Thesis Review

Name of PhD student: Karolina Rojek-Sito

Affiliation of PhD student: Laboratory of Emotions Neurobiology of the Nencki Institute of Experimental Biology, Polish Academy of Sciences

Name of supervisor: Dr. Ewelina Knapska, PhD., DSc, Professor of the Nencki Institute

Title of the doctoral dissertation: **Central amygdala - ventral tegmental area – cortical circuits mediate initiation and maintenance of social interaction**

Name of reviewer: Balazs Hangya MD PhD

Affiliation of reviewer: Lendület Laboratory of Systems Neuroscience, Institute of Experimental Medicine

Karolina Rojek-Sito submitted a doctoral dissertation titled "Central amygdala - ventral tegmental area – cortical circuits mediate initiation and maintenance of social interaction" consisting of Abstract, Introduction (28 pages), Research aims, Methods (22 pages), Results (38 pages), Discussion (13 pages), Summary and conclusions, and a list of 265 References, 148 pages in total. The dissertation meets formal standards and the sections are organized in a balanced manner.

The Introduction section reviews previous literature on social interaction, interactions between the neural circuits underlying social functions and reward processing, and key components of the neural circuit that was in the focus of the PhD studies. The Introduction is thorough and properly sets the stage for the interpretation of the experiments performed by the candidate, without undue detours into tangential topics.

In the Research aims section, the candidate poses two questions: whether initiation and maintenance of social interaction utilizes the same neural circuits, and whether the neural circuits involved in motivation for food vs. social reward are separable. These are important, timely and open scientific questions that merit the investigations described in this thesis work.

The Methods section properly describes the applied techniques with the appropriate level of scientific detail, illustrated by figures. As a minor criticism, some figure legends lack the details to fully interpret the figures (e.g. Figs. 9-10 - what was the labelling, where, compared to a reference atlas, was the image taken, etc.). More details for the ANOVA tests would be desirable, including all statistics on both main effects and interaction terms.

The Results section consists of four parts, each addressing different circuit elements (pathways or neuron groups) of the neural network thought to underly social motivation and reward processing. The Candidate first investigated the roles of the ACC-CeA and OFC-CeA pathways in the initiation and maintenance of social interaction in rats. The Candidate shows that the ACC innervates more 'social cells' (cells activated by social contact) than the OFC in the CeA. Unfortunately, the corresponding figure only shows cell counts as bar graphs and no microscopy images. As a weakness, this is generally true to the anatomical experiments of the thesis: micrographs only appear in the Methods and do not properly illustrate the anatomical results. Adding proper, specific anatomical images would both strengthen these results and demonstrate the skills of the Candidate. Next, the Candidate used a chemogenetic approach to separately inhibit the ACC-CeA and OFC-CeA pathways. While the former only affected maintenance, the latter inhibited both maintenance and initiation of social interaction. Although this suggests the existence of separatable circuits for these social functions, one caveat is that ACC-CeA inhibition also seems to decrease social initiation according to Figure 19. While this effect was not significant in the Candidates sample, that could be due to insufficient statistical power for this test. Thus, further, more thorough investigation would be needed to draw firm conclusions. That said, the direct statistical comparisons of the DREADD effect on social initiation with respect to the two pathways revealed a statistical difference between the ACC-CeA and OFC-CeA pathway, lending some additional support to the Candidate's conclusions. The Candidate also found that locomotor activity did not change in consequence of these manipulations. In this regard, it is not fully clear how 'activity' (in pixels/10 minutes?) was measured and some additional measures such as average speed, distance travelled, etc. could provide a more accurate picture. Finally, inhibiting the ACC-CeA and OFC-CeA pathways also decreased motivation for food reward.

In the second part, the Candidate focused on the social cells in CeA by using c-fos-dependent constructs. This elegant design allowed the Candidate to demonstrate that activating social cells suppressed motivation for food reward. Interestingly, if my understanding is correct, inhibition of social cells activated by positive social interaction also decreased motivation for food reward, which

observation would probably require further experiments to better understand. All in all, these results are nicely presented. However, it is unclear whether all optogenetic experiments have eYFP-controls, that is, controls using viral constructs only expressing the fluorophor and not the optogenetic actuator, undergoing photostimulation (e.g. in Fig. 32). The presented 'no light' control does not control for potential non-specific effects of photostimulation (e.g. through the eye, or through non-infected cells); therefore, these controls have, righteously, become part of the standards for these experiments. Still in this section, the Candidate measured neurotransmitter content in different areas following manipulations, and found, among others, important changes in cortical dopamine levels and VTA GABA levels. It would be interesting to see whether these two are correlated, when measured from the same animals. This experiment goes beyond the usual ideas and provided important insights, thus, I consider this a very valuable part of the study.

Third, the Candidate investigated the CeA-VTA projection. Chemogenetic inhibition of this projection seemed to decrease both the initiation and maintenance of social interactions; however, the effect on the initiation is not fully clear, as the initiation of social interactions showed a parallel increase in the partner rats, and it is not clearly presented in Fig. 35A whether the CeA-VTA: c21 group showed a significant decrease compared to controls (although Fig. 38 seems to confirm the result). The CeA-VTA projection was found to innervate mostly GABAergic VTA neurons: again, staining results in the form of fluoromicrographs would have been desirable. Also, although the CeA output is thought to be GABAergic, it would be nice to confirm the hypothesized GABAergic nature of the CeA-VTA projection.

In the last part, the role of two VTA-cortical projection in controlling social interaction are studied, namely the VTA-ACC and VTA OFC projections. Chemogenetic activation of these pathways increased social interactions: initiation in case of ACC and maintenance in case of OFC. However, activation of VTA-ACC non-significantly increased maintenance and activation of VTA-OFC non-significantly increased initiation. This again raises the general warning that statistically non-significant effects do not directly translate to a lack of effect, especially that the direct comparisons did not show much difference in this particular case. Also, the lack of effects in Fig. 48 is confusing, as it seems to be at odds with Fig. 43.

Finally, the Candidate appropriately discusses the potential ramifications of their results in the Discussion and Summary and conclusion sessions. The potential role of a disinhibitory CeA-VTA GABA-VTA DA circuit differentially affecting ACC and OFC is particularly interesting. The Candidate provides summary figures that greatly help summarize the results, but adding one more figure on the assumed circuit (above) would further highlight this important result.

The dissertation ends with a comprehensive list of citations, which are appropriate for the topics discussed in the thesis work.

The language of the dissertation is clear and contains only few typographical errors (e.g. p.55. 'wl conducted'; p. 108, line 1 should read 'allowed me/us to investigate'; p.63. 'U-Mann Whitney test' should read 'Mann-Whitney U-test'; p.77. 'the method's validity' should be omitted; p. 114 last line should read 'lever pressing' instead of 'level pressing').

Main strengths of the dissertation:

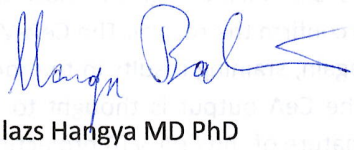
- It provides a comprehensive set of experiments aimed at dissecting a specific circuit underlying social initiation and maintenance.
- The text is coherent, clear and the results are clearly presented and demonstrated by figures.
- The findings are interesting and important in furthering our understanding of neural control of social functions.

Main weaknesses of the dissertation:

- I advocate more cautious interpretation of statistically non-significant differences ('negative results').
- Quality anatomical figures (microscopy) are largely missing.
- Some figures/figure legends lack the necessary details.

In summary, these weaknesses are minor compared to the strengths of the dissertation. The dissertation provides novel insights into an important scientific problem, and the doctoral dissertation demonstrates the Ph.D. student's overall theoretical knowledge of the field and ability to conduct independent scientific work. Therefore, I congratulate the Candidate for this thorough and solid piece of work.

In summary, the doctoral dissertation presents important novel scientific results and meets all required standards of doctoral theses in neuroscience. Therefore, I propose the Candidate student be admitted to the subsequent stages of the doctoral defense.



Balazs Hangya MD PhD

Lendulet Laboratory of Systems Neuroscience

Institute of Experimental Medicine

H-1083 Budapest, Szigony u. 43., Hungary

Anna Blasiak, PhD
Jagiellonian University
Department of Neurophysiology and Chronobiology
Gronostajowa 9
30-387 Krakow

Kraków dn. 23.08.2023

Review of the doctoral dissertation of Karolina Rojek-Sito,

entitled "**Central amygdala - ventral tegmental area – cortical circuits mediate initiation and maintenance of social interaction**" supervised by Ewelina Knapska, PhD, Assoc. Prof.

The doctoral thesis of Karolina Rojek-Sito addresses a profoundly significant and timely topic: social interactions and their relationship with the brain reward system, with a primary focus on the neuronal circuits governing the initiation and maintenance of social interactions. This subject matter is of utmost importance in our modern and digitized society, where disrupted social interactions are associated with the overwhelming presence of mobile devices, social media platforms, and other technological advancements that were designed to facilitate human bonding and coexistence. Now we understand that these and other factors have led to serious disturbances in social interactions, the repercussions of which are observed at various stages of human development, notably in children and adolescents who in extreme cases are paying the highest price for these questionable improvements. Therefore, understanding the complex neural mechanisms and neurocircuits underlying social interactions is crucial not only for the enhancement of our understanding of fundamental brain processes but also lays the foundation for possible future targeted interventions and therapeutic strategies for individuals struggling with social interaction related disorders.

In the dissertation submitted for review, the Author has adhered to the typical structure found in academic theses, characterized by a clearly delineated introduction, the stated objectives of the work, an overview of the methods employed, presentation of the results, their subsequent discussion, and a comprehensive bibliography.

In the Abstract, the Author highlights very important aspect of her work, which is definition of the circuits regulating appetitive social behaviors by their functional connectivity with other brain structures and not by the markers they express. At the same time she indicates that "such defined neuronal circuits could serve as therapeutic targets for rescuing social deficits". It would be very interesting to hear more details on the Author's idea regarding the specific approaches/techniques that can be currently used in the human clinical studies aimed at rescuing social deficits.

AB

The list of Abbreviation explains the most commonly used abbreviations in the dissertation, although some of the listed elements are explained based on their function, and they are not expansions of the specific abbreviation (e.g. DIO is explained as a "reading frame constructs", and the abbreviations stands for double-floxed inverse open reading frame), some are not fully explained (e.g. CREB is **cAMP** response element binding protein) or Polish instead of English words are used (like NA - noradrenalina).

In the Introduction of her doctoral thesis, the Author summarizes the significance of social interactions and their relationship to socio-cognitive development. She highlights the pivotal role of social interactions in group cooperation and competition, as well as their importance in cognitive development and the proper assimilation of external environmental information. Furthermore, within the Introduction, the Author comprehensively and accessibly describes the neuroanatomical components and brain circuits associated with social interactions, which have been investigated in this study. An important element here is the description of social interaction dysfunctions. Another valuable component of the dissertation Introduction is the section concerning the methods employed, in which the Author presents the key techniques used throughout the course of the doctoral work. The description is very clear and indicates the Author's familiarity and understanding of the methods used. It seems that a minor inaccuracy appeared in the description of the action of inhibitory DREADDS, and referring to them as factors inhibiting synaptic vesicle release and "mildly **depolarizing** soma" membrane (as a consequence of hM4Di activation, **hyperpolarization** of the cell membrane is expected and observed). In general, hM4D/CNO is commonly employed as both a suppressor of neuronal electrical activity *and* an inhibitor of synaptic vesicle release. Nonetheless, specific engineering can enable the refinement of hM4D for selective synaptic silencing. Overall, Introduction is a well-developed section of the thesis, with content that is appropriate for the rest of the work. The thoughtful and mature presentation of topics relevant to the thesis and overall stylistic correctness, make reading this part of the work very interesting.

The aims of Karolina Rojek-Sito's doctoral dissertation have been clearly defined. The Author of the thesis has undertaken the task of elucidating the neuronal circuits involved in initiation and maintenance of social interaction and verify whether there is a dependency between social interaction and food reward-related circuits. To achieve the goals of her doctoral research, state-of-the-art techniques utilized in neurobiology were employed, including optogenetics, chemogenetics, and genetically modified rats.

The section of the reviewed doctoral thesis that describes the applied Methods in general is well-written and gives a good picture of the used procedures. Enrichment of this section of the dissertation with diagrams depicting the applied procedures and analyses, especially those

related to social behaviors, greatly facilitates the comprehension of the methodological approach. At the same time, the chapter concerning the statistical methods employed is rather brief and it lacks information about the tests used to verify whether the analyzed data meet the assumptions of ANOVA (apart from possible deviations from the normal data distribution, which have been checked, there is no information regarding the verification of homogeneity of variance or the independence of the analyzed data).

Additional explanations would enhance the value of this section of the work:

- What was the reason for subjecting the rats to two surgeries instead of injecting all viral vectors (and/or PHA-L) during a single procedure?
- Were the placements/reconstructions of the injection site verified/done?
- Was the functional expression of viral vectors delivered DREADDs and ChR2 verified (for example, using ex vivo electrophysiological recordings)?
- In the description of the immunohistochemical procedure (c-fos studies), it was mentioned that both primary and secondary antibodies were obtained from rabbits. Is this indeed the case, and if not, what was the host? Also, what was the host for the secondary antibody used for anti-GFP, anti-Gad67, anti-mCherry, and anti-Th staining? Also, providing examples of staining (in all cases) at lower magnification, would enable an initial assessment of the specificity of the antibodies used, which has not been otherwise verified.
- What was the size and position of the analyzed ROIs? In how many slices from a single brain, the cells were counted?
- In case of HPLC experiment, no technical details, except for the description of the tissue harvesting, were provided.
- Despite described in the dissertation advantages over CNO, c21 also exhibits off-target effects at a dose as low as 1 mg/kg (doi: 10.1371/journal.pone.0238156, [illegible]), and a three times higher dose was used in the experiments described in the doctoral thesis. Among the described in the literature side effects of higher doses of c21, is a strong increase in dopaminergic substantia nigra neuronal activity in control animals. In the opinion of the doctoral candidate, could this have had any impact on the obtained results?

In the Results section, which comprises the most extensive part of the dissertation, the Author describes results of series of experiments showing that manipulation of specific projections within studied complex neural network exert distinct effects on the initiation and maintenance of social

A/B

contact. This study divides social interaction into specific components: initiation, representing the motivation to approach a cage partner, and maintenance, representing positive responses to the partner's attempts to initiate contact and the continuation of the social interaction. In addition, it introduces a third dimension; "blocking," representing instances of reluctance or unwillingness to participate in social interactions. Beyond these dimensions, this study expands its scope to investigate the selectivity of neuronal circuits in the context of motivation, employing a progressive ratio lever-pressing test to evaluate their role in food reward related motivation control.

The presented findings demonstrate that the OFC-CeA, CeA-VTA, and VTA-ACC projections play a pivotal role in initiating social contact, whereas the ACC-CeA, OFC-CeA, and CeA-VTA projections are crucial for sustaining social interactions. Another critical finding described in this doctoral dissertation indicates that CeA-VTA pathway, along with dopaminergic VTA-ACC/OFC projections, play a key role in the control of social interactions, and comprise largely separate neuronal component from circuits involved in the control of food intake motivation.

The results described in the work are convincing, and the use of sophisticated techniques enabled specific manipulations of selected neuronal circuits. Providing additional explanations would enhance the understanding and interpretation of the presented results:

- Further explanations are needed for the statistical analysis and the graphs that depict the number of CeA neurons receiving ACC or OFC projections activated by social interaction. The parametric statistical test (t-test) used to compare the counted cells requires the assumption of normal data distribution. How was the normality of distribution assessed when dealing with a low number of individual data points (3 or 4)?
- Some improvements could be made in the figure descriptions, which are occasionally imprecise and contain shortcuts (e.g., in some descriptions, the data presented in the graphs are described as 'rats,' while in others, the figure legend accurately refers to depicted data). Additionally, the identical descriptions of the axes in A and B panels of Figs. 17 and 18 make it challenging to distinguish the differences between them. The charts legends in Fig. 21-23 are nor clear (pink backgrounds indicating c21 or NaCl injections are only present in the 'test' sections, yet the color coding suggests that NaCl and c21 were also injected during the "baseline").
- The use of a highly sensitive and demanding technique, relying on optogenetic manipulation of the CeA neurons expressing c-fos-dependent ChR2, enabled the functional characterization of CeA outputs activated by social interactions and food reward. It would be beneficial

to present raw data (e.g., microscopic documentation) showing baseline and induced levels of reporter protein, as well as changes in endogenous c-fos expression in the analyzed tissue.

- Despite pointing out the potential negative consequences of optostimulation, the control animals did not receive light stimulation. In the Author's opinion, could this have influenced the obtained results?
- One of many interesting results presented in the reviewed PhD thesis was observation that activation of either the VTA-ACC or VTA-OFC dopaminergic projections leads to hypersociability. Given that different areas of the VTA, especially in the medio-lateral axis, are implicated in controlling various aspects of reward and salience processing, it would be interesting to examine the anatomical distribution of VTA neurons innervating the studied areas.

The significance of the results obtained during the doctoral thesis research is discussed and summarized by the Author in a well-written Discussion. In the opening sections of the discussion, the author explains, citing relevant literature, the rationale for studying social interactions following a 21-day social separation, indicating that such isolation can increase the motivation of rats to interact and reduce the variability in their responses.

- However, it's important to note that social isolation constitutes a significant stressor for rats, and studies in humans have shown changes in the activity of the ACC during social isolation (e.g., DOI: 10.1126/science.108913). How might this stress component potentially influence the results?

The chemogenetic approach used in the study allowed for the modulation of ACC/OFC neurons innervating CeA and CeA neurons innervating VTA. However, it's important to consider that c21-induced changes in neuronal signaling likely occurred not only at the level of the studied structures (CeA/VTA) but also in other structures innervated by neurons of the ACC/OFC-CeA and CeA-VTA axes. A valuable addition to the Discussion would be the identification of neuronal circuits (if known from the literature or mapped based on the material acquired during the described studies) whose activity could have also been modulated during the chemogenetic experiments and the potential influence or involvement of this modulation on the observed results.

The diagrams included in the Discussion, illustrating the key discoveries described in Karolina Rojek-Sito's doctoral dissertation, significantly enrich this section of the work and facilitate the comprehension of the discussed results. Numerous references to the literature (265 citations throughout the thesis) enhance the value of the dissertation. Noteworthy is the broad perspective on

AB

the literature related to the topic of the work, not only in terms of the number of cited articles but also regarding the time frames within which the referenced works fall (from 1965 to 2023, with the majority of references focusing on the most recent literature).

Summarizing, in her comprehensive doctoral dissertation, Karolina Rojek-Sito, using projection- and neuron-specific manipulations, described the complex neuronal mechanisms governing social interactions. Employing state-of-the-art techniques, the Author characterized the role of the complex network constituted by ACC, OFC, CeA and VTA, and revealed that these neuronal elements are critical anatomical substrate underlying the control of specific social behaviors. These highly important findings shed light on previously unknown neuronal circuits controlling various aspects of social interaction. Presented and wisely discussed results clearly distinguish the circuits responsible for the initiation of social interactions from those that facilitate their maintenance. Notably, the presented results indicate that the circuits underlying social interactions only partially overlap with previously documented circuits regulating food reward, significantly increasing our understanding of the complex neural basis of social behavior.

The minor editorial errors and the information I have highlighted as potential additions to the dissertation are shortcomings that, as a reviewer, I am obligated to address. However, they do not diminish my overall high evaluation of Karolina Rojek-Sito's dissertation. Consequently, I conclude that the doctoral thesis of Karolina Rojek-Sito fully meets the requirements specified in Article 187 of the Act of July 20, 2018, on Higher Education and Science (Journal of Laws of 2021, item 478, 619, 1630).

Rozprawa doktorska Karoliny Rojek-Sito w pełni spełnia warunki określone w artykule 187 Ustawy z dnia 20 lipca 2018 r. Prawo o szkolnictwie wyższym i nauce (Dz. U. z 2021 r. poz. 478, 619, 1630).

Anna Białek

25.08.2023

EVALUATION OF THE PhD THESIS BY Karolina Rojek-Sito

Central amygdala- ventral tegmental area – cortical circuits mediate initiation and maintenance of social interaction.

Conclusion of the evaluation: Positive.

Please find below the reasoning of my evaluation, of what I think is an exceptional PhD thesis, not only for its structure and formal presentation, but for the rigor, clarity and novelty of the results obtained.

As the author states herself, one very interesting approach of this thesis is that she manages to define circuits regulating appetitive social behaviours (social vs food) by their functional connectivity with other structures and not by the molecular identity of the cells, as most of other studies do.

The introduction is very well written and frames the problems addressed in a vast context of previous contributions. The first part balances exquisitely the big picture, with human and cognitive psychology concepts with experimental data and basic neuroscience research, in many different species. I really enjoyed reading the introduction of this thesis, framing what is normal behaviour and what can be wrong in different conditions, such as autism spectrum disorder.

The second part of the introduction regarding neuroanatomical components of the social brain is a very complete and up to date presentation of what we know of several parts of the social network. It reflects the maturity and comprehensive knowledge of the candidate regarding her thesis theme. The introduction finishes with an interesting section discussing the differences between social and non-social reward, and how all these processes might be impaired in conditions with dysfunctional social interactions.

The research aims are clear and concise, being the objective of this work to dissect the different neural circuits engaged in the initiation vs maintenance of social interactions, and to study whether these circuits relate to social information, or they are modulated by general reward information, i.e. not specifically social. Thus, this thesis addresses a very interesting and important question: how social

interactions are instantiated at the level of multiple neural circuits, and further performs a very elegant and detailed set of loss and gain of function experiments in specific circuits.

The experiments and methodological approaches to address this important question are exceptional, and the dataset generated is clean and robust. This is a heroic amount of work, very exhaustive which provides an extremely valuable dataset. From these experiments and analyses, the author claims to identify partially different circuits that are involved in different aspects of social interactions. I would like to congratulate the PhD candidate, to have devoted so many efforts in dissecting specific aspects of social behaviour. These are not easy questions to tackle, and are not usually addressed, favouring simplistic approaches to social interactions, which are not good for the field. I thus really appreciate the efforts that the author has dedicated to make sense of this complex data set and present such an interesting PhD thesis work.

The PhD candidate starts by demonstrating the important role of the central amygdala (CeA) in highly motivated social interactions, i.e. after social isolation. Using a combination of neuroanatomy and optogenetic tagging and reactivation of behaviourally salient cells, she proves the existence of “social cells” in CeA. These cells are active during social interactions and when optogenetically re-activated, they increase the motivation to interact with conspecifics. Interestingly, CeA social cells are not coding for general reward or motivation, as they are specific for social interaction and not active, for example, during food-seeking tasks. She describes that the existence of an overlap between cells active for social or food rewards is not complete. Furthermore, the functional study of the projections coming from and projecting to CeA, and the neurochemical profiles obtained after social or food reward situations, reveals that the circuits are specifically involved in social events and independently encoding the initiation versus maintenance aspects of a social interaction.

Using viral strategies to chemogenetically silence specific neural circuits in a sequential manner, the author shows that dopamine neurons in the ventral tegmental area (VTA) are crucial for the initiation of a social interaction. The present PhD thesis expands previous findings and relates dopamine function in the VTA to the motivation to initiate an interaction through its projections to cortical areas (anterior cingulate cortex – ACC – and orbitofrontal cortex – OFC). Dopamine neurons in the VTA projecting to both ACC and OFC, and the projection from OFC to the CeA are necessary for the initiation of a social interaction, but not required for its maintenance. On the other hand, the maintenance of social interactions requires the activity of the neurons from ACC that project to CeA, and from there to the VTA.

The thesis finishes by a discussion section, where the author highlights her main findings and puts them in context, providing also additional information of the rationale of the experimental design.

In conclusion, this PhD thesis provides the first evidence that distinct neural circuitries mediate different aspects of dynamic social interactions. This is a very important discovery that opens the door to interesting new questions that might be addressed in the future by the host laboratory or inspire other scientists. Some of these possible questions are described by the author, in the last section of the thesis. The thesis is very well written and presented and represents a clear advance of the state-of-the-art in the field of social neuroscience.

Therefore, I recommend to the Scientific Council of the Nencki Institute to admit Mrs. Karolina Rojek-Sito to further stages of the procedure for conferral of a doctoral degree.

To whom it might concern,

I was asked to provide my opinion on whether this doctoral thesis should be considered as outstanding. In my opinion, there is no doubt that it deserves this recognition.

As explained in my report, this PhD thesis represents a clear advance in the knowledge in the field of social neuroscience. The results obtained are robust and interesting, and arise from very elegant experimental design, to address a very complex and unexplored question.

I am aware that the results presented in this PhD thesis are in press in a very relevant peer review journal in the field, which further support my opinion that this thesis deserves the recognition as outstanding.

In conclusion, yes, I recommend the recognition of this thesis as outstanding.

Kind regards

Cristina Marquez, PhD