Biochemical Society studentship report 2008

The role of protein kinase A targeting in neurotransmitter release

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Background:

cAMP-dependent protein kinase or protein kinase A (PKA) is a much studied enzyme which phosphorylates various protein targets involving many different signalling pathways, and acts to regulate the activity of other enzymes, ion channels, chromosomal proteins, and transcription factors. In the cerebellum, the region of the brain important in coordination and motor learning, PKA activity enhances neurotransmitter release from the presynaptic nerve terminal. This phenomenon is thought to be an important cellular mechanism in learning and memory. A-kinase anchoring proteins (AKAPs) are a class of multivalent protein scaffolds which co-localise PKA and other cAMP-related enzymes, such as phosphodiesterase and phosphatases, to specific regions in the cell (Fig. 1). Dr. Evans' lab has previously found that AKAP2 is enriched in the presynaptic terminals of the cerebellum, and is therefore likely to be involved in PKA targeting. We investigated the role of AKAP2 in cerebellar presynaptic neurotransmitter release, with the specific aims as described below:

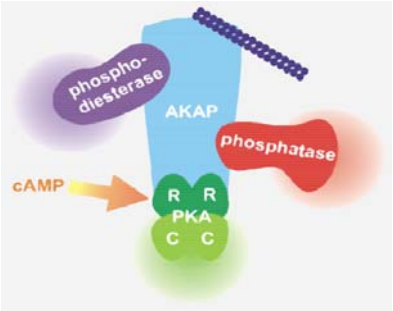

Figure 1. AKAP signalling. AKAPs have 3 major features: (i) sub-cellular localisation e.g. tethering to the cytoskeleton, (ii) interaction with regulatory subunits via an amphipathic helix, and (iii) binding sites for other cAMP related signalling molecules.

Aims:

1. Design and construct vectors containing silencing RNAs against the rat AKAP2 mRNA
2. Validate the silencing vectors in heterologous cells expressing AKAP2
3. Express AKAP2 silencing vectors in cultured neurons to examine the role of AKAP2 in PKA targeting in living synapses.

Description of work:

3 possible shRNA-encoding oligos were designed and cloned into the pSUPER.GFP siRNA expression vector. The GFP-shRNA constructs were transformed into XL10-Gold *E. coli* and successfully transformed clones were selected using antibiotic resistance. The vectors were confirmed to contain the inserts by restriction digest and gel electrophoresis and then checked by DNA sequencing. The silencing vectors were then propagated in bacterial cultures, purified, and used to transfect mouse and rat cells as described in the following section.

Results:

Validation of the silencing vectors was carried out by co-transfection into 3T3 mouse fibroblast cells along with the pMH-AKAP2 plasmid. The degree of silencing in these cells was quantified by immunofluorescence staining of the HA-tagged rat AKAP2 (encoded by the pMH plasmid) and GFP. As the control, pMH-AKAP2 was co-transfected with the empty pSUPER.GFP vector. 100 cells from each treatment group were classified as showing only green fluorescence (GFP), only red fluorescence (AKAP2), or both. In the group transfected with the shRNA3 vector, we observed a significant reduction in the number of cells that were co-stained with green and red fluorescence, suggesting that cells transcribing shRNA-3 have a reduced expression of AKAP2 (Fig. 2).

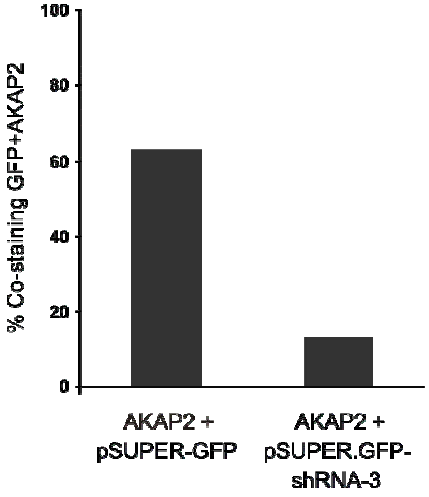

Figure 2. Silencing of HA-AKAP2 expression in 3T3 cells. Plot showing the % of cells co-stained with green fluorescence (GFP) and red fluorescence (HA-AKAP2). GFP is a marker for expression of the shRNA silencing vector. Control, n=100; silencing vector, n=101.

Following this, we transfected the shRNA-3 silencing vector into B104 rat neuroblastoma cells, which were confirmed to express endogenous AKAP2 by Western blotting and immunofluorescence. We found that the AKAP2 fluorescence intensity in cells transfected with shRNA-3 was only 40% of that in untransfected cells (Fig. 3). This suggests that shRNA-3 is successfully silencing expression of AKAP2.

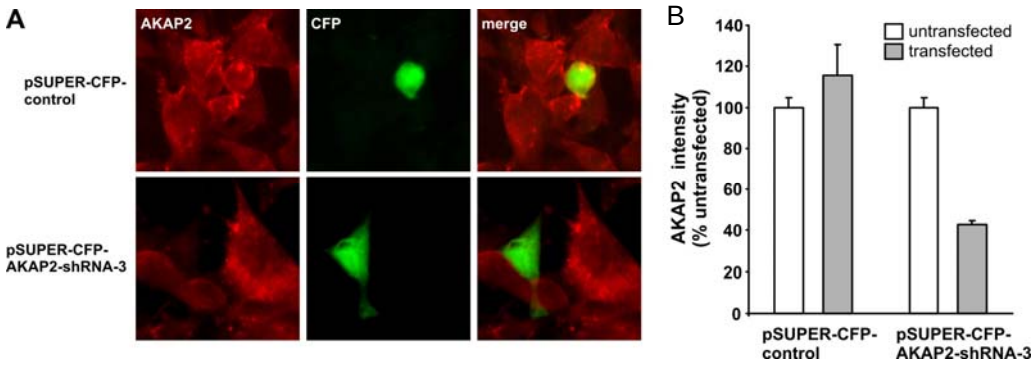

Figure 3. Silencing of endogenous AKAP2 expression in B104 cells. A) Immunostaining showing the presence of AKAP2 and CFP. CFP is encoded on the silencing vector and is used as a marker for shRNA-3. B) Graph comparing the red fluorescence (AKAP2) intensity of untransfected cells and cells transfected with control plasmid or silencing vector. The intensity is expressed as a percentage of the fluorescence of the untransfected cells.

Future directions:

The shRNA-3 silencing vector could be further validated by Western blotting to directly compare the levels of AKAP2 in cells with and without shRNA. We could then progress into cultured rat neurons as mentioned in the third aim. The transfection of cerebellar granular neurons would allow us to determine the effects, if any, on neurotransmission. This would be achieved with the use of fluorescent dyes, which, together with a real-time live cell imaging assay, give us the ability to monitor synaptic vesicle fusion in individual synapses. An inhibition or reduction in neurotransmitter release would indicate that AKAP2 is essential for cerebellar signalling, like that occurring in learning and memory. In addition, the AKAP2 mRNA transcript is known to undergo alternative splicing, resulting in the production of several different isoforms. Instead of a global knockdown of AKAP2 as effected by our shRNA-3 construct, the design of shRNAs to achieve targeted knock down of one or more of these isoforms at a time would help to shed light on their respective functions.

Departures from original proposal:

We were unable to investigate all the aims in the initial proposal. We managed to design, construct, and validate a silencing vector, but due to time constraints and recurring bacterial infections of my tissue cultures, have not yet examined the role of AKAP2 in neurons.

Value of studentship to the student:

This studentship has left me feeling far more comfortable and confident in a lab environment, and not just because of the many new techniques I've learnt. The firsthand experience of the scientific process – including planning, executing and optimising an experiment, collecting and interpreting the data, troubleshooting, and finally presenting the results – I have found to be invaluable now that I've started my placement year. One thing that surprised me (and now I'm glad I know) is that even procedures which seem simple in theory may not always turn out as expected, and the same applies to the best planned experiments. To me, research sometimes seems like a long, drawn out test of my problem solving and troubleshooting skills, but perseverance will eventually pay off, even if it does lead down a couple of dead ends first. This experience has definitely helped to reinforce my interest in scientific research and motivated me to seriously consider a PhD.

Value of studentship to the lab:

This studentship has been invaluable to the lab in terms of getting the technique of plasmid-based shRNA silencing set up and optimised. The results that Kevin generated have been used as proof of principle preliminary data in a Project grant application to the Wellcome Trust, which aims to continue Kevin's project to ascertain the function of AKAP2 in presynaptic plasticity. Final year undergraduate project students in the Evans lab this autumn will also make use of reagents that Kevin generated. Therefore, despite not fulfilling all of the original aims, Kevin's legacy will help future students and researchers in the lab.