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Neuronal activity and the expression of clathrin-assembly protein AP180

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ABSTRACT

The clathrin-assembly protein AP180 is known to promote the assembly of clathrin-coated vesicles in the neuron. However, it is unknown whether the expression of AP180 is influenced by neuronal activity. In this study, we report that chronic depolarization results in a reduction of AP180 from hippocampal neurons, while acute depolarization causes a dispersed synaptic distribution of AP180. Activity-induced effects are observed only for AP180, but not for the structurally-related clathrin-assembly proteins CALM, epsin1, or HIP1. These findings suggest that AP180 levels and synaptic distribution are highly sensitive to neuronal activity.

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1. Introduction

Clathrin-coated vesicles are highly versatile trafficking organelles fulfilling many functions such as internalizing extracellular molecules, transducing intracellular signaling, and recycling synaptic vesicle components at the synapse. As a consequence, the clathrin-coated vesicle is equipped with extensive and well-coordinated regulatory proteins. Some of these regulatory proteins direct the construction of clathrin-coated vesicles, whereas others control the functions of the vesicles.

AP180 is one such regulatory protein [1–3]. Biochemical studies have shown that AP180 promotes the assembly of clathrin-coated vesicles as well as restraining their small uniform sizes [4,5]. Functional studies carried out in invertebrates have demonstrated that disruption of AP180 causes malformed synaptic vesicles and dysfunctional synaptic release [6–8]. However, neither the regulation nor the functional role of AP180 in vertebrate or mammalian neurons is well understood.

In previous studies, we found that AP180 is highly enriched in the presynaptic terminals of rat hippocampal neurons [9,10]. To understand the functional significance of AP180 in hippocampal neurons, it is necessary to know not only how AP180 acts in synaptic release, but also whether neuronal activity regulates AP180. In this study, we examined AP180 protein expression in chronically or acutely depolarized neurons. We also compared the expression of AP180 in these neurons to the expression of several other clathrin regulatory proteins that have the structure organization similar to AP180, including CALM (clathrin assembly lymphoid myeloid protein) [11,12], epsin1 [13,14], and HIP1 (huntingtin-interacting protein 1) [15,16]. We have found that neuronal activity specifically affects AP180 expression and synaptic localization.

2. Materials and methods

2.1. Animals and cell culture

All animal procedures were approved by the National Institute on Aging Animal Care and Use Committee and compiled with the NIH Guide for Care and Use of Laboratory Animals. Timed pregnant female Sprague–Dawley rats were used as the source of hippocampal cells (embryonic day 17–18). Cultures of hippocampal neurons were prepared as described previously [3,17]. Dissociated neurons were grown in Neurobasal medium containing B27 (Invitrogen).

2.2. Reagents

The following antibodies were used: mouse monoclonal anti-AP180 (clone AP180-I; Sigma) [4,9,10,18]; goat polyclonal anti-CALM (sc6433; Santa Cruz Biotechnology) [19]; rabbit polyclonal anti-epsin1 (a gift from Dr. Linton M. Traub) [20,21]; rabbit polyclonal anti-HIP1 (also a gift from Dr. Linton M. Traub) [16,21]; mouse monoclonal anti-synaptophysin (Sigma); and rabbit polyclonal anti-synapsin I (Chemicon International). Tetrodotoxin and bicuculline were from Sigma.

2.3. Immunoblots

Cell lysates were prepared as described [22]. Immunobotting was performed using standard procedures. The following dilutions

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of the antibodies were used: AP180, 1:5000; CALM, 1:500; epsin1, 1:500; HIP1, 1:500; synaptophysin, 1:5000.

2.4. Immunolabeling and image analysis

Immunofluorescence labeling was carried out as previously described [3].The AP180 antibody was used at 1:5000; the synapsin 1 antibody was used at 1:2500. The labeled neurons were examined using a 40X objective on a Zeiss LSM510 laser scanning confocal microscope. The confocal acquisition settings were kept the same for those samples when quantification was performed. The brightness, contrast and levels of the images were minimally adjusted (in Adobe Photoshop 8.0) for those images presented. No additional digital image processing was performed. Puncta counts were performed using ImageJ. The average intensity of synaptic and non-synaptic AP180 or synapsin 1 labeling was determined using imageJ and the synaptic/non-synaptic ratio was obtained.

3. Results and discussion

Our goal was to examine whether neuronal activity has effects on the expression of AP180. We used cultured hippocampal neurons to assess the expression level and pattern of AP180. Neuronal activity was evoked by KCl, or silenced by sodium channel blocker tetrodotoxin (TTX). The AP180 antibody used throughout this study has been characterized and described in several previous studies [4,10,18].

AP180 begins to be expressed by embryonic neurons as soon as they acquire a neuronal fate and its level of expression rapidly increases during development [18]. In cultured hippocampal neurons, AP180 is readily detectable as early as 1 div (days *in vitro*) and it remains at a high level in 7 div- and 14 div-neurons [22]. We began the study by measuring AP180 in 7 div hippocampal neurons that had been incubated for 24 h with moderately high potassium (10 mM KCl), TTX (2 μM), or the $GABA_A$ receptor blocker bicuculline (10 μM). We found that the levels of AP180 did not vary between the untreated control neurons, the neurons treated with 10 mM KCl, or the neurons silenced by TTX (Fig. 1). Moreover, the level of AP180 did not change in the bicuculline-treated neurons (Fig. 1A). We also measured the level of CALM, a clathrin-assembly protein that has a domain structure similar to AP180 and that is ubiquitously expressed by all cells including the neurons [10,11]. None of the above treatments affected the expression level of CALM in these neurons (Fig. 1A).

Immunofluorescence labeling of the neurons revealed that AP180 was expressed and distributed as discrete puncta, consistent with the synaptic locations of the protein [10]. The punctate expression pattern of AP180 appeared to be identical in the control neurons and the neurons that had been exposed to KCl or TTX (Fig. 1B).

In our previous study [3] and additional pilot experiments, we found that while cultured hippocampal neurons grow axons and dendrites as early as ~2 div, they do not develop fully functional synapses until ~10 div. Therefore, we used 14 div neurons containing functionally mature synapses to further characterize the effects of neuronal activity on AP180 expression. Unlike the immature neurons described above (Fig. 1), KCl treatment in the mature neurons resulted in a moderate reduction in AP180 expression (Fig. 2A). Co-treatment with KCl and TTX partially prevented this reduction, whereas TTX alone did not cause any changes (Fig. 2A). Unlike AP180, the level of CALM was unaffected by any of the treatments.

We next asked whether a higher concentration of KCl would further reduce AP180 levels in these neurons. We elevated KCl from 10 mM to 35 mM, a concentration known to strongly depolarize neurons. We found that the neurons exposed to 35 mM KCl had drastically reduced AP180 levels (Fig. 2B). While the magnitude of the reduction varied among experiments, the 35 mM KCl-treated neurons always had significantly lower AP180 levels than the control neurons did, as evidenced by the immunoblots (Fig. 2B). Co-incubating KCl with TTX nearly completely prevented the loss of AP180. In contrast to AP180, the CALM level was not reduced in the 35 mM KCl-treated neurons (Fig. 2B). Furthermore, we measured the levels of two additional clathrin-assembly proteins, epsin1 and HIP1, and found them unaffected by high KCl (Fig. 2B). Similarly, high KCl also failed to change the expression of the synaptic vesicle protein synaptophysin. That the levels of these other proteins were unaffected in high KCl-treated neurons suggests that the KCl-induced reduction is specific to AP180.

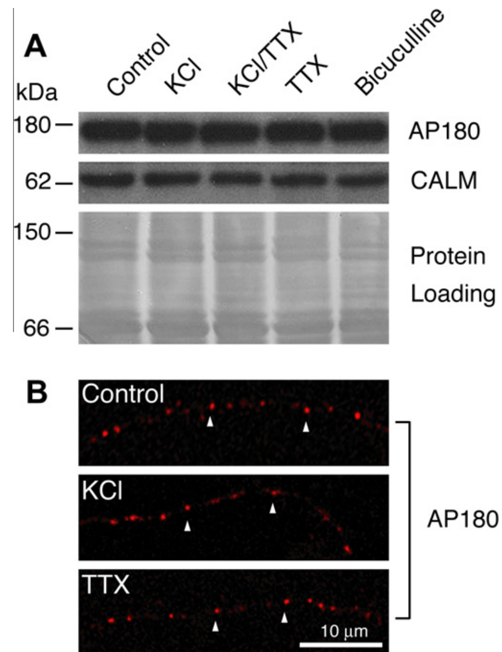

Fig. 1. Exposure to depolarizing or activity-suppressing agents does not affect levels or localization of AP180 in immature hippocampal neurons. (A) Hippocampal neurons (7 div) were treated with KCl (10 mM), TTX (2 μM), or bicuculline (10 μM) for 24 h. Immunoblot analysis shows the expression levels of AP180 and CALM in these neurons. The nitrocellulose membrane stained with Coomassie Blue shows even protein loading among the samples. (B) Immunofluorescence labeling shows the punctate expression pattern of AP180.

Analysis of the neurons by immunofluorescence staining confirmed that treatment with high KCl reduces AP180 levels. Unlike the control neurons, where AP180 was seen as bright puncta dotted along the axons, the AP180-puncta in the KCl-treated neurons were scarce and barely visible (Fig. 2C). Quantitative analysis revealed that the number of AP180-puncta was significantly lower in the KCl-treated neurons (49 ± 11% vs 97 ± 5% in control neurons, $n = 20$ neurons). The TTX-treated neurons did not show any changes in the expression pattern of AP180.

We next investigated the acute effect of KCl. We treated the neurons with 35 mM KCl for 10 min instead of 24 h. The total level of AP180 was unchanged (Fig. 3A). However, immunofluorescence staining revealed diffuse AP180 distribution in the KCl-treated neurons (Fig. 3B). Unlike the cleanly separated AP180 puncta along the axons in the control neurons, AP180 immunostaining in the acutely KCl-treated neurons was often seen smeared between the puncta (Fig. 3B). Double immunostaining with antibodies against AP180 and the synaptic marker synapsin I demonstrated co-localized AP180 puncta with synapsin I-positive synaptic puncta in the

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