Prague, 29th April 2014

Dear committee members,

It is a pleasure for me to evaluate the thesis "Studies of membrane protein structure and function using polarization microscopy" presented by Alex Bondar, MSc. I am aware that this work deals with highly relevant biological questions. On top of this, original technology was developed by the applicant and the team based on linear dichroism in combination with 2 photon microscopy that allow to address these questions.

The fact, that the technology was described in such a highly prestigious journal as Nature Methods is, reflects the quality of the research, as well as the need of development of such techniques. These are crucial for exploration of complex biological systems. Indeed, the work that followed this original methodological paper did in the pace of the studies of Alex Bondar continue in logical direction. Next paper documents the usefulness of this technology in shedding more light on so far unsettled fundamental question about the GPCR-G protein relationship prior to the receptor activation. Also, the fate of the G proteins upon the receptor activation is logical continuation of the studies.

It would be redundant to do just another review in this place, since the publications that are fundamental for the thesis were reviewed in the highly respectable journals prior the publication. Thus I have just few questions, or suggestions, that exceed the scope of the thesis, but yet might be of interest hopefully:

1. Several approaches using fluorescent tags and energy transfer were used to study the fate of activated G-proteins as well as their association with the GPCRs prior the agonist application (pre-coupling). Can you summarize the approaches and link the outcomes with the techniques?

2. Should we assume, that the model describing GPCR signaling as a multiple conformational state equilibrium system, in which the agonists promotes one or more of the active states, than even without agonist, certain pool of receptors should be in the active state. Thus, these receptors that reach active state even without the agonist should be interacting with the G-proteins. Did any of these studies use inverse agonist prior to agonist application?

3. For the adrenergic receptors, did you use different bias agonists in the studies following the fate of the G-proteins during their activation?

As a conclusion, I propose that Alex Bondar, MSc.deserves the Ph.D. title based on the presented thesis.

Doc. MUDr. Jaroslav Blahoš, Ph.D.

opponent

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Opponents review: PhD thesis

„Studies of membrane protein structure and function using polarization microscopy"

from Alexey Bondar, MSc, University of South Bohemia. In Ceske Budejovice, Faculty of Science

Cellular functions are controlled by many different proteins including those localized at the plasma membrane. An important subset of these membrane-targeted proteins is dedicated to transduce and integrate the information transported via extracellular mediators inside cells. Many of these membrane proteins, specifically those that are integral membrane proteins serve as receptors of hormones, neurotransmitters and drugs. In his Ph.D thesis Alexey Bondar presents novel and elegant methods to study the activity or assembly of such membrane associated proteins in single intact cells by means of polarization microscopy. In this thesis, he first introduced general principles of light microscopy specifically those that are suited to study function of cellular proteins, such as FRET, BRET and FRAP. He than elegantly explained the physical and theoretical principles of two-photon excited Fluorescence polarization linear dichroism microscopy (FP-LD). In case of ordered orientations of fluorophores, alterations of the

orientation for instance due to interactions of the fluorophore carrying protein with other proteins will lead to a change in the linear dichroism, which can be monitored by microscopy. Alexey Bondar and the Lazar lab utilized this principle idea to image the function of membrane proteins in intact cells. Their concept was to utilize the ordering nature of membranes, which allow for a restricted orientation of molecules carrying a fluorophore. Experimental validation of this concept demonstrated a much stronger dichroic ratio upon 2P-excitiation compared to 1P-excitation. Furthermore, it could be demonstrated that a flexible membrane attachment anchor of the GFP did not allow for anisotropic effects. These experimental findings, which were published in *Nature Methods* served as a basis for Alexey Bondars work on heterotrimeric G proteins. He based on the fact, that heterotrimeric G proteins work as a functional complex of Gα-subunits and Gβγ subunits, which exhibit high affinity binding in the inactive state thereby burying their surfaces needed for interaction with effectors. Upon receptor mediated activation of these G proteins the high affinity binding is attenuated and these G protein subunits either dissociate or rearrange in a way that allows exposition of surfaces on these G proteins that allow for interaction with effectors. In his thesis Alexey Bondar describes his studies on the measurement of G protein activity based on alterations on 2P-LD. Here the concept was that GFP variants fused to the Gα-subunit of heterotrimeric G proteins exhibit a different orientation if its carrying alpha-subunit is bound to a Gβγ subunit. Indeed he demonstrated that this is the case at least for certain constructs he obtained from my group and also Dr. Tinkers as well as Dr. Lamberts group. Most importantly Alexey Bondar could demonstrate that upon receptor mediated G protein activation the LD ratio was reduced, in line with G protein dissociation or at least higher flexibility of the orientation of the fluorophore carrying subunit. This initial work on imaging G protein activity in living cells has also been published in the Nature methods paper 2011. In a second study, published recently in *JBC,* Alexey Bondar tried to solve the problem of whether G protein subunits dissociate or rearrange during activation. The hypothesis of G protein rearrangement has been put forward by my own research more than a decade ago, when we established the first FRET assays for mammalian G proteins and observed much to our surprise an increase in FRET between Gαi and Gβγ upon activation. Other groups using BRET approaches have published similar results. However the interpretation of these results were not undisputed, since mobility measurements by the Lambert group showed that activated

G protein subunits exhibited clearly less stable interactions compared to inactive ones. Alexey Bondar addressed this controversial issue by his novel LD measurement approach. He found that in dependence of the fluorophore insertion site on the Gα subunit and the Gα-subtype not only the LD ratio was different, but also the potency of Gβγ subunits to alter the LD ratio differed. His conclusion was that in line with the reported results on Gi protein subunits there is not much alteration of the LD upon activation if the fluorophore is inserted between AA91 and 92, suggesting indeed that these subunits do not really dissociate upon activation. However different results were obtained with Gαo subunits and Gi subunits that were fused at their N-terminus to CFP followed by a membrane anchoring GAP43 domain. In these cases Gβγ increased the LD ratio and agonist mediated activation of these G proteins partially reversed this effect. Therefore the authors concluded that possibly the insertion of fluorophores at Pos 91 in $G\alpha i_{1,2,3}$ leads to the observed "non-dissociating" behaviour of Gai subunits. Analysis of Gi/o-mediated GIRK current activation demonstrated that independent of the labelling site on Gα-subunits that these G proteins are capable of activating GIRK channels via their Gβγ-subunits. However testing for Gβγ-mediated membrane translocation of the C-terminus of GRK3 he found that specifically those Gαi constructs that carried a fluorophore at position 91 were not able to mediate this Gβγ dependent translocation. Therefore he concluded that in respect to function these constructs are not equal to wilde type. The experimental evidence Alexey Bondar presents is quite solid and his interpretation might hold true, however specifically the GRK3-CT translocation assay he performed would have profited from important controls: The translocation of GRK3-CT to the membrane is a relative assay that depends on the ratio of available Gβγ subunits and GRK3-CT molecules expressed. The available Gβγ subunits will depend on the expression level of Gα subunits, therefore it would have been important to control relative expression levels in these experiments.

In the third, yet unpublished part of his work Alexey Bondar applied 2P-LD measurements in order to study receptor / G protein interactions. His results on $alpha_{2A}$ adrenergic and $GABA_B$ receptors confirm results from the majority of recent publications regarding this matter: Both receptors did not exhibit any detectable precoupling to any member of the Gi/o family. Even though the results of this study are not exceptionally new, they were obtained with a different independent method and therefore represent are valuable contribution to the field.

Overall the studies presented in Alexey Bondars thesis are of very high quality and the experimental approach of exceptional novelty. Therefore some minor points that could be criticized such as the missing expression controls and missing clarities in respect to controlling plasmids (did he sequenced all used constructs etc.) do not significantly diminish the scientific achievements and overall quality of this thesis. Therefore, I strongly recommend acceptance of this thesis for the defence to the thesis committee and the faculty of science of the University of South Bohemia.

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Nové Hrady, 30.4.2013

Oponentský posudek doktorské disertační práce

Alexey Bondar: Studies of membrane protein structure and function using polarization microscopy

In his PhD thesis Alexey Bondar tries to understand G protein signal transduction using two-photon polarization microscopy (2ppm), demonstrating that interactions between G protein subunits and thus the G protein activation and can be studied by this technique in live cells. The thesis also includes a huge part of technological development, as the studied system is the very first studied by 2ppm and serves as a proof of concept. Nevertheless, the reported result that Gi/o proteins do not pre-couple to their cognate G-protein coupled receptors (GPCRs) is of high physiological relevance, as it was assumed earlier that binding between inactive G proteins and GPCRs is responsible for the specificity and fast kinetics of the interaction between these proteins upon activation.

The thesis itself can be separated into four parts: 44 pages of a comprehensive introduction into the field and the methodology, followed by a detailed 10 page "Materials and Methods" section. The core part then are three papers that back up the thesis and are adapted to form the "Results and Discussion" section. The thesis finally concludes with a 6-page "Conclusion" section that discusses and sums-up the three main findings of the candidate's scientific work: 1. 2PPM is a very promising novel technique to study membrane protein structure and function in live cells. 2. Dissociated G α_{GTP} and G$\beta\gamma$ protein subunits are the major form of G proteins and finally 3. Gi/o proteins do not pre-couple to GPCRs in the active state. introduction of the observed systems, including a summary and discussion of results for each studied system, and the attached publications to back up the thesis.

The thesis is very nicely structured and easy to read. After starting with a few philosophical thoughts about "Seeing is believing" and the role of membrane proteins in general, the reader is introduced into the aims of the research work right away. So already on page 6 the reader has a very clear picture what the thesis will be about. This is followed by a detailed introduction into various microscopy techniques, fluorescence and the essential physics needed to understand the thesis. Finally the biological system is introduced and the reader is updated with state-of-the art knowledge with respect to G proteins. I also appreciate the "Materials and methods" section that goes beyond the information given in the individual papers.

The thesis contains a huge amount of experimental work of a remarkable broadness, from molecular biology, Western blotting, electrophysiology, microscopy (2ppm, FRET, confocal) up to image processing. The candidate to date published or submitted 6 papers, on four of which he is the first author. Three papers are included in the thesis and make the "Results and Discussion" section while the additional three are very closely related to the methodology development and in my opinion could have been included in the thesis easily, however, I also understand the reasoning for not including them as they do not contribute to the G protein study directly. It is necessary to note that one of the included papers has been published as a full paper in Nature Methods with an impact factor of 23.6 and has gained to date already 12 citations, which is remarkable for an experimental paper that

describes a novel method. The main physiological finding has been published in JBC, which is one of the leading journals in the field.

Questions for the defense that should be addressed by the candidate:

1. Is it possible to estimate the detection limit in terms of local intracellular concentration of the fluorescent protein

2. The reported results exclude the pre-coupling model, and argue for the presence of a mechanism that ensures fast and efficient GPCR signal transduction. This could be probably managed by either kinetic scaffolding or spatial confinement of molecules to membrane micro-domains, as is mentioned in the thesis. Do you have any evidence for the one or other model? What is your personal opinion on the probability of each model?

3. Before succeeding to get functional fluorescence-tagged G proteins you probably tried various linkers/constructs. How hard it is to get a working construct and how large do you estimate the risk, that the fluorescence-tag alters protein functionality and that the resulting construct behaves different from wild-type?

4. One paper has been submitted to PLOS one recently. Is there already any feedback from the reviewers?

5. Two papers have been included as manuscripts ready for submission. Have they been submitted already and if yes, to which journals?

Finally, it is my pleasure to state that Alexey Bondar until now conducted internationally recognized high quality science. The well written thesis that tells a thrilling story and the publications that back up the thesis show, without leaving any doubts, that the applicant fulfills all criteria for being awarded a PhD degree not only from the University of South Bohemia, but from any University in the world. Therefore I can fully recommend Alexey Bondar for being awarded the PhD degree with honors.

(Český doplněk: Alexey Bondar jásně prokázal tvůrčí schopností, prácé bez sebemenších pochybů splňuje požadavky kládené na disertační přáce v oboru biofyziky)

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