Translocation of CaM kinase II to synaptic sites *in vivo*

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The idea that calcium/calmodulin-dependent protein kinase II (CaMKII) is strategically localized to excitatory synapses to exert its important role in long-term potentiation and other forms of neuronal plasticity[1] is supported by the binding of CaMKII to isolated postsynaptic densities (PSD) in biochemical assays[2] and by the finding in cultured neurons that PSD clusters of green fluorescent protein (GFP)-tagged CaMKII form in response to glutamate application or direct electrical stimulation[3,4]. The observation that CaMKII also forms large clusters in response to ischemic insults[5], however, questions the physiological relevance of such translocations. Here we show that in intact zebrafish, repeated sensory stimulation resulted in reproducible and reversible translocation of GFP-CaMKII to the PSD in an identified interneuron in a sensorimotor circuit.

To study stimulus-induced translocation *in vivo*, we expressed GFP-tagged CaMKII cDNA in intact zebrafish using microinjection of single-cell embryos[6]. We used the zebrafish HuC promoter[7] because it drives high levels of expression in, among other neuronal cell types, a commissural interneuron, the commissural primary ascending (CoPA) cell. CoPA interneurons are morphologically distinct, with long dendrites extending both rostrally and caudally[8,9], which facilitate imaging long stretches of postsynaptic membrane in a single optical section. More importantly, the dendrites and somata of CoPA neurons are contacted by processes of glutamatergic Rohon Beard (RB) cells (**Fig. 1a**), the primary sensory neurons that convey information from the skin to the spinal cord[10]. The morphological contacts probably represent functional synapses for two reasons. First, in frog tadpoles, intracellular recordings with dye injections show that dorsolateral commissural interneurons, which are morphologically and anatomically similar to CoPA cells[8], receive synaptic contacts from RB neurons[11]. Stimulation through this circuit causes a bending away from the skin stimulus in frog tadpoles[12]. Second, when zebrafish CoPA neurons are back-filled *in vivo* with the calcium indicator calcium green dextran (CGD), a brief train of electrical stimuli to the skin results in a fluorescence increase of CGD in the CoPA cell (data not shown), indicating that RB cells can activate CoPA neurons. Thus, in this zebrafish circuit, RB sensory neurons innervating the skin excite CoPA interneurons that, in turn, relay excitation to the contralateral spinal cord via their commissural axons.

Intact fish expressing GFP-CaMKII in CoPA cells were imaged using confocal microscopy after paralysis with injected α-bungarotoxin (Sigma, St. Louis, Missouri). Images of the CoPA dendrite and/or soma were collected at 2–3 s intervals. After 5–10 baseline images were obtained, a brief burst of high-frequency current pulses was applied to the skin in the region of the labeled CoPA neuron. The skin stimulation led to an accumulation of bright, oval, submembranous patches, roughly 1 μm long and 0.3 μm wide, distributed non-uniformly along the CoPA dendrite (**Fig. 1b** and **Supplementary Movie 1** online). Of 84 electrical stimulation experiments performed, 66 resulted in translocation of CaMKII. The fastest appearance of clear GFP-CaMKII patches was captured at 2 s after stimulation (**Fig. 1c**). The actual onset of translocation, however, may have occurred earlier. Movement artifact associated with the stimulation limited our ability to analyze the first and second post-stimulation images. In contrast to the rapid onset of translocation, the clusters decayed over a period of ~3 minutes (**Fig. 1d**), similar to the time frame of recovery observed in *in vitro* studies of GFP-tagged CaMKII translocation[3,4]. Reversible translocation was inducible for up to 15 trials in the same cell (data not shown). Electrical stimulation of embryos co-injected with cDNAs encoding GFP-CaMKII and a red fluorescent protein, DsRed, resulted in submembranous patches of GFP-CaMKII alone, indicating that the redistribution was probably due to the CaMKII moiety (**Fig. 1c** and **Supplementary Movies 2** and **3**).

To confirm that the translocation was mediated by synaptic transmission, rather than by direct stimulation of the CoPA cell,

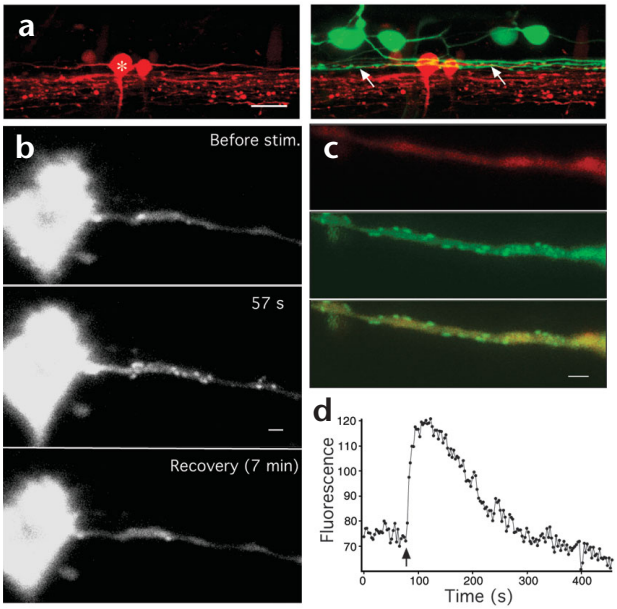

Fig. 1. Skin stimulation causes reversible translocation of CaMKII in CoPA cell dendrites of intact two-day old zebrafish. (**a**) Morphological contacts (arrows, right) between an axon of a RB sensory cell expressing GFP (green, right) and the soma (asterisk, left) and dendrites of a CoPA cell filled with tetramethylrhodamine dextran (Molecular Probes, Eugene, Oregon) (red, left and right). (**b**) Distribution of GFP-CaMKII before, 57 s after and 7 min after stimulation (100 pulses, 0.2 ms each, at 100 Hz and 48 μA). (**c**) GFP-CaMKII (green, middle and bottom) and DsRed (red, top and bottom) after stimulation (60 pulses, 0.2 ms each, at 50 Hz and 32 μA). (**d**) Time course of translocation and recovery of GFP-CaMKII in an individual CoPA cell measured by change in fluorescence intensity (in arbitrary units). Arrow marks time of stimulation. Scale bars, 20 μm (**a**), 2 μm (**b**) and 2 μm (**c**). The Institutional Animal Care and Use Committee of the State University of New York at Stony Brook approved all experiments.

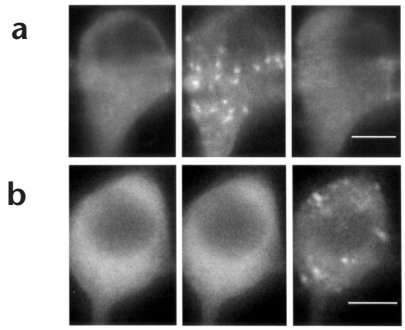

Fig. 2. Translocation of CaMKII *in vivo* is mediated by synaptic transmission. (**a**) Distribution of GFP-CaMKII in a CoPA cell soma before (left) and ~1 min after (middle) application of a 500 μM glutamate solution containing 20 μM glycine. Right, recovery to a uniform distribution ~3.5 min after washout of the glutamate solution. (**b**) GFP-CaMKII remains homogeneous in the presence of 300 μM APV (left) and ~3 min after application of a 500 μM glutamate solution containing 20 μM glycine and 300 μM APV (middle). After washout of APV, exposure of the same cell to 500 μM glutamate results in GFP-CaMKII clusters (shown ~3.5 min after treatment, right). Scale bars (**a** and **b**), 5 μm.

glutamate was applied to the spinal cord in an intact fish by partial removal of the overlying skin. After glutamate exposure, GFP-CaMKII formed transient clusters in the CoPA cell that were similar in size and pattern to those induced by skin stimulation (**Fig. 2a**). Glutamate-induced translocation was blocked in the presence of APV, a specific *N*-methyl-D-aspartate receptor (NMDAR) antagonist (**Fig. 2b**). When APV was removed and the same cell was exposed to glutamate, GFP-CaMKII formed clusters. These results indicate that the observed translocations resulted from activation of the NMDAR, consistent with previous reports that the NMDAR is important for synaptic translocation[3].

Is CaMKII translocation *in vivo* targeted to synaptic sites? To address this question, we co-injected embryos with cDNAs encoding CaMKII fused to yellow fluorescent protein (YFP) and PSD95 fused to cyan fluorescent protein (CFP). PSD95, a prominent component of the PSD, was tagged to mark synaptic sites (**Fig. 3a**). Upon electrical stimulation of the skin, YFP-CaMKII formed clusters that largely overlapped with PSD95-CFP. This colocalization showed that the translocated CaMKII had accumulated at synapses.

We also determined whether the translocation of GFP-CaMKII was reproducible in successive trials by re-stimulating the same site on the skin after recovery from the first stimulus. We counted the number of clusters before and after stimulation over an approximate length of 20–25 μm. Under these conditions, we observed significant overlap of GFP-CaMKII patches after each train of stimuli. Ten of eleven clusters that were observed in the first trial reappeared in the second trial, along with the emergence of two clusters that were not seen previously (**Fig. 3b**). In two other multiple-trial experiments in which minimal movement artifact permitted careful mapping of puncta, 15 of 17 and 12 of 14 clusters that were formed in the first trial returned in the second trial, along with 3 and 6 new clusters, respectively. Although not all clusters reappeared in the second trials, the high percentage of reproducibility suggests that CaMKII was targeted specifically to synapses that were activated through sensory stimulation.

Taken together, our results indicate that translocation of a kinase strongly implicated in synaptic plasticity[1] can be tracked reproducibly in an intact vertebrate responding to a physiological stimulus. The amenability of zebrafish to imaging the movement of tagged molecules should permit the monitoring of active synapses underlying plasticity *in vivo*.

Note: Supplementary information is available on the Nature Neuroscience website.

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Competing interests statement

The authors declare that they have no competing financial interests.

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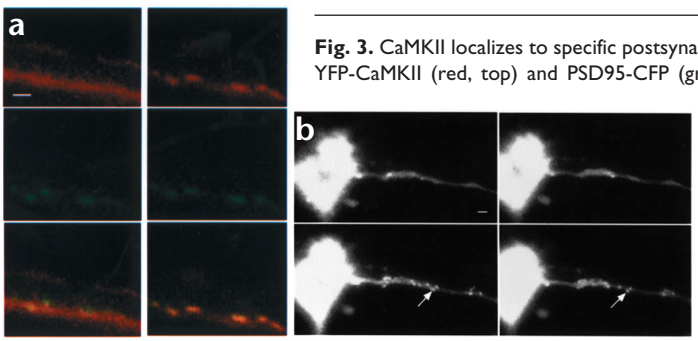

Fig. 3. CaMKII localizes to specific postsynaptic sites. (**a**) Left, pre-stimulation images of a dendrite labeled with YFP-CaMKII (red, top) and PSD95-CFP (green, middle), with overlay (bottom). Right, after stimulation (30 pulses, 0.2 ms each, at 100 Hz and 30 μA), YFP-CaMKII forms clusters (top) that coincide (bottom) with sites marked by PSD95-CFP (middle). (**b**) Re-translocation of GFP-CaMKII to largely the same dendritic sites in response to successive stimuli (100 pulses, 0.2 ms each, at 100 Hz and 48 μA). Top panels show pre-stimulation images of each trial. Bottom panels show images acquired 57 s after stimulation in trial 1 (left) and 24 s after stimulation in trial 2 (right). The period between stimuli in the two trials was ~5 min. Arrows indicate examples of a reproduced cluster of fluorescence. Scale bars, 1 μm (**a**) and 2 μm (**b**).