Molecular and Cellular Neuroscience 76 (2016) 33–41

Contents lists available at ScienceDirect

Molecular and Cellular Neuroscience

journal homepage: www.elsevier.com/locate/ymcne

Presynaptic CamKII regulates activity-dependent axon terminal growth

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ARTICLE INFO

Article history:
Received 6 February 2016
Revised 12 August 2016
Accepted 22 August 2016
Available online 24 August 2016

Keywords:
CamKII
Activity-dependent axon terminal growth
NMJ
miRNA pathway

ABSTRACT

Spaced synaptic depolarization induces rapid axon terminal growth and the formation of new synaptic boutons at the *Drosophila* larval neuromuscular junction (NMJ). Here, we identify a novel presynaptic function for the Calcium/Calmodulin-dependent Kinase II (CamKII) protein in the control of activity-dependent synaptic growth. Consistent with this function, we find that both total and phosphorylated CamKII (p-CamKII) are enriched in axon terminals. Interestingly, p-CamKII appears to be enriched at the presynaptic axon terminal membrane. Moreover, levels of total CamKII protein within presynaptic boutons globally increase within one hour following stimulation. These effects correlate with the activity-dependent formation of new presynaptic boutons. The increase in presynaptic CamKII levels is inhibited by treatment with cyclohexamide suggesting a protein-synthesis dependent mechanism. We have previously found that acute spaced stimulation rapidly downregulates levels of neuronal microRNAs (miRNAs) that are required for the control of activity-dependent axon terminal growth at this synapse. The rapid activity-dependent accumulation of CamKII protein within axon terminals is inhibited by overexpression of activity-regulated miR-289 in motor neurons. Experiments in vitro using a *CamKII* translational reporter show that miR-289 can directly repress the translation of CamKII via a sequence motif found within the *CamKII* 3′ untranslated region (UTR). Collectively, our studies support the idea that presynaptic CamKII acts downstream of synaptic stimulation and the miRNA pathway to control rapid activity-dependent changes in synapse structure.

1. Introduction

The processes of synaptogenesis and long-term synaptic plasticity involve extensive structural remodeling on both the pre- and postsynaptic side of the synapse. This remodeling is essential for the proper development and function of the nervous system. The axon growth cone is highly dynamic and responds to signals in the surrounding environment directing it to grow towards a target region and ultimately form a synapse on a specific target cell. Importantly, these synapses continue to remodel throughout development and during activity-dependent plasticity. Mechanistically, changes in axon terminals can occur very rapidly (on the order of minutes) and at sites that can be extremely distant (hundreds to thousands of microns) from the cell body. To facilitate these rapid changes, local machinery needs to already be in place within the growth cone and presynaptic boutons. Proteins localized to axon terminals include highly conserved components of signal transduction pathways. These mechanisms have been extensively characterized. It has also been shown that specific mRNAs are packaged into transport ribonucleoprotein particles (RNPs) and actively transported into distal axons. These mRNAs are released from repression and subsequently translated in the axonal compartment in response to a local stimulus (Gumy et al., 2014). Local translation provides a mechanism by which axons can rapidly alter their protein composition without requiring direct communication with the nucleus (Jung et al., 2012).

One of the most important secondary messengers in axon growth and guidance is calcium (Sutherland et al., 2014). Increased intracellular Ca^{2+} levels binds to calmodulin (CaM) resulting in the activation of Ca^{2+}/CaM-dependent enzymes including calcineurin (CaN), protein kinase A (PKA), and the Calcium/Calmodulin-dependent Kinase II (CamKII) (Faas et al., 2011). In the growth cone, activation of CamKII and PKA promotes attraction and dual inhibition switches this attraction to repulsion (Wen et al., 2004). In presynaptic nerve terminals, a major target for phosphorylation by CamKII is synapsin. The reversible association of synapsin with synaptic vesicles helps to facilitate vesicle clustering and presynaptic plasticity and is controlled by phosphorylation at CamKII and PKA phosphorylation sites (Hosaka et al., 1999; Stefani et al., 1997). Recent studies from the Littleton lab (Vasin et al., 2014) have identified a PKA/synapsin-dependent mechanism that is required at the larval *Drosophila* neuromuscular junction (NMJ) to regulate the rapid budding and outgrowth of new presynaptic boutons in response to acute spaced depolarization. While several other signaling mechanisms have been implicated in this process (Ataman et al., 2008; Koon et al., 2011; Korkut et al., 2009; Korkut et al., 2013) little is

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http://dx.doi.org/10.1016/j.mcn.2016.08.007
1044-7431/

known about the role of presynaptic CamKII. Furthermore, even less is known about the upstream mechanisms that are involved in the control of activity-dependent presynaptic bouton outgrowth and, more specifically, precisely how these upstream mechanisms are linked to local presynaptic signaling events (Freeman et al., 2011; Nesler et al., 2013; Pradhan et al., 2012).

In mammals and flies, CamKII expression can be post-transcriptionally regulated at the level of translation. The activity-dependant translation of the *CamKII* mRNA in *Drosophila* olfactory projection neuron (PN) dendrites requires components of the microRNA (miRNA)-containing RNA induced silencing complex (RISC) (Ashraf et al., 2006). Similar results have been observed in mammalian hippocampal neurons (Banerjee et al., 2009). In both cases, this is facilitated via the rapid activity-dependent degradation of the SDE3 helicase Armitage (MOV10 in mammals). Degradation of Armitage/MOV10, and potentially other RISC components, is thought to destabilize the apparatus required for miRNA-mediated mRNA regulation (Ashraf et al., 2006; Banerjee et al., 2009). Consistent with this hypothesis, rapid degradation of miRNAs occurs in mammalian neurons in response to activity (Krol et al., 2010).

Similarly, we have shown that spaced stimulation rapidly downregulates levels of five miRNAs in *Drosophila* larval ventral ganglia (Nesler et al., 2013). We demonstrated that three of these miRNAs (miRs-8, -289, and -958) control rapid presynaptic bouton growth at the larval NMJ. We focus here on CamKII because the fly *CamKII* 3′ untranslated region (UTR) contains two putative binding sites for activity-regulated miR-289 (Ashraf et al., 2006). This suggests that 1) the CamKII protein might be required to control activity-dependent axon terminal growth, and 2) the *CamKII* mRNA may be a downstream target for regulation by neuronal miR-289.

In this study, we show that knockdown of *CamKII* within the presynaptic compartment using transgenic RNAi disrupts activity-dependent presynaptic growth. We demonstrate that phosphorylated CamKII (p-CamKII) is enriched at the presynaptic axon terminal membrane. We also find that spaced stimulation rapidly leads to a global increase in total CamKII protein levels within axon terminals. This increase can be blocked by treatment with either the translational inhibitor cyclohexamide or presynaptic overexpression of miR-289. Together, this suggests a translation-dependent mechanism. Using an in vitro translational reporter fused to the *CamKII* 3′UTR, we show that *CamKII* expression is downregulated by miR-289 via one binding site. Collectively, these data provide support for the idea that CamKII is acting downstream of activity-regulated miRNAs to control rapid activity-dependent presynaptic plasticity.

2. Materials and methods

2.1. Fly strains

All *Drosophila* stocks were cultured at 25 °C on standard Bloomington medium. Stocks were obtained from the following sources: *Canton-S*, w^{1118} *(Iso31)*, *UAS-CamKII*^{Ala}, *UAS-CamKII*$^{R3(WT)}$, *UAS-CamKII*^{T287A}, *UAS-CamKII*^{T287D}, and *C380-Gal4* (Bloomington *Drosophila* Stock Center); *UAS-CamKII*^{v38930} and *UAS-CamKII*^{v47280} long hairpin RNAi lines (Vienna *Drosophila* Resource Center) (Dietzl et al., 2007); *UAS-CamKII:EYFP-CamKII 3'UTR* and *UAS-CamKII:EYFP-NUT 3′UTR* were gifts from S. Kunes (Ashraf et al., 2006); *UAS-mCherry:miR-289 pri-miRNA* was from Barbee lab stocks (Nesler et al., 2013).

2.2. Activity paradigm

The acute spaced synaptic depolarization assay was done exactly as we have previously described (Nesler et al., 2013). Where indicated, cyclohexamide (100 mM) was added to normal HL3 haemolymph-like dissection buffer during the entire rest phase. Following the rest phase, larvae were checked to make sure that they were alive and then processed for NMJ analysis.

2.3. Immunohistochemistry

NMJ dissection, immunostaining, and the quantification of boutons were done as we have previously described (Nesler et al., 2013; Pradhan et al., 2012). We used the following primary antibodies: mouse anti-CamKII 1:2000 (Takamatsu et al., 2003) (Cosmo), rabbit anti-CamKII 1:4000 (Koh et al., 1999), anti-DLG 1:100 (4F3; deposited to the Developmental Studies Hybridoma Bank by C. Goodman), anti-DVGLUT 1:10,000 (Daniels et al., 2004), anti-pT287 CamKII 1:150 (Santa Cruz), and anti-GFP 1:2500 (TP401; Torrey Pines). Secondary antibodies conjugated to Alexa Fluor® 488, 568, and 633 (Molecular Probes) were used at a concentration of 1:500. Antibodies against HRP conjugated to Dylight™ 594 and 647 (Jackson Labs) were used at 1:500 and added with secondary antibodies. All images were acquired on an Olympus FV1000 scanning confocal microscope using either a 60× (N.A. 1.42) or 100× objective (N.A. = 1.4). Unless otherwise indicated, images presented have been combined using FV1000 software from confocal stacks collected at intervals of 0.4 μM.

2.4. Quantitative immunofluorescence and western analysis

For quantitative confocal microscopy of CamKII immunofluorescence, larvae from 0× and 5× high K^+ treatment groups were dissected on the same day and then processed for immunochemistry in the same dish to ensure that antibody staining was consistent. All images were acquired using identical settings on the scanning confocal microscope. Initial settings were established for each paired experiment by thresholding the red and green channels to the brightest NMJs identified in the 5× high K^+ treatment group. Following imaging, the intensity of CamKII fluorescence relative to either HRP or DvGLUT was analyzed using two distinct methods to identify regions of interest (ROIs) using ImageJ v1.45 open source software (NIH). The initial steps in both processes were identical. First, RGB images were split into their corresponding channels and then uniformly zoomed to 150% for analysis. Next, the ROI manager was opened to compile a list of ROIs as defined by either boutons demarked by HRP or DvGLUT positive puncta. For HRP images specifically, ROIs were selected in the HRP channel (red) by using the "freehand selections tool" carefully tracing around each individual bouton and adding them to the ROI list. Alternatively, for DvGLUT puncta, the ROIs were selected using a thresholding approach. First, the image in the DvGLUT channel (red) was auto-thresholded. Next, the DvGLUT puncta were selected and added to the ROI manager for measurement. Then the image was closed and re-opened, colors split again, keeping all of the data points in the ROI manager. This was done so that the red channel was no longer thresholded due to the fact that thresholding the image eliminates the variability in puncta intensity. In both cases, ROIs were superimposed on the CamKII channel (green). Measurements were taken for both the green and red channels using the "mean gray value" in ImageJ. Following quantification for the entire data set, a mean value for green intensity (CamKII) and red intensity (HRP or DvGLUT, depending on the experiment) was calculated for each NMJ. This was done so that the data was not biased in favor or those NMJs containing a greater number of ROIs. Finally, a ratio was calculated (green/red) for each NMJ and recorded. This data was then exported for statistical analysis.

For quantitative western blotting, the CNS (ventral ganglion plus optic lobes minus eye imaginal discs) was dissected from 15 larvae from 0× and 5× high K^+ treatment groups (each). This CNS tissue was homogenized directly in 2× Laemmli sample buffer (BioRad), clarified by centrifugation, and the entire supernatant separated by SDS-PAGE. The following primary antibodies were used: anti-CamKII 1:4000 (Cosmo) and anti-actin 1:1000 (Cell Signaling). Secondary antibodies conjugated to HRP (Cell Signaling Technology) were used at a dilution of 1:1000. Band intensities from scanned images were determined using ImageJ (NIH).