The Journal of Experimental Secondary Science

RESEARCH ARTICLE

Identification of AKAP150 in Presynaptic Axon Terminals of the Basolateral Amygdala

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Abstract

A-kinase Anchoring Proteins (AKAP) are responsible for coordinating cellular processes by binding cAMP-dependent protein kinase A (PKA) and various signaling enzymes to cellular organelles and membranes. Our work involved studying one member of the AKAP family, AKAP150, in the rat brain. The absence of AKAP150 disturbs neuronal processes; AKAP150 knockout mice displayed impaired learning and memory, reduced anxiety, impaired motor skills, lower strength, and increased seizure resistance. In the amgydala, AKAP150 is especially important for auditory fear conditioning and memory consolidation. AKAP150 is also needed for long-term potentiation of synaptic communication to occur. Every tissue contains AKAP150, but its specific sub-cellular localization has remained unknown. To determine the localization of AKAP150, immunohistochemistry was used to detect and label AKAP150 in rat brain tissue. This tissue was mounted onto a block that was cut into ultrathin sections, which were then placed onto grids and imaged with an electron microscope. Previous research has focused on AKAP150 in post-synaptic processes such as dendritic shafts and spines. However, our analysis of EM images indicates that AKAP150 is also found in presynaptic axon terminals. This study suggests that future research into the function of AKAP150 should also consider its role in presynaptic processes such as neurotransmitter release.

Introduction

A-Kinase Anchoring Proteins (AKAPs) are a family of intracellular scaffolding proteins that tether the cAMP-dependent protein kinase (PKA) holoenzyme to cellular membranes and organelles and orchestrate many other second-messenger mediated processes[5]. There are forty-three known members of the AKAP family that target PKA to different sites. One specific member of this AKAP family is AKAP150, which is known in humans as AKAP79[3]. This particular AKAP is highly expressed in neurons[4].

The action of AKAPs and PKA on cellular processes is to regulate the phosphorylation of other proteins, some of which are associated with synaptic plasticity and memory consolidation. Both PKA and AKAP150 proteins are found in the lateral amygdala, a region of the brain known to be critical for the acquisition and consolidation of fear memories. Moita and colleagues[2] have shown that AKAP-PKA complexes are essential for fear conditioning to occur. Using a specific peptide that blocks PKA from anchoring onto AKAPs effectively, they found that when the blocking peptide interfered with PKA and AKAP interactions, acquisition of fear was unaffected, but consolidation of longer-term fear memory did not occur. The study of AKAP and fear conditioning was one of the first that indicated AKAP targeting of PKA is essential for the success of some neuronal processes[2].

AKAP150 must be present for certain neuronal processes to occur; inhibiting neuron excitability and regulating synaptic strength are examples of these processes. Loss of AKAP150 results in a loss of the PKA holoenzyme from hippocampal dendritic spines, and this can result in changes in synaptic transmission and deficits in memory. AKAP150 knockout mice, which have been genetically altered to not contain AKAP150 in any of their cells, displayed a loss in motor coordination and strength. These knockout mice also suffered from impaired learning, memory, and spatial memory but displayed reduced anxiety and increased seizure resistance. Studying and comparing the AKAP150 knockout mice versus wild type mice show that AKAP150 is capable of regulating neuronal signaling events in vivo[3].

Mutations in AKAP150 can disrupt dendritic signaling complexes and lead to electrophysiological and behavioral phenotypes in mice[4]. When AKAP150 knockout mice were compared with mutant mice that were deficient in the PKA binding domain of AKAP150 (D36), it was shown that PKA localization was altered in dendrites of the hippocampus and

striatum in both the knockout mice and D36 mice. The altered localization in these mice of PKA suggests that other neuronal AKAPs are unable to compensate for the loss of PKA binding to AKAP150 to maintain a normal PKA distribution. In D36 mice, learning and memory deficits were more severe, indicating that AKAP150 is responsible for targeting PKA to dendritic spines[4].

In addition, all AKAPs play an essential role within the hippocampus through long-term potentiation, which occurs when nerves that are continually stimulated produce stronger signals and effects. When AKAP is blocked, long-term potentiation is lost. The PKA-mediated phosphorylation is necessary for long-term potentiation induction and for long-term potentiation to maintain itself[1].

Before the work of Lilly and colleagues[1], the location of AKAP150 had long remained unknown. They found that within the rat hippocampal CA1 pyramidal cell, AKAP150 was often seen near asymmetric synapses, which is consistent with the fact that AKAP150 frequently interacts with excitatory postsynaptic processes. AKAP150 was seen postsynaptically and near excitatory/asymmetric synapses within the rat hippocampus while mostly being concentrated in the striatum and olfactory tubercle.

The main objective of this study is to determine the localization of AKAP150 in neurons in the rat basolateral amygdala (BLA), the region of the brain associated with fear and anxiety behavior. AKAP150 has been shown to be critical for both of these processes[2]. Lilly and colleagues[1] found that within the hippocampus, AKAP150 was largely concentrated within post-synaptic processes such as dendritic spines. Thus, we anticipated to largely find AKAP150 in postsynaptic processes within the rat basolateral amygdala.

Materials and Methods

Sprague Dawley rats were perfused with paraformaldehyde, the brains were removed, and cut into thin sections on the freezing, sliding microtome. Sections were thawed with ice-cold phosphate buffered saline (PBS). Three five-minute rinses with done with PBS. Sections were then placed in blocking serum comprised of 3% normal goat serum, 1% bovine serum albumin, 0.1% glycine, and 0.1% lysine and left on a rotator for one hour at room temperature. A primary antiserum solution was made with AKAP 150 (C-20), a purified goat polyclonal antibody from Santa Cruz Biotechnology, Inc. In order to confirm that the antibody used in this study was specific, Western blots were performed on brain extract from wild type mice and mice in which the AKAP150 gene had been knocked out (kind gift of Drs. Weisenhause and McKnight). The results are shown in Figure 1.

Sections were placed into the primary antibody solution and left at four degrees Celsius overnight. Sections were rinsed three times for five-minutes each in PBS. A secondary antibody solution was made with Biotin-SP-conjugated AffinipURE Donkey Anti-goat IgG (Jackson ImmunoResearch Laboratories, Inc). Sections were placed in the secondary antibody solution for one hour and then rinsed with PBS. Sections were placed into the Avidin/Biotinylated Peroxidase Complex (ABC; Vector) solution for one hour and then rinsed with PBS. Sections were transferred into diaminobenzidine (DAB) solution for ten minutes and rinsed twice for ten minutes each in PBS and twice for ten minutes each in phosphate buffer without saline (PB). The peroxidase of this ABC reagent catalyzes the diaminobenzidine (DAB) reaction, and the DAB then settles into the tissue as the label that we search for during electron microscopy. Next, sections were post-fixed in a 1% osmium tetroxide and PB for twenty minutes and then washed with PB. The sections were placed into a series of ethanol rinses for dehydration – five minutes at 50% ethanol, thirty-five minutes at 70% ethanol and 1% Uranyl acetate (to add contrast to the tissue), five minutes at 70% ethanol, two rinses of five minutes each at 95% percent ethanol, three changes of five minutes each at 100% ethanol, and three changes at five minutes each of propylene oxide. Once dehydrated, these sections were set into a 2:1 mixture of propylene oxide and Durcupan resin for thirty minutes, then a 1:1 mixture for thirty minutes, and finally a 1:2 mixture for thirty minutes. Sections were placed into pure plastic overnight.

Sections were flat embedded onto slides. Lead blocks were placed on top of the slides, and the slides were placed into an oven at sixty degrees Celsius for twenty-four hours.

Squares about .3 millimeters by .3 millimeters were cut from the amygdala section of the brain with a surgical-feather scalpel. We mounted the cut tissue onto blocks of resin, cleaned with one-hundred percent

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ethanol, with either superglue or Durcupan resin.

The blocks were cut into a into a trapezoid shape with the tissue sitting at the top and the overall dimensions no larger than .75 millimeters wide and no more than 0.5 millimeters tall.

After approaching with the diamond knife, the knife was filled with distilled and filtered water. Water was drawn out with a syringe until the top of the water surface became flat. Then the ultramicrotome was used to cut ribbons of ultrathin (approximately 70 nm thick) sections. These ribbons of sections were broken apart with an eyelash and transferred onto a grid. Excess water was blotted off with filter paper before the grid was placed into a grid box. The grids were allowed to dry overnight.

In order to provide contrast for cell membranes, we prepared a solution of lead citrate, 19.2 mL of distilled water, and .532 grams of lead nitrate. To this, we added 9 mL of sodium hydroxide, which turned the overall solution clear. The grids were placed into the staining box, and we filled this box with the lead citrate solution and let it cover the grids for five minutes. After five minutes, we submerged the grids into a distilled water solution for another five minutes. After this, we transplanted the grids into another distilled water solution for another five minutes. The grids then were thoroughly dried off to prevent any excess lead from remaining on the grids.

All necessary preparations (loading specimen, centering the beam, making a gain reference, warming the grid) were made. We took micrographs of the DAB label which was seen as a dark, fluffy substance under the microscope. The tissue was scanned and images of DAB label taken whenever found. Care was taken to avoid overlap in scanning the tissue and imaging a particular profile twice. The micrographs were individually analyzed for the location of AKAP150 label.

Results

145 micrographs taken on the transmission electron microscope were analyzed for AKAP150. The location of AKAP150 is indicated by DAB staining, which can be seen in micrographs as a dark, fluffy substance. By examining our electron microscopy images, we found AKAP150 located in post-synaptic processes. This result is consistent with previous hippocampal culture studies (Lilly et al., 2005). These presynaptic processes included dendritic shafts (Figure 2A) and spines (Figure 2B, 2C). Many of these spines were in contact with axon terminals received asymmetric that likely represent glutermatergic inputs to these dendrites.

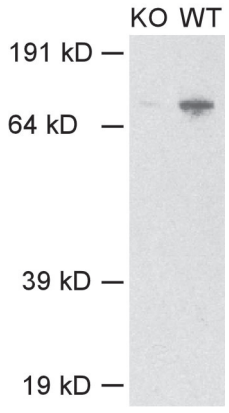

Figure 1. Western blot to test specificity for AKAP 150 (C-20). The first lane is the knockout sample, and the second lane is the wild type sample.

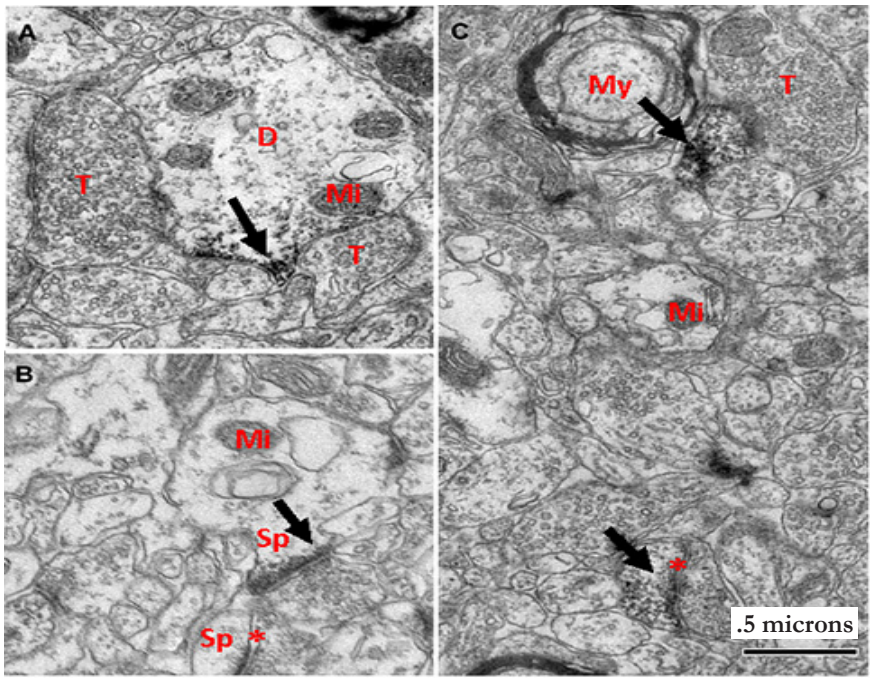

Figure 2. AKAP150 in post-synaptic dendritic shafts and spines. (A) AKAP150 in a dendritic shaft. (B) AKAP150 in a stubby spine. (C) AKAP150 in two spines D – Dendrite, Mi – Mitochondria, Sp – Spine, T- Axon Terminal, My – Myelinated process, * – Synapse, ► – Vesicles

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Unexpectedly, AKAP150 was also observed in presynaptic processes in significant quantities. This was determined through analysis of micrographs from profiles of axon terminals that contained DAB label (Figure 3). This DAB label was associated with membrane and plasma membrane surfaces not directly in contact with symmetric synapses. Preliminary quantification of data has shown that the occurrence of AKAP150 in presynaptic axon terminals is significant and not a rare occurrence; for every 47 dendritic spines that contain AKAP150, there are 31 axon terminals that contain AKAP150.

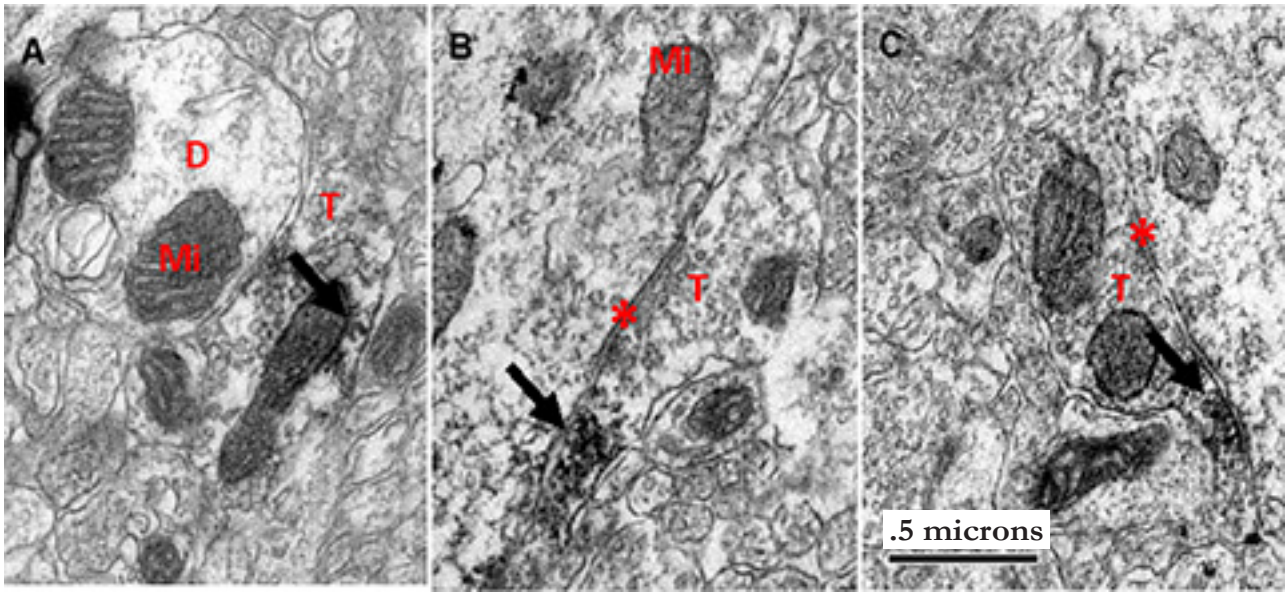

Figure 3. AKAP150 in pre-synaptic axon terminals. D – Dendrite, Mi – Mitochondria, T – Axon Terminal, * - Synapse

	Spines	Axon Terminals	Other (Dendritic shafts, preterminal axons, glial processes)
Number of times AKAP150 observed inside	47	31	108
Percentage	25.269%	16.667%	58.065%

Figure 4. Distribution of AKAP150 in neural processes within the rodent BLA

Discussion

Our results represent a novel finding because we found that AKAP150 is located in a neuronal compartment that it was previously not thought to exist inside. This indicates that future studies of AKAP150 functions should not be restricted to its postsynaptic actions. Rather research should broaden to include studies of AKAP150 in presynaptic processes, such as a possible role in presynaptic neurotransmitter release.

Further quantification of our data needs to continue. Ongoing work will determine how stress alters AKAP150 distribution within the BLA. Extreme stress can cause long-term changes in behavior, which can be seen in individuals who suffer from post-traumatic stress. This altered behavior ultimately stems from changes in the brain and in neural function in relevant brain regions. We wish to determine the differences in the molecular architecture

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of signaling proteins such as AKAP150 that may underlie this altered behavior. By gaining knowledge of the distribution of AKAP150 in a stressed brain versus an unstressed brain, we hope to suggest novel drug treatments for stress disorders.

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Acknowledgements

This work was conducted as part of the Institute on Neuroscience, an eight-week summer research program sponsored by the Center for Behavioral Neuroscience (CBN) and directed by Dr. Chris Goode and Dr. Kyle Frantz, and supported by a Merit Award from the Office of Research and Development, Department of Veterans Affairs to ECM. The author gratefully acknowledges her parents for driving almost a hundred miles each day during the summer to transport her to and from Yerkes, to Marcelia Maddox for her excellent technical assistance, to Dr. Walt Hubert for his guidance, and to Dana Golowich, the Post AP Advanced Research director at the Center for Advanced Studies at Wheeler High School, for notifying the author about the Journal of Experimental Secondary Science and encouraging her to submit her paper.