

Short communication

p600 STABILIZES MICROTUBULES TO PREVENT THE AGGREGATION OF CaMKII α DURING PHOTOCONDUCTIVE STIMULATION

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Abstract: The large microtubule-associated/Ca²⁺-signalling protein p600 (also known as UBR4) is required for hippocampal neuronal survival upon Ca²⁺ dyshomeostasis induced by glutamate treatment. During this process, p600 prevents aggregation of the Ca²⁺/calmodulin-dependent kinase II α (CaMKII α), a proxy of neuronal death, via direct binding to calmodulin in a microtubule-independent manner. Using photoconductive stimulation coupled with live imaging of single neurons, we identified a distinct mechanism of prevention of CaMKII α aggregation by p600. Upon direct depolarization, CaMKII α translocates to microtubules. In the absence of p600, this translocation is interrupted in favour of a sustained self-aggregation that is prevented by the microtubule-stabilizing drug paclitaxel. Thus, during photoconductive stimulation, p600 prevents the aggregation of CaMKII α by stabilizing microtubules. The effectiveness of this stabilization for preventing CaMKII α aggregation during direct depolarization but not during glutamate treatment suggests a model wherein p600 has two modes of action depending on the source of cytosolic Ca²⁺.

Keywords: P600, UBR4, Ca²⁺, Ca²⁺/calmodulin-dependent kinase II α , Microtubules, Paclitaxel

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Abbreviations used: CaM – calmodulin, CaMKII – Ca²⁺/calmodulin-dependent kinase II, GFP – green fluorescent protein, NMDA – N-methyl-D-aspartate, AMPA – α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, DMSO – dimethyl sulfoxide

INTRODUCTION

Ca²⁺/calmodulin-dependent kinase II (CaMKII) accounts for nearly 1% of the total protein in the mammalian brain [1]. By virtue of its toroidal arrangement, its subunits are able to phosphorylate one another, entering into a sustained period of kinase activity. This molecular hysteresis and accompanying signalling activity has led to the hypothesis that CaMKII is a medium of Ca²⁺-dependent memory [2]. The localization and activity of CaMKII is tightly linked to Ca²⁺-mediated neuronal survival. Under ischemic conditions *in vivo*, CaMKII rapidly enters a particulate state wherein it loses kinase activity for a prolonged period [3–5]. Experimental reproductions of the lowered pH, uncoupled oxidative phosphorylation, and Ca²⁺ overload characteristic of the ischemic brain induce the α isoform of CaMKII (CaMKII α) to form similar long-lasting self-aggregates [6–10]. While these various reports of pathological inactivation and self-aggregation are likely to be observations of the same phenomenon, this is still uncertain, as is the role of this pathological behaviour. Furthermore, not only does CaMKII respond to Ca²⁺ influx, its activation state also controls it: CaMKII inhibition causes sufficient glutamate-induced Ca²⁺ entry to cause neuronal death under ambient culture activity [11]. Thus, studies of CaMKII, with its extraordinarily high neuronal expression, its role in synaptic plasticity and memory, and its direct regulation of neuronal survival, are of paramount importance as proxies of neuronal well-being and outcome.

p600 (or UBR4) is a large multifunctional protein (600 kDa) that plays an important role in neurite outgrowth, neuronal migration, protein degradation, Ca²⁺ signalling, cell adhesion, cell survival, and autophagy [12–16]. p600 is enriched in the brain, particularly in neurons, and has at least two microtubule-stabilizing domains [12].

We recently reported that depletion of p600 by RNAi significantly increases the proportion of neurons showing CaMKII α aggregation upon glutamate-induced Ca²⁺ entry in hippocampal cultured neurons [17]. p600 was found to form a complex with CaM and CaMKII α , mediated by a direct and atypical interaction between p600 and CaM. Specific disruption of this interaction resulted in neuronal death under ambient activity, and potentiated CaMKII α aggregation following the application of mild doses of exogenous glutamate. Using an array of inhibitors, it was determined that the primary contributors to CaMKII α aggregation were Ca²⁺ influx through N-methyl-D-aspartate receptors (NMDA) and metabotropic release of Ca²⁺ through inositol 1,4,5-trisphosphate receptors.

Importantly, pre-incubation with the CaMKII inhibitors KN62 or KN93 failed to prevent the aggregation of CaMKII α , demonstrating that the aggregation was CaM-independent and distinct from the translocation to synapses [18]. Furthermore, stabilization of microtubules with paclitaxel had no effect, demonstrating that p600 did not prevent CaMKII α aggregation via its microtubule-stabilizing function. Intriguingly, inhibition of voltage-gated L-type Ca²⁺ channels modestly rescued the aggregation of CaMKII α . This suggested

that p600 could modulate CaMKII α aggregation upon direct depolarization. To further investigate this finding, we analyzed the spatio-temporal dynamics of CaMKII α aggregation induced by photoconductive stimulation, a technology that allows for the direct depolarization of single neurons coupled with live imaging.

MATERIALS AND METHODS

Cell culture, transfection, and photoconductive stimulation

Dissociated cultures of rat primary hippocampal neurons were prepared on silicon wafers for photoconductive stimulation as described previously [19, 20]. Neurons were co-transfected with GFP-CaMKII α and either control or p600 RNAi vectors (molar ratio 1:2) at DIV10 [17]. The day following transfection, the silicon wafers were mounted in a specialized photoconductive stimulation apparatus [20] and immersed in a pre-warmed bath solution consisting of 135 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 3 mM CaCl₂, 10 mM d-glucose, and 10 mM HEPES (pH 7.3). For trials with paclitaxel, neurons were pre-incubated for 15 min with 100 nM paclitaxel (Sigma) or a DMSO vehicle (1:10,000). Individual neurons that expressed GFP-CaMKII α were selected. Under constant light intensity, the neurons were depolarized with a 4 V square wave at 100 Hz, 5 ms pulse duration. High-resolution images were captured at ~1.4 frames per second, using a 100x water-immersion objective on an Olympus BX61WI microscope and a Watec N102 integrating CCD camera.

Quantification, curve fitting and statistical analysis

Neurons were depolarized and 5 min later categorized as displaying GFP-CaMKII α aggregation or not (Fig. 1). Neurons that did not show GFP-CaMKII α aggregation after the initial depolarization were depolarized for 30 s to validate the experimental setup, and those that did not show GFP-CaMKII α aggregation after that were not used for curve fitting or statistical analysis. The data presented in Fig. 1 are fit to a Boltzmann distribution. For accurate statistical analysis, the data were treated as discrete. The mean and standard error were determined by iteratively fitting a cumulative mass function to the data. The results were used to compare the datasets using Student's t test.

Quantification of single-cell aggregation

Using ImageJ, the degree of aggregation of GFP-CaMKII α was determined in each frame of the video by taking intensity thresholds. The automatic particle analyzing function was then used to count the average number of particles. Thirty eight different intensity thresholds were used, covering the range of intensities over which the image of the soma was not saturated. The results are plotted in the graph in Fig. 2C.

Dendrite analysis

In Fig. 2D, the images are brightness and contrast adjusted to clearly show the localization of the aggregates. The trace in the final panel was created using the

threshold-adjusting function of ImageJ, wherein the threshold was adjusted to show the initial synaptic distribution of GFP-CaMKII α . The outline was then automatically traced and overlaid on the final frame for comparison.

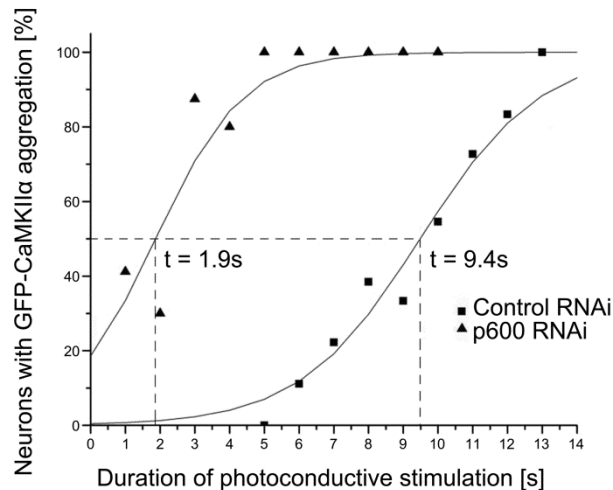


Fig. 1. Depletion of p600 reduces the length of depolarization required to cause neurons to enter a state wherein GFP-CaMKII α is aggregated. Neurons were depolarized for the indicated time, allowed 5 min to respond, and scored for the presence or absence of GFP-CaMKII α aggregation. When fit to a Boltzmann distribution and compared against one another, the data show that depletion of p600 by RNAi causes a mean 80% reduction in the length of depolarization required to cause GFP-CaMKII α to aggregate (control RNAi, $n = 78$ neurons; p600 RNAi, $n = 50$ neurons; $t_3 = 17.7$, $p < 0.001$ by Student's t test).

RESULTS

Depletion of p600 lowers the threshold of GFP-CaMKII α aggregation following photoconductive stimulation

Our previous findings showed that inhibiting L-type Ca $^{2+}$ channels had a significant effect in preventing CaMKII α aggregation following the bath application of glutamate and glycine [17]. Based on that, we assessed if direct depolarization of single neurons could cause CaMKII α aggregation. We constructed a dose-response curve wherein single neurons were depolarized by photoconductive stimulation [19, 20] for a given time, incubated for an additional 5 min, then scored for the presence or absence of GFP-CaMKII α aggregates.

When transfected with a control RNAi, neurons formed aggregates after an average of 9.49 ± 0.24 s (\pm SEM, $n = 78$) of depolarization. We then assessed if knockdown of p600 would potentiate this effect. When p600 is knocked down by RNAi, neurons form aggregates after an average of only 1.77 ± 0.36 s ($n = 50$), resulting in a left-shifted dose-response curve ($t_3 = 17.7$, $p < 0.001$ by Student's t test). Thus, the absence of p600 predisposes neurons to undergo CaMKII α aggregation after direct depolarization.

Depletion of p60 interrupts the cytoskeletal localization of GFP-CaMKII α with irreversible aggregation

A recent study showed that during KCl depolarization, CaMKII α undergoes a rapid and sustained translocation to microtubules [21]. Since the microtubule translocation and self-aggregation of CaMKII α can both be caused by depolarization, but are mutually exclusive behaviours, we sought to compare the time-course and localization of GFP-CaMKII α during neuronal depolarization. We co-transfected single neurons with GFP-CaMKII α and control or p60 RNAi, depolarized them at 0 s (for 5 s), and filmed for 5 min.

We live-imaged 9 control RNAi-transfected neurons and 11 p60 RNAi-transfected neurons. Fig. 2 shows representative examples. Fig. 2A and Suppl. movies 1 and 2 in Supplementary material at <http://dx.doi.org/10.2478/s11658-014-0201-9> depict a neuron co-transfected with control RNAi wherein GFP-CaMKII α is at first relatively diffuse, but within 1 min, it translocates to the microtubules. This initial translocation is largely sustained for the remainder of the experiment.

By contrast, a neuron transfected with p60 RNAi (Fig. 2B, Suppl. movies 3 and 4) shows the same translocation of GFP-CaMKII α to the microtubules, but this process is ostensibly less pronounced, and is interrupted by a sustained aggregation of GFP-CaMKII α . The degree and time-course of aggregation of these representative neurons is quantified and plotted below. Figs 1 and 2 together show that there is a duration of depolarization after which the microtubule translocation of GFP-CaMKII α is interrupted by self-aggregation, and that p60 sets the threshold at which the former behaviour transitions to the latter.

Stabilization of microtubules rescues the aggregation of GFP-CaMKII α after direct depolarization

p60 directly binds and stabilizes microtubules [12]. We hypothesized that neurons in which p60 is depleted might have a less stable population of microtubules, making the translocation of GFP-CaMKII α to these microtubules less probable. By replacing the microtubule-stabilizing function of p60 with 100 nM paclitaxel, we were able to significantly decrease the proportion of neurons showing GFP-CaMKII α aggregation. At 5 s of depolarization, only 37.5% of neurons with p60 knockdown showed aggregation (6/16 with 100 nM paclitaxel, 37/37 with the vehicle control 0.01% DMSO; $p = 0.002$ according to the two-tailed Fisher's exact test). We conclude that during direct depolarization, p60 prevents GFP-CaMKII α aggregation by stabilizing microtubules.

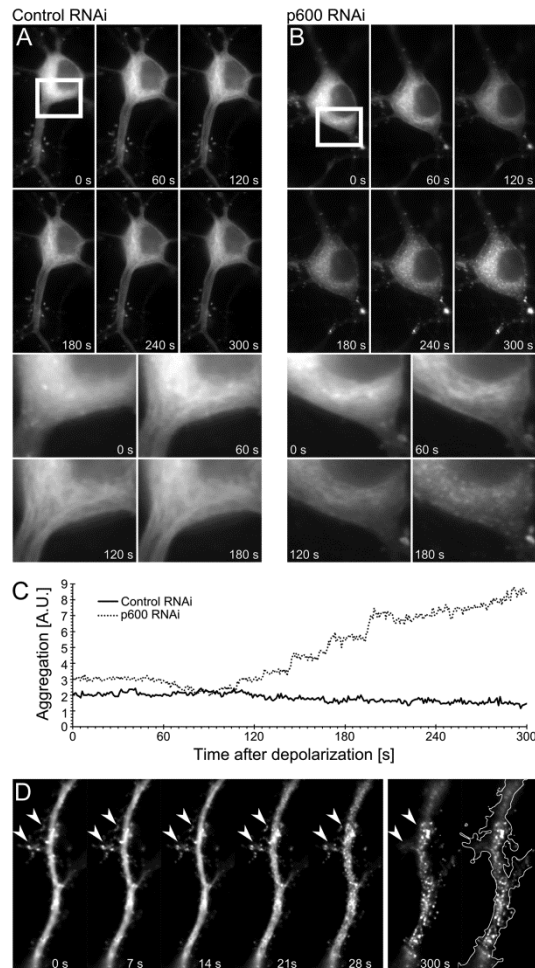


Fig. 2. In the absence of p600, the cytoskeletal localization of GFP-CaMKII α is interrupted by a long-lasting self-aggregation. These are representative images. A and B – In a neuron transfected with control RNAi, upon depolarization, GFP-CaMKII α undergoes a rapid and sustained translocation to the microtubules. When p600 is depleted, the cytoskeletal translocation of GFP-CaMKII α is interrupted by a sustained aggregation. C – Frame-by-frame quantification of these movies shows that the aggregation of GFP-CaMKII α takes place between 1 and 5 min after depolarization. D – Imaging of a single primary dendrite shows that the aggregation of GFP-CaMKII α takes place primarily in the dendritic shaft.

GFP-CaMKII α aggregation occurs primarily in the dendritic shaft

Lastly, we sought to compare the spatial dynamics of the CaMKII α aggregation seen here to the synaptic translocation of CaMKII α [18]. We used our live-imaging model to take high-resolution footage of the primary dendrite during depolarization. Fig. 2D and Suppl. movie 5 show that during depolarization, the aggregation of GFP-CaMKII α takes place primarily in the dendritic shaft.

The overlay (on the final panel, in white) of the initial distribution of GFP-CaMKII α over the final aggregated distribution illustrates that the aggregation involves a net (though incomplete) evacuation of dendritic protrusions. While we cannot conclude that the aggregation takes place exclusively outside of the synapses, we can reasonably claim that the aggregation of GFP-CaMKII α seen in this study is not a synaptic phenomenon.

DISCUSSION

These data suggest a model wherein CaMKII α translocates to microtubules during induced depolarization, and with a longer depolarization it begins to self-aggregate. In this model, p600 stabilizes microtubules in order to set the threshold at which CaMKII α switches from microtubule translocation to self-aggregation. The notion that p600 regulates the localization of CaMKII α by stabilizing microtubules is novel, and complements our previous study showing that p600 prevents CaMKII aggregation through binding to CaM in a microtubule-independent manner [17]. The effectiveness of microtubule stabilization for preventing CaMKII α aggregation during direct depolarization, but not during glutamate treatment, led to a model wherein p600 has two modes of action depending on which channels are activated.

Based on the conservative assumption that the aggregation we have observed here is driven by Ca²⁺, as it has been in every experimental paradigm so far, there are two possible mechanisms by which the microtubule-associated function of p600 could prevent CaMKII α aggregation. The first general model is that the concentration of cytoplasmic Ca²⁺ is unaffected by the actions of p600, and that the response of CaMKII to the same stimulus is altered. Based on the interaction between p600 and CaMKII α and the ubiquitous intracellular expression of both [17], it is possible that during voltage-dependent Ca²⁺ influx, CaMKII α would normally target to p600 or to the stable microtubules it sustains. Depletion of p600 could remove these docking sites and thereby favour the self-aggregation of CaMKII α .

The second possibility is that p600 regulates Ca²⁺ influx. Furukawa and Mattson [22] demonstrated that the stabilization of microtubules by paclitaxel can in fact restrict excitotoxic Ca²⁺ entry, and that microtubule destabilization causes neuronal death. Thus in addition to more well-known mechanisms by which cytosolic Ca²⁺ affects microtubule stability [24, 23], microtubule stability in turn affects the levels of cytosolic Ca²⁺. By analogy to that study, p600 may stabilize microtubules during direct depolarization, restricting Ca²⁺ entry and preventing CaMKII α aggregation. Though Furukawa and Mattson did not find that Ca²⁺ entry by KCl depolarization was mitigated by paclitaxel pre-treatment, they did find that the effect of microtubule stabilization on Ca²⁺ entry was channel-specific. This precedent of channel-specificity could offer means to reconcile the effectiveness of microtubule stabilization in direct depolarization that was not found after glutamate/glycine treatment.

The finding that p600 acts differently to prevent CaMKII α aggregation when faced with different sources of Ca²⁺ strongly suggests a heterogeneous subcellular response by p600. Efforts to distinguish between these two general models will begin with detailed measurements of local cytosolic Ca²⁺ concentrations, and will require minute observations of microtubule dynamics when p600 is depleted.

It is noteworthy that the aggregation of CaMKII α is distinct from its translocation to synapses [18]. In this report, the aggregation takes place both in the soma and the dendrites (Fig. 2B and D), as opposed to the purely synaptic targeting seen in the former case. Interestingly, the endogenous CaMKII inhibitor, CaMKII-N, resides exclusively in the dendritic shaft and soma [25]. Apart from the movement away from the synapses and into the dendritic shaft, there is a case for the inactivation of CaMKII. Firstly, the *in vitro* and *in vivo* inactivation of CaMKII follows a similar time-course with a completion time of about 5 min, which has been closely correlated to the aggregation and movement to the particulate fraction [4, 6, 26]. This time-course of inactivation and aggregation is strikingly similar to the aggregation quantified in Fig. 2C. Secondly, our previous work has shown that the depletion of p600 or disruption of the p600/CaM interaction not only causes CaMKII α aggregation, but also causes neuronal death [17]. Since CaMKII tends to both inactivate and aggregate in excitotoxic conditions [4], the ultimately fatal outcome of p600 depletion suggests that the aggregation of CaMKII α seen in this study also involves an inactivation.

Though the purpose of the CaMKII α aggregates remains unknown, entry into this state can reasonably be supposed to preclude other physiological behaviours of CaMKII α . Even allowing for the slight possibility that CaMKII α in its aggregated form is able to interact with its usual binding partners, we can assume that these interactions would be greatly diminished on account of its sequestration in aggregates. For example, CaMKII directly phosphorylates the GluR1 subunit of the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor in order to potentiate synaptic strength [28, 27]. The implied inactivation of its kinase activity, the reduced mobility/larger size of the aggregates, and the net synaptic evacuation we observed (Fig. 2D) combine to make it unlikely that CaMKII α aggregates are able to phosphorylate the AMPA receptor in any significant way. Similarly, aggregated CaMKII α is unlikely able to maintain its usual role in binding and modulating the properties of NMDA receptors and L-type channels [29, 30]. The combined evidence strongly suggests that in its aggregated form, CaMKII α is unable to continue in its role as a modulator of Ca²⁺-signalling and synaptic plasticity. As a direct regulator of CaMKII α aggregation, p600 is thereby a hypothetical regulator of these processes. The consequence of interrupting the microtubule binding of CaMKII α is more difficult to predict since it is not fully clear what function(s) the CaMKII α /microtubule interaction serves. CaMKII, predominantly the α -isoform, associates with microtubule preparations, though it is not clear to what element

of microtubules it binds [31, 32]. However, it has been shown that CaMKII α directly phosphorylates α -tubulin near the C-terminus [31, 32], thereby favouring the depolymerisation of microtubules [32]. This association and phosphorylation has even been proposed to be a means of directly encoding information on microtubules [33]. The only study of the functional consequences of the CaMKII α /microtubule interaction [21] offered strong evidence that an interaction between CaMKII α and dendritic microtubules increases the surface expression of AMPA receptors and spine remodelling. This study also showed that this translocation of CaMKII α requires stable microtubules [21]. Although the intermediate mechanism is speculative, their result supports the model wherein activated CaMKII α translocates to microtubules to ultimately potentiate synaptic strength. Therefore, p600 could be a regulator of synaptic strength by regulating the transition between the microtubules translocation and aggregation of CaMKII α .

While CaMKII α aggregation can be experimentally induced by all manner of pharmacological insults, the depletion of p600 or the mere disruption of the p600/calmodulin interaction [17] remains the only known means of inducing a neuron to enter this state by its own activity. p600 regulates the localization of CaMKII α , but it is also required for neuronal survival [17].

While a general understanding of the role of CaMKII α aggregation in neurons remains elusive, it is a topic that warrants further study both because of the importance of the enzyme itself, and as a proxy of neuronal death. This study also offers the first indication that the self-aggregation CaMKII α is dependent on microtubule stability, and it introduces p600 as the mediator of this effect. Since Ca²⁺ overload, microtubule instability and p600 depletion have all been independently shown to cause neuronal death [17, 34, 35], this study puts us closer to a unified model wherein p600 is an interface between microtubule dynamics, Ca²⁺ signalling, and neuronal survival.

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