Salus University

Annual Progress Report: 2009 Formula Grant

Reporting Period

January 1, 2010 – June 30, 2010

Formula Grant Overview

The Salus University received $59,678 in formula funds for the grant award period January 1, 2010 through April 30, 2011. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Mechanisms of Signal Transduction and Degenerative Diseases in the Retina – The general long-term purpose of this high-risk pilot project is to obtain new knowledge for the subsequent in-depth study of the intricate mechanisms triggering photoreceptor death. Human congenital retinal diseases causing blindness often arise from deregulated signal transduction mechanisms in rods and cones. Our main purpose is to evaluate how intracellular signal transduction messengers, cAMP and cGMP, contribute to retinal degeneration in a mammalian retina. The specific aim in the planned pilot study is to develop a new, presently unavailable, transgenic mouse model that would produce cAMP through a controlled light-sensitive mechanism in vivo. The outcome from this proof-of-concept project can then be used for leveraging funds to further expand the study on a larger scale.

Anticipated Duration of Project

1/1/2010 – 4/30/2011

Project Overview

The broad objective of the study is to evaluate how different cyclic nucleotides contribute to the degenerative processes in retinal rods and cones. The leading cause for many types of congenital blindness, such as retinitis pigmentosa, Leber congenital amaurosis, dominant rod and cone dystrophies, is the death of photoreceptors triggered by mutations in the proteins that constitute normal signaling pathways. Deregulation of signal transduction in these cases affects intracellular concentrations of cyclic nucleotides, cAMP and cGMP. While a number of studies, including several in vivo and in vitro models reported by the PI, clearly confirm the involvement of an abnormal cGMP synthesis as a culprit in some of the human disease-causing mutations, the role of cAMP is much less clear, despite the indication of its possible involvement. There is biochemical evidence that cAMP concentrations change in some types of degenerating retinas, but the main question remains whether this is a feed-back response caused to mitigate the degenerative process or a part of the cell destruction mechanism itself. One of the main problems for the evaluation of the cAMP as the potential trigger of the photoreceptor cell death lies with

the complexity of the cAMP versus cGMP analysis in vivo. The cGMP synthesis is extremely powerful in rods and cones compared to that of cAMP, while the destruction of both nucleotides is sensitive to light and can be catalyzed by the same enzyme, PDE6. We reason that in order to specifically evaluate the possible role of cAMP in photoreceptor signaling and death, it is necessary to manipulate with the cAMP concentrations in living mouse photoreceptors without interference from the cGMP synthesis. Therefore, we plan in the specific aims of this project to express in mouse rods a mutated form of guanylyl cyclase turned into adenylate cyclase. In a genetic background lacking the normal endogenous photoreceptor guanylyl cyclase, this would specifically allow for regulation of cAMP synthesis in rods by the same light- and calcium-sensitive mechanisms that would normally control cGMP synthesis.

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Other Participating Researchers

Andrey Savchenko, PhD – employed by Salus University

Expected Research Outcomes and Benefits

At the end-point of the pilot study initiated with this high-risk application, we expect to produce a new genetic mouse model suitable for in-depth study of the intricate role of cyclic nucleotides disease-related retinal degenerative processes. The use of such a model should have a two-fold benefit: i) it may help to answer questions about the specific role of cAMP in photoreceptor signaling and death affected by mutations found in human patients; ii) it will likely create a new, presently unavailable, research tool for manipulating the cAMP concentrations in living photoreceptor neurons at will.

Summary of Research Completed

With the accordance to the longer-term objectives of this project, we need to build and express in mouse rods a transgenic DNA construct that would impart to the photoreceptor cell the capabilities of cAMP synthesis. In the same manner as the rod regulates the synthesis of c GMP by retinal guanylyl cyclase (RetGC) by Ca2+ sensor proteins (GCAPs), we design the cAMP synthesis to be regulated by light via the same GCAP-mediated Ca2+ feedback.

Activity on this pilot project started May 1, 2010 and since that time the following progress has been achieved.

We employed the paradigm developed by Ramamurthy et al., in order to convert RetGC1 into adenylyl cyclase through a site-directed mutagenesis. We therefore have made the substitutions,

E925K and C997D, in the catalytic domain of a human RetGC1 required to switch the specificity of the catalytic center of the enzyme from utilizing Mg2+GTP to Mg2+ATP as a substrate (Ramamurthy et al., 2001). The site-directed mutagenesis was performed using a standard "splicing by overlap extension" technique (Gordon and Pease, 1989), and the cDNA coding for the mutated enzyme (RetGC1AC) was subcloned into the pRCCMV vector and verified by DNA sequencing. An extremely high GC-content of the RetGC1 cDNA was a challenge that we had to overcome before we optimized the PCR reaction and ended up with the properly subcloned product.

Before we can proceed with making the actual transgenic construct, we have to verify that the mutant enzyme continues to bind GCAP and indeed has changed its catalytic properties as required for the objectives of the project. Therefore, the two following experiments were conducted.

GCAP1 binding by RetGC1AC in living transfected cells:
We used our previously developed method to test the binding of GCAP1 fused with a green fluorescent protein (GCAP1-GFP) to RetGC1 expressed in HEK293 cells. When expressed without RetGC, GCAP1-GFP is diffusely distributed throughout the cytoplasm and the nucleus alike as a soluble protein, but when co-expressed with RetGC1 it changes its localization to the membrane-bound and clears from the nucleus (Peshenko et al., 2008, 2010). Since we replaced only two amino acid residues in the catalytic center of the cyclase we expected that this modification should not hamper GCAP-binding capacity of the mutated enzyme, RetGC1AC. Indeed, the experiment described in Fig. 1 now directly demonstrates that both wild type RetGC1 and RetGC1AC equally well make GCAP1-GFP localize to the membranes.

Catalytic activity of RetGC1AC:
We have tested the activity of RetGC1AC in the presence of either P-32 alpha-GTP or P-32 alpha-ATP as a substrate. We find that as predicted, RetGC1AC lost the cGMP producing activity and catalyze cAMP production instead. Importantly, RetGC1AC activity was strictly GCAP-dependent (Fig. 2).

Therefore, we can now proceed to the next step of the plan and make the RetGC1AC into a transgenic construct containing rhodopsin promoter for the transgenic expression in a mouse retina.

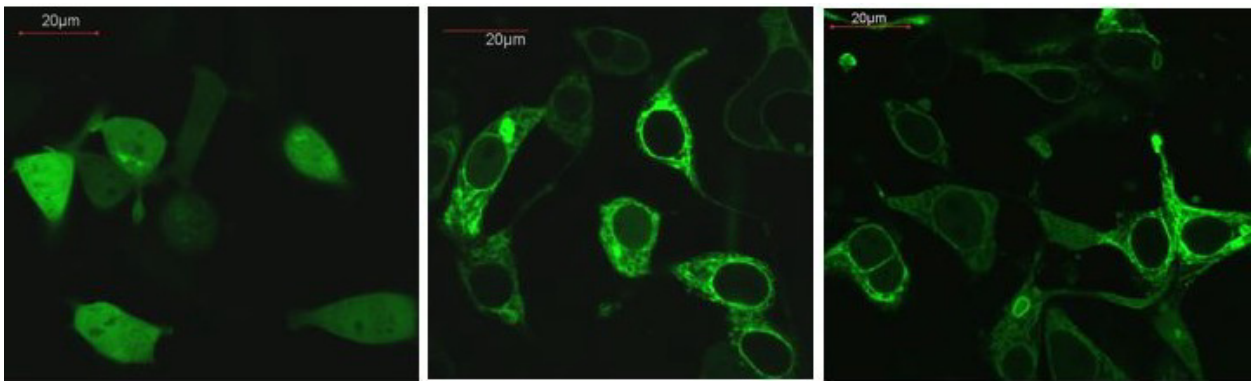

Figure 1. GCAP1-GFP fluorescence is distributed diffusely throughout the transfected HEK293 cells when co-expressed with a negative-control RetGC1 construct truncated at kinase homology domain (*left panel*), but changes its localization to the endoplasmic reticulum containing the membrane RetGC1 when co-expressed with wild type RetGC1 (*middle panel*). It maintains the same membrane-associated pattern when co-expressed with the RetGC1AC mutant (*right panel*).

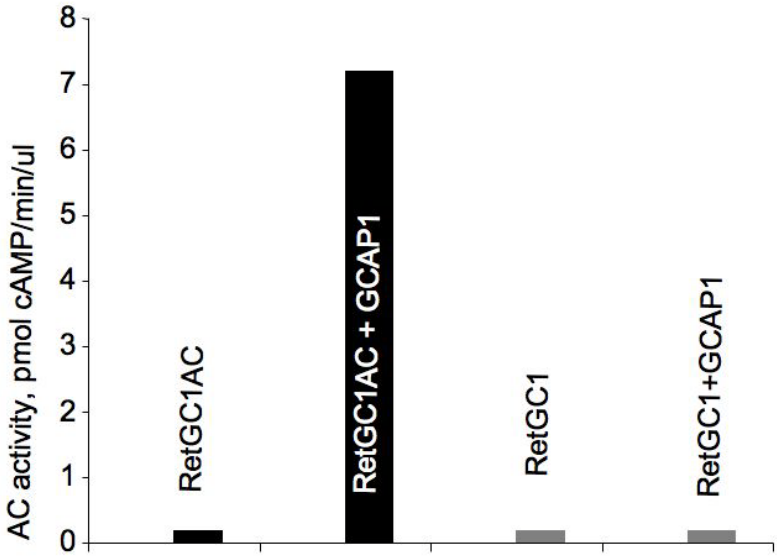

Figure 2. RetGC1AC, but not the wild-type RetGC demonstrates GCAP-stimulated adenylyl cyclase activity. Membranes isolated from transfected HEK293 cells were assayed in vitro in the absence or in the presence of recombinant GCAP1. The amount of P-32 cAMP was determined using thin-layer chromatography.