the PN-RCaMP1h sensor (Figure 2C-D). Together these data are consistent with a model in which mAKAPα is required for the recruitment of PKA to the mAKAPα-nesprin-1α perinuclear compartment, but not for the release of Ca^{2+} into that compartment.

Perinuclear Ca^{2+} dynamics and PKA activity depends on L-type Ca^{2+} channel activity

We investigated which voltage-gated channels might contribute to Ca^{2+} fluxes in the mAKAPα perinuclear compartment. Preincubation of hippocampal neurons with the L-type Ca^{2+} channel blocker nifedipine inhibited the Ca^{2+} transients detected by the parent diffusely localized RCaMP1h sensor 51% in amplitude and that detected by the PN-RCaMP1h sensor 92% in amplitude (Figure 3A,B,E,F). Preincubation with the N-type Ca^{2+} channel blocker conotoxin GVIA inhibited the Ca^{2+} transients detected by the parent diffusely localized RCaMP1h sensor 55% in amplitude and that detected by the PN-RCaMP1h sensor 60% in amplitude. Accordingly, nifedipine, but not conotoxin GVIA, significantly inhibited KCl-dependent PKA transients detected by the PN-AKAR4-1α sensor (Figure 3C,D). Preincubation of the neurons with Ca^{2+} channel blockers did not alter the lack of response of the parent AKAR4 sensor to KCl depolarization (Figure 3G,H). Taken together, these results suggest that Ca^{2+}-dependent PKA activity in the mAKAPα-nesprin-1α compartment is preferentially dependent upon L-type channel activity.

Chelation of Calcium at the nuclear envelope inhibits activity-induced cAMP change.

As KCl-mediated neuronal depolarization activated perinuclear PKA via an L-type Ca^{2+} channel-dependent mechanism, we next considered whether the Ca^{2+} influx promoting PKA activity was local to mAKAPα signalosomes or elsewhere in the cell besides that compartment. Carp parvalbumin-β is a high affinity (K_a = 29 nM) Ca^{2+}-binding protein with 10^4-fold Ca^{2+} selectivity over Mg^{2+} (Wang et al., 2013). To deplete the perinuclear compartment of Ca^{2+}, we expressed a parvalbumin-β-nesprin-1α fusion protein tagged with either GFP or mCherry to allow confirmation of intracellular localization (Figure 4A). Co-expression of Parv-GFP-nesprin reduced ~82% the amplitude of the Ca^{2+} transient induced by depolarization in the perinuclear compartment, but did not affect Ca^{2+} transients detected by the diffusely localized parent RCaMP1h sensor (Figure 4B-E), demonstrating that nesprin-1α localized parvalbumin could only reduce [Ca^{2+}] in that compartment. Importantly, expression of the mCherry-Parv-nesprin fusion protein suppressed 66% KCl-induced PKA activity detected by PN-AKAR4 (Figure 4F-G), demonstrating that elevation of [Ca^{2+}] within the perinuclear compartment is required for full activation of PKA in mAKAPα signalosomes.

Perinuclear Ca^{2+} is required for activity-dependent neurite extension.

Given that elevated perinuclear [Ca^{2+}] was required for activation of mAKAPα-bound PKA, that we previously showed to regulate axon outgrowth (Boczek et al., 2019), we then asked if selective chelation of perinuclear Ca^{2+} would inhibit axon outgrowth. Hippocampal neurons were transfected with plasmids to co-express either Parv-GFP-nesprin or control GFP-nesprin with mCherry, that served as a whole cell marker (Fig 5A). Measurement of the longest neurite showed that in the absence of KCl, axon length was similar for GFP-nesprin and Parv-GFP-nesprin expressing neurons. KCl stimulation for 2 days induced a 15% increase in axon extension for control GFP-nesprin neurons (Fig. 5B,C). In contrast, KCl-stimulation induced no

increase in axon extension for neurons expressing Parv-GFP-nesprin, demonstrating that perinuclear Ca^{2+} signaling is necessary for activity-enhanced neurite extension.

Discussion

Using live cell imaging of primary hippocampal neurons, mAKAPα-bound PKA at the nuclear envelope is shown here to be activated by KCl-mediated depolarization via a mechanism requiring L-type Ca^{2+} channel activity and local increases in [Ca^{2+}], promoting neurite extension (Figure 6). This study extends prior observations regarding mAKAPα signalosomes and neurite extension, including 1) that mAKAPα expression and perinuclear localization is important for neurite extension *in vitro* (Wang et al., 2015; Boczek et al., 2019); 2) that elevated cAMP at mAKAPα signalosomes is sufficient and necessary to induce hippocampal neurite extension *in vitro* (Boczek et al., 2019); and 3) that displacement of the mAKAPα-bound phosphodiesterase PDE4D3 results in elevated perinuclear cAMP levels and increased neurite extension (Boczek et al., 2019). Surprisingly, KCl-mediated membrane depolarization, which induced neurite outgrowth, increased PKA activity detected with the localized PN-AKAR4 sensor, but not the diffusely expressed parent AKAR4 sensor, despite using a strong KCl stimulus. This was in contrast to prior findings that forskolin activated PKA detected with both sensors (Boczek et al., 2019). Further, relatively high levels (40 mM) of KCl was required for perinuclear PKA activation, consistent with previous findings that mAKAPα-dependent perinuclear signaling is linked to signaling in stressed, but not healthy neurons (Wang et al., 2015). Neuronal activity, modeled *in vitro* by KCl-mediated depolarization, is a major determinant of CNS neurite extension and neuronal survival, and, moreover, induces these processes via cAMP/PKA-dependent mechanisms (Lipton, 1986; Shen et al., 1999; Goldberg et al., 2002; Corredor et al., 2012). Given additional prior findings regarding the role of mAKAPα signalosomes in retinal ganglion cell survival (Wang et al., 2015; Boczek et al., 2019), we suggest that the data shown herein

support a model in which the perinuclear, mAKAPα cAMP compartment is a major node in the intracellular signaling network controlling both axon extension and neuroprotection.

Imaging of neurons expressing a nuclear envelope-localized parvalbumin fusion protein suggests that Ca^{2+} influx induced by KCl depolarization must include elevation of Ca^{2+} at the nuclear envelope in order for mAKAPα-bound PKA to be fully activated. In addition, depletion of Ca^{2+} at the nuclear envelope inhibited KCl-stimulated axon elongation, demonstrating the functional consequence of Ca^{2+} signaling within the perinuclear compartment. Notably, activation of L-type Ca^{2+} channels appears critical for this process, consistent with the previously recognized role of these channels in regulating neuronal gene expression (Wild and Dell'Acqua, 2018). L-type Ca^{2+} channels have also been linked to hippocampal survival signaling in response to iron toxicity (Bostanci and Bagirici, 2013) and have been contrasted with NMDA-mediated Ca^{2+} entry and induction of cell death in hippocampal neurons (Stanika et al., 2012), although neither of these studies examined Ca^{2+} or cAMP signaling at the perinuclear region. Together with our data demonstrating dependence on L-type Ca^{2+} channels for perinuclear Ca^{2+} and cAMP signaling, and previous observations identifying the importance of this compartment for neuronal survival and axon growth (Boczek et al., 2019), these examples support a model in which specific Ca^{2+} signaling pathways converge on mAKAPα at the nuclear envelope to support survival and growth signaling. We cannot exclude that L-type Ca^{2+} channels at sites remote from the nuclear envelope regulate mAKAPα-bound PKA, including dendritic channels important for excitation-transcription coupling (Oliveria et al., 2007). However, our data are consistent with a model in which local influx through L-type Ca^{2+} channels that are near the nucleus confer compartment-specific activation. L-type channels are enriched on the soma of hippocampal neurons (Hell et al., 1993). The Dell'Acqua laboratory has elegantly demonstrated that somatic L-type channels induce nuclear factor of activated T-cells type 3 (NFATc3) transcription factor nuclear translocation via activation of the phosphatase calcineurin (Wild et

al., 2019). While NFATc3 translocation in neurons does not appear to be dependent upon ryanodine receptors that confer Ca^{2+}-induced Ca^{2+} release from intracellular stores (Wild et al., 2019), mAKAPβ in striated myocytes has been shown to bind ryanodine receptors, and L-type channels can induce ryanodine receptor opening and release of stored Ca^{2+} (Marx et al., 2000; Kapiloff et al., 2001; Ruehr et al., 2003). Whether ryanodine receptors that have been detected at neuronal nuclear envelope and can regulate nuclear Ca^{2+} participate in elevating perinuclear Ca^{2+} fluxes at mAKAPα signalosomes will be subject of future studies (Walton et al., 1991; Kumar et al., 2008).

Our findings imply that a Ca^{2+}-dependent adenylyl cyclase (AC) is responsible for local synthesis of cAMP in the mAKAPα compartment (Fig. 6). It is formally possible that Ca^{2+}-activates perinuclear cAMP via inhibition of a local cAMP phosphodiesterase, albeit the phosphodiesterase that regulates the mAKAPα compartment PDE4D3 is not known to be inhibited by Ca^{2+} signaling. Instead, the binding of an adenylyl cyclase by mAKAPα could confer this local regulation. AC1, AC3, and AC8 are activated by Ca^{2+}/calmodulin, and AC10 (soluble AC) by Ca^{2+} and bicarbonate (Sadana and Dessauer, 2009). mAKAP has been shown to bind AC5 and AC2, but not AC1 and AC6 when co-expressed in heterologous cells (Kapiloff et al., 2009). Other ACs were not tested for mAKAP binding. mAKAP residues 245-340 binds directly the conserved C1 and C2 catalytic domains of AC5, such that the specificity in mAKAP-AC binding presumably depends upon AC sequences not conserved among isoforms. While activating ACs 1-8, forskolin does not activate AC9 and AC10 (Sadana and Dessauer, 2009). As both forskolin and KCl stimulate PN-AKAR4 in hippocampal neurons (as shown here and in Boczek et al. (Boczek et al., 2019)), one might predict that AC3 or AC8 (but not AC1) is responsible for mAKAPα-associated PKA activity. However, forskolin should broadly activate transmembrane ACs in neurons, potentially resulting in a non-specific, non-physiologic diffuse cAMP activation, including in the mAKAPα compartment. AC10 can promote retinal ganglion

cell neurite extension and survival *in vitro* (Corredor et al., 2012), and thus AC10 could participate in mAKAPα signalosomes. On the other hand, AC1/AC8 double knock-out did not affect retinal ganglion cell axon growth, but did inhibit the forskolin-potentiated survival of these neurons *in vitro* (Corredor et al., 2012). In addition, other adenylyl cyclases are regulated indirectly by Ca^{2+}-dependent protein kinases and phosphatases. Provocatively, while AC1/8 double knock-out reduced KCl-dependent cyclase activity in hippocampal neurons ~60%, KCl could also activate adenylyl cyclase in these cells via activation of calcineurin (Chan et al., 2005). As mAKAPβ binds active calcineurin promoting the dephosphorylation of NFATc and MEF2 transcription factors (Dodge-Kafka et al., 2019), it is possible that KCl and Ca^{2+} activates adenylyl cyclase in the mAKAPα compartment via a calcineurin-dependent pathway. The identification of the adenylyl cyclase(s) critical for perinuclear cAMP-dependent neurite extension and neuroprotection will require future studies involving specific interference with the expression (RNAi) of individual cyclases and PN-AKAR4 imaging. While it remains to be established in neurons, given the prominent role of mAKAPβ signalosomes in the control of stress-regulated cardiac myocyte gene expression (Dodge-Kafka et al., 2019), cAMP-dependent signaling at mAKAPα signalosomes presumably regulates neuronal gene expression controlling neurite extension. Future studies will be directed at the discovery of mechanisms by which activity-dependent cAMP signaling at mAKAPα signalosomes promote hippocampal neuron neurite outgrowth *in vitro*. Additionally future studies should explore whether organization of signaling downstream of physiologic (e.g. synaptic) signaling is involved in other phenotypes including homeostatic regulation of activity. Meanwhile, the identification of a cAMP compartment that can promote axon growth and neuroprotection suggests that further study of this compartment is warranted in terms of both basic mechanism and potential translational relevance.

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433 **Figure Legends**

434 Figure 1**. Depolarization selectively activates PKA signaling at the nuclear envelope. A**,
435 Sensors used in this study. In AKAR4, phosphorylation of the LRRATLVD peptide by PKA
436 results in FHA1 phospho-peptide binding and increased cerulean-cpVenus FRET (Depry et al.,
437 2011). In RCaMP1h, Ca^{2+} induces the binding of the M13 peptide by the mutant calmodulin
438 domain (mCaM), increasing mRuby fluorescence (Hirabayashi et al., 2017). In the perinuclear-
439 localized sensors PN-AKAR4 and PN-RCaMP1h, nesprin-1α contains 5 spectrin repeats (SR)
440 and a transmembrane KASH domain that localizes the protein to the nuclear envelope via
441 binding to SUN domain proteins (Pare et al., 2005a). **B,** Grayscale CFP images of hippocampal
442 neurons expressing AKAR4 or PN-AKAR4. Scale bar, 10 µm. **C-D**, Hippocampal neurons were
443 transfected with PN-AKAR4 and PN-RCaMP1h or AKAR4 and RCaMP1h expression plasmids.
444 Representative traces (smoothed with Prism) and pseudocolor images showing FRET (AKAR4)
445 or intensity (RCaMP1h) responses to 40 mM KCl introduced by perfusion. Images were
446 obtained simultaneously for AKAR4 and RCaMP1h and for PN-AKAR4 and PN-CaMP1h. Here
447 and below a cytosolic region of interest in the soma was measured for the non-localized AKAR4
448 and RcAMP1h sensors. Scale bar, 10 µm. See Figure 3 for quantification of average responses.
449 E, Averaged trace for PN-AKAR4 response to increasing KCl concentration (10 mM, 30 mM and
450 40 mM). Solid line and shaded area indicate mean and s.e.m. respectively. n=9 from 4
451 independent experiments. F, PN-AKAR4 amplitude to different KCl concentrations. Black bars
452 indicate mean values. Datasets were compared by one-way ANOVA and Tukey's post-hoc
453 testing. **$p \leqslant 0.01$, ***$p \leqslant 0.001$.

454 Figure 2. **Activity-induced perinuclear PKA activity is mAKAPα-dependent.** Hippocampal
455 neurons transfected with PN-AKAR4 and PN-RCaMP1h expression plasmids and infected with
456 adenovirus for control or mAKAP shRNA were stimulated with 40 mM KCl. *n* = 15 for both
457 shRNA and include data from 3 experiments using separate hippocampal neuron cultures. **A**,

458 Averaged traces for PN-AKAR4. **B**, Amplitude of PN-AKAR4 traces. **C**, Averaged traces for PN-
459 RCaMP1h. **D**, Amplitude of PN-RCaMP1h traces. Data in A,C are mean ± s.e.m.; black bars in
460 B,D indicate mean values. Datasets were normally distributed and compared by unpaired *t*-tests.
461 ***p $\leqslant$ 0.001.

462 Figure 3. **Perinuclear cAMP is regulated by L-type Ca^{2+} channels**. Hippocampal neurons
463 expressing PN-AKAR4 and PN-CaMP1h or AKAR4 and RCaMP1h were pre-incubated with 10
464 μM nifedipine (Nif, *n* = 19, 18 for parent and PN-sensors, respectively), 0.5 μM conotoxin GVIA
465 (Con, *n* = 15, 36), or no inhibitor control (Ctrl, *n* = 14, 14) before stimulation with 40 mM KCl
466 (bar). **A**, Averaged traces for PN-RCaMP1h. **B,** Amplitude of PN-RCaMP1h traces. **C**, Averaged
467 traces for PN-AKAR4. **D**, Amplitude of PN-AKAR4 traces. **E**, Averaged traces for RCaMP1h. **F,**
468 Amplitude of RCaMP1h traces. **G**, Averaged traces for AKAR4. **H**, Amplitude of AKAR4 traces.
469 Traces show mean ± s.e.m. and are normalized to initial baseline values (R_0 or I_0); black bars in
470 B,D,F,H indicate mean values. Datasets were compared by Kruskal-Wallis and Dunn's post-hoc
471 testing. *p $\leqslant$ 0.05, **p $\leqslant$ 0.01, ***p $\leqslant$ 0.001.

472 Figure 4. **Perinuclear Ca^{2+} is required for cAMP elevation at the nuclear envelope. A**,
473 *Cyprinus carpio* β-parvalbumin – nesprin -1α fusion proteins. Nesprin-1α contains 5 spectrin
474 repeats (SR) and a transmembrane KASH domain that localizes the protein to the nuclear
475 envelope via binding to SUN domain proteins (Pare et al., 2005a). **B,C**, Hippocampal neurons
476 expressing PN-RCaMP1h and either Parv-GFP-nesprin (Parv, *n* = 15) or control GFP-nesprin
477 (Ctrl, *n* = 15) were stimulated with 40 mM KCl. **D,E**, Neurons expressing RCaMP1h and either
478 Parv-GFP-nesprin (Parv, *n* = 26) or control GFP-nesprin (Ctrl, *n* = 23) were stimulated with 40
479 mM KCl. **F,G**, Neurons expressing PN-AKAR4 and either mCherry-Parv-nesprin (Parv, *n* = 26)
480 or control mCherry-nesprin (Ctrl, *n* = 18) were stimulated with 40 mM KCl. Traces show mean ±

s.e.m. and are normalized to initial baseline values (R_0 or I_0); black bars in C,E,F indicate mean values. Datasets compared by Mann-Whitney *U*-test. ***$p \leq 0.001$.

Figure 5. **Perinuclear Ca^{2+} regulates neurite extension. A,** Images of hippocampal neurons expressing mCherry and Parv-GFP-nesprin-1α and stained with Hoechst nuclear stain in representative neurite extension assay. Scale bar, 50 µm. **B,** Grayscale images of mCherry fluorescence for hippocampal neurons expressing mCherry and either GFP-nesprin Parv-GFP-nesprin and cultured for 2 d in defined media +/- 40 mM KCl. Scale bar, 50 µm. **C**, Means of three independent experiments (differently colored symbols) and average mean (bars) for lengths of the longest neurite are shown, *$p \leqslant 0.05$ as determined by matched 2-way ANOVA and Tukey post-hoc testing.

Figure 6. **Model of cAMP signaling regulation in the perinuclear compartment.**

Depolarization of the plasma membrane in hippocampal neurons triggers the opening of L-type Ca^{2+} channels leading to increased perinuclear [Ca^{2+}], and activation of Ca^{2+}-dependent adenylyl cyclase. Perinuclear cAMP binds PKA regulatory (R) subunits, activating PKA catalytic (C) subunits at mAKAPα signalosomes. PKA-phosphorylated effectors that remain to be identified regulate gene expression promoting axon extension. LTCC, L-type Ca^{2+} channels. AC, adenylyl cyclase. PKA, protein kinase A.