Kraków, 12.02.2024

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Candidate's name and surname: Ilke Guntan
PhD Thesis Title: Characterisation of circadian dynamics of the transcription factor Zbtbt14 in naïve and epileptic mice
Thesis Supervisor: prof. dr hab. Katarzyna Łukasiuk
Reviewer: dr hab. Milena Damulewicz

THESIS EVALUATION

1. **Scientific merit of the thesis**

a. Originality of the research*:*

The circadian clock plays an essential role in the adaptation of organisms to environmental conditions. Specific cell types in the brain undergo rhythmic changes in their protein levels, which sustain daily neuronal plasticity, and regulate brain activity and its functions. It has been extensively documented that the disruption of the clock enhances the progression of numerous diseases, including, but not limited to cancer, cardiac disease, neurodegenerative diseases, and others. Additionally, there are strong evidences that epileptic episodes are connected with circadian disruption. The main objectives of the work that was submitted for revision were to ascertain the circadian dynamics of the transcription factor Zbtb14 in the brains of both control and epileptic mice.
Zbtb14 is expressed in many tissues, and it plays a role as both a positive and negative regulator of specific gene expression. It was shown that promoters of clock-controlled genes have a Zbtb14-binding motif. This motif was also overrepresented in the promoter regions of genes, with a changed expression pattern in the ventral hippocampus in the epilepsy model. Based on this information, I consider the subject of the work to be original, and the results obtained to be a good extension of the current knowledge in the field.

b. Scientific merit of the chapters*:*

The dissertation's scientific significance is derived from a novel and intriguing subject, coupled with a precise examination of commercially available anti-Zbtb14 antibodies. A detailed description of antibody validation has great value. Description of daily fluctuations in the levels of Zbtb14 protein and subcellular distribution in the hippocampus marks the commencement of a new chapter in the field. Furthermore, an investigation of the protein levels differences between control and epileptic mice provides important information about the possible role of the circadian clock in epileptogenesis. The presented results are very important, however I have some concerns which I depicted below.
In my opinion, some of the described findings are overinterpreted. For example, in the thesis, the expression of Zbtb14 was measured only under LD12:12 conditions. Statistically significant

differences between time points suggest daily changes, however it is not enough to interpret that these rhythms are circadian. To confirm clock-dependent regulation, experiments need to be performed in constant darkness (rhythms are still observed) and in clock mutants (in most cases there is no rhythmicity). However, in the thesis, there is no discussion of how these rhythms could be regulated, i.e., by light or other environmental conditions. Similar in epileptic study – only two time points are checked and compared between control and epileptic mice. Observed changes in protein levels are interpreted as changes in rhythmicity, but there is no information whether mice after treatment with pilocarpine were still rhythmic (PER expression, locomotor activity), there is also no discussion about how the rhythm could be changed (arrhythmicity, phase shift, changes in the amplitude).

2. **Substantial merit of the thesis and critical notes:**

Chapter 1 - Introduction takes 11 pages and describes very briefly the circadian clock, epilepsy, the Zbtb14 transcription factor, and the role of the hippocampus in epilepsy and circadian rhythms. I have concerns about citations, as this part is based mostly on reviews, not on original papers. This citation style causes many misunderstandings, i.e., the term “circadian” was not created by Vitaterna in 2001, but by Franz Halberg in 1959, light as Zeitgeber was described by Jurgen Aschoff in 1960, not by Redfern in 1991. There is no information about the scientists, who described the first clock genes and mechanisms: Konopka, Benzer, Takahashi or the work of Michael Rosbash, Michael Young and Jeffrey Young which was honoured with the Nobel Prize. In this chapter, it would be good to add information about the rhythms observed in the brain, such as neuronal plasticity, daily changes in neurotransmitters release, etc. In the chapter 1.3, which describes the link between clock and hippocampus, only daily changes in energy needs are described. What about other circadian rhythms in this structure? Only the central oscillator is mentioned in the thesis, what about peripheral oscillators located in the brain? What is the role of melatonin in the regulation of hippocampus physiology? What are the known molecular reasons of epilepsy? What is the role of glia in epilepsy?

In Chapter 2 – the Doctoral Student presents the aim of this study, which is to investigate circadian changes in Zbtb14 levels in the hippocampus of naïve and epileptic mice. Hypotheses are clear, however, they could be more precise.

Chapter 3 (Materials and Methods) is well prepared. It presents a complete list of chemicals, reagents, and recipes used in experiments, with the name of the manufacturer and catalog number. However, the host animal is missing for few antibodies on the list. In my opinion there is no need to repeat the same information many times, like that a red light was used to collect samples during the dark phase, number of animals in cage, etc.

On page 23, paragraph 2, there is information that ZT0 in the Nencki Animal Facility is at 7AM and in paragraph 3 that ZT0 is at 7.30AM, this should be specified. In chronobiology this information is very important.

As I’m not familiar with epilepsy induction in mice, could The Doctoral Student explain what is the rationale of collecting material three months after epilepsy induction. After recovery from the initial period of seizure activity, pilocarpine-treated animals develop spontaneous seizures a few weeks later. During this latent period prior to the development of spontaneous seizures,

the hippocampus, undergoes many changes. Are these spontaneous seizures still observed after three months?

The methods are described in details. However, I'm not convinced of the control for immunostaining and western blot. Why were sections/membranes incubated with recombinant protein, followed by secondary antibody? Why instead of this procedure, recombinant protein was not run on the gel as a positive control?

Description of double staining is confusing: "…biotinylated anti-rabbit secondary antibody was diluted as primary secondary antibody solution. Then it was incubated with fluorescein Avidin D. Next, it was incubated with a secondary antibody (which one?) the following day the secondary antibody anti-NeuN was used. And finally, Alexa fluor anti-mouse secondary was used as secondary". In this protocol there is one primary antibody and a lot of secondary antibodies. The definition of primary antibody is that they bind to the antigen, which means that anti NeuN and anti-GFAP are primary, not secondary. What does it mean "primary secondary"?

One of the techniques used in the thesis was the quantification of the density of Zbtb14-positive cells. What is the justification for counting Zbtb14-positive cells at different time points? Is there any possibility that cells in the hippocampus can switch on and off the expression of this TF at a specific time point? In my opinion, it would be better to measure fluorescence intensity per cell, which could give not-direct information about protein level.

In the Method section, there is description of NISSL staining, however there are no results for this technique. Which experiment was performed with NISSL staining?

In the Chapter 4, the results are presented. This part takes 65 pages, however the first 56 pages describe antibody optimization with detailed protocols. Nine different commercially available antibody were checked using western blot and immunostaining, which is hard work. The data presentation here is very clear, every figure has representative pictures with a short summary table showing changes in the basic protocol.

For this Chapter I have a few questions: it is not clear to me why after first unsuccessful trial with selected antibody (no signal, non-specific signal, etc.) it was continuously characterized. Why during protocol optimization different strategy was used for every antibody – antigen retrieval, aceton fixation, etc. In case there is no signal, the easiest way is to increase the concentration of primary antibody or to lengthen incubation time, up to few days – why this optimization was not used?

Comments for Immunostaining: In the figure description showing microscope images, the scale is missing. To have better resolution and less background signal, it is better to use a confocal microscope instead of a fluorescent one. The other way to have a more specific signal in the brain tissue is to use red-shifted fluorescence, which is associated with reduced background autofluorescence and better tissue penetration. To confirm there is no staining in the astrocytes it would be good to use higher magnification. It is not explained why staining for NeuN was done at 3PM and for GFAP at 3AM? To conclude that there is no expression in glia it would be good to check different time points as the level can oscillate with the daily pattern.

The data in Fig. 4.33 show representative images of anti-Zbtb14 immunostaining at three time points. What is the rationale for focusing on these specific time points (in the previous data, 11AM was included). On the representative images of somatosensory cortex, the fluorescence

intensity seems to be higher at 3AM than 11PM. Is there any difference in the fluorescence intensity between time points? Are there observed changes in the location of protein from the nuclear to the cytoplasmatic compartment at different time points?

Comments for Western blot: It is not explained why protein isolated from the heart was used as a positive control and not recombinant protein used as immunogen during antibody production? Why the dot blot is the last step of western blot optimization. It would be faster and easier to start with this technique to pre-select antibody for the next trials. During optimization, different protocols were used, however I'm not sure why, just after the first trial, the procedure with primary antibody omitting was not used? What is the rationale for additional trial in which antibody or recombinant protein were stored separately overnight at 4 degrees and then mixed and used for incubation with the membrane? Please comment on the result presented in figure 4.2 B ii – why is there no signal on this membrane? Figure 4.14iii suggests that the secondary antibody NA931 gives a nonspecific signal, so what is the rationale that it was continuously used in the next trials (Figure 4.17, the same picture is used in Figures 4.14 and 4.17). After a long and detailed procedure of antibody validation, the selected one used for Western blot showed one band around 55 kDa. However, in Fig. 4.34. in the nuclear extract there are 3 bands for Zbtb14. Please comment.

In figure 4.29 on the left - in the ELU1, there are two bands, please comment. It is not clear to me what is the purpose of immunoprecipitation, as purified protein was not used for further analysis. Is this another way of antibody validation?

RNAseq data are very interesting, and broadly commented in the discussion part. However, what is the rationale for doing this experiment on rats, while all others were done on mice?

My biggest concern is about the circadian aspect described in the thesis. To be called circadian the rhythm must show an endogenous free-running period that lasts about 24h and it must be clock-dependent. In this thesis all experiments were done only under LD12:12 (12h of light, 12h of darkness) conditions, in effect observed statistically significant differences between time points indicate daily changes, but there is no proof they are circadian. To confirm their circadian nature, experiments in DD (constant darkness) and with clock mutants needs to be done. The other aspect is circadian terminology used in the thesis. First of all in chronobiology Zeitgeber time (ZT) is used as a quantification of time, where ZT0 means the time when the lights are on, and ZT12 means the time when the lights are off. Using ZTs simplifies data presentation and analysis, as ZTs do not depend on local time or the specific rules in animals facility. In this thesis all time points are presented at local time, which is confusing. In addition mice were kept in two different animal facilities with different time of lights-on. In this way, samples collected at 3PM correspond to ZT8 in one place and ZT7,5 in the second one. Information at page 96 "The data are presented starting from when the light was turned on, i.e. 11AM" is confusing, as it suggests that 11AM corresponds to ZT0, which is not true. There is a difference between the circadian clock and the circadian cycle – in the text PhD Candidate uses there terms as equals, i.e. "The circadian cycle is crucial for the entrainment of the self-regulated endogenous clock of the organism, which creates the circadian rhythm within the organism". Clock is entrained by Zeitgebers, not by circadian cycles. The other example: "arrhythmic circadian expression of genes…" – expression is circadian or arrhythmic, it cannot be both at the same time.

It was shown in the thesis that there is daily rhythmicity in the Zbtb14 protein level in the cytoplasm and nucleus. This result is very interesting, the maximal level of protein was

observed in the middle of the day, followed by the minimum at the beginning of the night, when in the nucleus the maximum was detected. This pattern is very clear and suggests also rhythmic transport to the nucleus. However, I don't agree with the conclusion that circadian dynamics of Zbtb14 (which is not confirmed) is perturbed by epilepsy. First of all, only two time points were checked. Second, it is difficult to compare data from studies in which nuclear and cytoplasmic fractions were measured separately with samples of whole tissue in epileptic study. Third, there is no data about possible changes in rhythmicity or molecular clock machinery after seizures.

The PhD Candidate suggests that transport can be controlled at the level of translation or degradation. This point could be discussed in details, because the level of protein is controlled by translation or degradation, but it is not clearly described how it works with transport.

The data presented in the thesis show that Zbtb14 reached the maximum level in the nucleus at the beginning of the night, which suggests that expression of target genes is enhanced during the night. This point could be discussed in details.

Editorial comments:

In Fig. 4.34 description in the legend "the data are expressed as multiples of the relative ratio at 11AM" is confusing, it would be more clear "the data are normalized to time point 11AM expressed as 1".

On page 46 there is description of western blot for Aviva antibodies with the phrase "the cells were incubated…", instead of "membrane was incubated…"

On page 99: What does it mean "the data are presented as multiples of the first time after the light was turned on"? The standard time point description in chronobiology is Zeitgeber time (ZT).

In the description of dot blot there is information that 3 samples were spotted and 4 samples are listed - the last one is doubled.

3. Final grade:

Despite my concerns I found this thesis to be interesting study, carried out with appropriate methods. The results are clearly presented and commented. PhD Student shows good theoretical background in the neurobiology. As a result, original insights into the link between the hippocampus and epilepsy have been obtained.

I, hereby, declare that the reviewed PhD thesis by **Ilke Guntan** meets the criteria pursuant to art. 187 of Act of 20 July 2018 on Academic Degrees and Academic Title and Title in the Arts (O.J. 2023, no 742 as amended) and request that the Research Discipline Council of the Nencki Institute of Experimental Biology, accepts **Ilke Guntan** for further stages of doctoral proceedings.

Rozprawa doktorska spełnia warunki określone w art. 187 Ustawy z dnia 20 lipca 2018 r. Prawo o szkolnictwie wyższym i nauce (Dz. U. z 2023 r. poz. 742 z późn. zm.). W związku z

powyższym, wnioskuję do Rady Naukowej Instytutu Biologii Doświadczalnej o dopuszczenie mgr **Ilke Guntan** do dalszych etapów postępowania w sprawie nadania stopnia doktora.

Dr hab. Milena Damulewicz

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Warszawa, 19.02.2024

EVALUATION OF THE DOCTORAL DISSERTATION OF İLKE GÜNTAN

entitled "Characterisation of Circadian Dynamics of the Transcription Factor Zbtb14 in Naïve and Epileptic Mice" performed at the Nencki Institute of Experimental Biology under the supervision of Prof. Katarzyna Łukasiuk

Epilepsy is a common and serious chronic brain condition comprising heterogenous syndromes and unprovoked, recurrent seizures that affect 7.6 per 1000 people across the globe. Nearly 75% of epilepsy cases emerge in childhood and is a lifelong condition. The high prevalence rates, mortality, and costs to society emphasize the urgency required to develop targeted therapeutics for drug-resistant epilepsy.

Bidirectional interactions between epilepsy and sleep have been already recognized and patients with epilepsy often have associated co-morbid major sleep disorders. On the other hand, sleep disruption is one of the major risk factors for the likelihood of recurrent seizures and the circadian rhythm seems to influence the timing and the severity of epileptic seizures. Moreover, the core-clock genes not only regulate the circadian rhythms but also could contribute to epileptic susceptibility. However, the molecular mechanisms of the relationship between circadian rhythms and epilepsy are not fully understood to date. Unravelling the underlying mechanism of these relations may lead to new treatment strategies for this disorder.

Circadian clock is an autoregulatory feedback loop system consisting of transcriptional activators and repressors which was clearly presented in Figure 1.1. in the assessed dissertation. Core circadian clock genes include *period* (*Per 1, 2, 3*), cryptochrome (*Cry 1, 2*), circadian output cycles kaput (*clock*), and brain and muscle ARNT-Like 1 (*bmal1*). When *clock* and *bmal1* are expressed, they heterodimerize before translocating back to the nucleus where they stimulate the expression of *Per 1, 2, 3*, and *Cry 1, 2*. *Per* and *Cry* heterodimerize before they translocate back to the nucleus to suppress the expression of *Clock/Bmal1*, thereby halting their own expression

in a negative feedback loop that takes approximately 24h to complete. Although altered expression of key clock genes is observed in human epileptic tissues, the molecular mechanisms responsible for these alterations remain poorly explored.

In examined Ph.D. study Author characterize the protein level of a transcription factor, member of the Zbtb protein family, zinc finger and BTB domain containing 14 (Zbtb14) in naïve mice and analyse the oscillatory pattern of this protein in the hippocampus of epileptic mice, to verify the hypothesis that oscillatory pattern of Zbtb14 is disrupted by epilepsy pathology. Therefore, in my opinion, the subject of dissertation is innovative and important for the development of research aimed at explaining the relationship between circadian rhythmicity abnormalities and epileptic seizures. Elucidation of this hypothesis may present new therapeutic avenues for these devastating conditions.

The dissertation has a classical layout and is divided into six major chapters: Introduction, Aim and Hypothesis, Materials and Methods, Results, Discussion, Conclusion and Limitations followed by Bibliography. It begins with the list of abbreviations followed by an Abstract in English and Polish.

In a short and compact Introduction, which is 11 pages long, the doctoral candidate a briefly discusses biological background of the circadian rhythm. Then, the second part of Introduction concerns various aspects of alterations in circadian clock gene expression in epilepsy and therefore could be titled e.g. *Alteration of circadian clock gene expression in animal models of epilepsy* instead of just *Epilepsy*. This subsection lacks even a brief description of the pilocarpine (Pilo) model of epilepsy, that was employed in this dissertation. Although the author described epilepsy induction in the Methods section, the model used should be thoroughly characterized in the Introduction. Over the years, many animal models have been developed to reflect the three phases of human epileptogenesis, the most frequently used involve systemic application of chemical convulsants – pilocarpine (Pilo) or kainic acid (KA). Both models replicate the main features of clinical temporal lobe epilepsy (TLE). However, Pilo has the ability to induce the status epilepticus (SE), faster than KA leading to a higher frequency of seizures, and a much earlier, more pronounced neuronal loss making it the preferred model for research into early changes in TLE. Then the author moves on to the role of the hippocampus in epilepsy and circadian rhythm, since the association between epilepsy and the hippocampus is well known and important. The next section contains a competent, comprehensive and well-balanced literature review on the role of Zbtb14 protein in the immune system regulation and transcription of key synaptic proteins.

The objectives of the work and hypothesis are clearly stated and followed by an extensively descriptive chapter of methodology, subdivided into two main sections, Materials and Methods. All reagents, chemicals, enzymes, and materials, kits, antibodies, antigens and sera, solutions and buffers as well as equipment used in the experiments were characterised with extreme care and detail. Clearly and well described methodology is based on immunochemical methods including immunofluorescence, Western blot, and immunoprecipitation techniques. Concerning methodology, I would like to ask the candidate, whether she took part in the epilepsy induction or whether received isolated brain structures. Additionally, candidate employed *in silico* analysis to gain insight into the potential transcriptional targets of the Zbtb14 protein.

The Results chapter (66 pages) is subdivided into three major sections, Antibody Optimisation, Immunoprecipitation Validation and Circadian Rhythm Experiments. The Discussion chapter of the dissertation contains 13 pages and is followed by Conclusion and Limitations. The Thesis contains overall 127 pages, including 36 Figures (1 in Introduction and 35 in Results) and 1 Table (in Results), and ends with bibliography, that contains well-selected and newest literature items.

Consistently pursuing her goals, the doctoral student begins her research with the testing of nine antibodies available on the market produced against the Zbtb14 protein: ab110904, ARP33497_P050, SAB2106303, SAB1400299, ARP38308_P050, SAB1402396, HPA070819, sc-514298, and HPA050758. In order to select the appropriate antibody, candidate performed extremely tedious and long-term research using both the standard protocols for immunofluorescence staining and Western blot analysis as well as modified manufacturers' protocols. This approach to the problem proves her great scientific maturity. Ultimately, she selected the sc-514298 antibody as a Zbtb14 protein specific that gives a band just above 55 kDa, higher than expected. Could the doctoral student explain why she observes the band at a different height than expected? She did not refer to this in dissertation. The specificity of the sc-514298 primary antibody was confirmed by dot blotting assay. Then, precipitation of the Zbtb14 protein with sc-514298 was confirmed with Western blot. Likewise, HPA050758 antibody detected Zbtb14 protein. Although this part of the research is very important, in reviewers' opinion, it is rather a methodological part and could be moved to the Materials and Methods section.

In the next, most interesting part of the dissertation, the doctoral student seeks answers to the following questions:

1. What type of cell does the Zbtb14 express in the brain?
2. Does subcellular localisation of the Zbtb14 protein change over the circadian cycle?
3. Does the Zbtb14 protein oscillate over the circadian cycle?
4. If the Zbtb14 protein oscillates over the circadian cycle, is the expression of Zbtb14 perturbed by epilepsy pathology?

Immunofluorescence double staining with the anti-Zbtb14 antibody HPA050758 and the anti-NeuN antibody ABN91 showed that Zbtb14 is mainly neuronal protein and not expressed in GFAP-positive astrocytes. The level of Zbtb14 protein significantly change over the circadian cycle in the brain region specific manner and oscillates in the ventral hippocampus but not in the dorsal hippocampus nor in the somatosensory cortex. The most important results showed that expression of Zbtb14 protein is affected be the temporal lobe epilepsy. At 11pm, expression of the Zbtb14 protein was significantly higher in epileptic animals than in controls. Moreover, circadian oscillation was observed exclusively in epileptic mice, the expression of the Zbtb14 protein in epileptic animals was significantly higher at 11 pm than at 3 pm, however, subcellular fractions (cytosol, nuclear fraction) were not examined.

Furthermore, the data in the *in vitro* model of epileptiform discharges revealed downregulated genes with ZF5 motifs on their promoters, that are involved in synaptic plasticity regulation and transmission.

Therefore, I would like to ask the doctoral student to make a comment, whether Zbtb14 protein could be responsible for synaptic malfunction in patients with epilepsy.

Having regard to the above, I conclude that these results provide new data and contribute to the progress of the knowledge about the rhythmic patterns in epileptic activity and seizure occurrence. Although the research requires continuation, the assessed doctoral dissertation is an original solution to a scientific problem and reveals changes in the Zbtb14 protein level in animal studies under epilepsy and circadian rhythm.

In this doctoral dissertation, the objectives were clearly formulated, which were achieved using appropriate research techniques and an established animal model. Obtained results are presented and discussed in an interesting and competent manner. The Introduction and Discussion are thoughtful and prove a broad knowledge of issues within the field of study. Ms. İlke Güntan introduced herself as an experienced researcher who is perfectly capable of designing and conducting experiments and drawing correct conclusions. Therefore, she has the ability to independently conduct scientific work. The dissertation presents the good theoretical knowledge of the doctoral student as well as ability to solve research problems. The work was prepared very carefully, I found only one editorial mistake, on page 101 Table 3.85 should be marked as 4.1.

Based on the conducted formal analysis, I conclude that the reviewed doctoral dissertation of M.Sc. İlke Güntan meets all of the criteria set for a doctoral dissertation (Rozprawa doktorska spełnia warunki określone w art. 187 Ustawy z dnia 20 lipca 2018 r. Prawo o szkolnictwie wyższym i nauce (Dz.U. z 2023 r., poz. 742 z późn. zm.)). On this basis, I submit to the Scientific Council of Nencki Institute of Experimental Biology in Warsaw for the admission of Ms İlke Güntan for further stages of the doctoral thesis.

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Warszawa, 2024-02-20

REVIEW

of the doctoral dissertation by İlke Güntan entitled: "Characterization of Circadian Dynamics of the Transcription Factor Zbtb14 in Naive and Epileptic Mice"

Doctoral thesis by İlke Güntan was conducted at the Laboratory of Epileptogenesis of the Nencki Institute of Experimental Biology Polish Academy of Sciences in Warsaw. The supervisor of this thesis is Professor Katarzyna Łukasiuk. The study received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie COFUND grant agreement no 665735 and Polish National Research Centre grant Harmonia agreement no UMO-2015/18/M/NZ3/00779.

Epilepsy and seizures are among the most common neurological disorders. Progress in the treatment of this disease associated with the introduction of many new antiepileptic drugs, as well as other treatment methods such as specific diets (ketogenic diet) and surgical methods, is undeniable and has allowed many patients to become seizure-free. However, a certain percentage of patients still fail to achieve full control over seizures. It seems that the reason for this situation is the not fully understood etiology of seizures and epileptic attacks.

The doctoral thesis submitted for review addresses the significance of the regulator of biological rhythms, specifically attempting to characterize the circadian dynamics of one of the identified and prominent transcription factors in epilepsy – the Zbtb14.

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The topic undertaken is very interesting, although the phenomenon of rhythmic occurrence of epileptic seizures has been known for a long time. It is a very important topic from the perspective of treating patients, in whom despite effective treatment for most of the time, periods of frequent seizures occur, which are not related to errors in drug administration or other provoking factors such as sleep deprivation or exposure to proconvulsive factors. Additionally, the issue of a certain tendency for more frequent occurrence of epileptic seizures at specific times of the day is also a subject of discussion, especially since in clinical conditions the diagnosis of a certain type of epileptic seizure takes into account their greater tendency to occur at specific times, for example, at night after falling asleep.

The doctoral thesis consists of 127 pages and has a classic layout. It includes of the following parts: abstracts (in Polish and English), introduction, aim of the work, materials and methods, results, discussion, conclusion and limitations, bibliography, and list of figures and tables. While the general layout raises no objections, unfortunately, the conclusions section contains more of a summary of the results than actual conclusions. I suggest creating bullet points outlining the conclusions drawn from the experiments. It would also be beneficial to provide numbering for the bibliography.

According to the declaration of the PhD student, experiments conducted at the Nencki Institute did not require the approval of the Bioethical Commission, whereas for experiments conducted in France, such approval was obtained. Taking these facts into account, it is requested to clearly indicate which experiments were conducted in Poland and which in France, preferably in the form of a table, and justify why experiments in Poland did not require approval from the Bioethical Commission. A copy of the approval from the Bioethical Commission at INSERM should also be attached to the thesis, and its number should be included as an appendix to the text of the thesis.

The introduction is divided into 4 main subchapters, in which circadian rhythms, mechanisms of epilepsy, the role of the hippocampus in epilepsy and circadian rhythm, and the Zbtb14 protein were discussed. The introduction is exhaustively written and provides a good justification for the chosen research topic. It also refers to the latest literature related to the topic. However, the PhD student did not avoid certain flaws in this part of the thesis. Some statements contained in the introduction are of a less scientific nature and rather refer to common knowledge, for example: "epilepsy is not contagious and can affect people of all ages." In my opinion, the doctoral thesis is a specialized work, in which including such statements is unnecessary. Moreover, the structure of the introduction should, in my opinion, have a slightly

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different order, starting with descriptions of epilepsy and then moving on to issues related to the regulation of circadian rhythms.

The next chapter is the section "Aim and Hypothesis," consisting of two subchapters. The aim of the research, as mentioned above, was an attempt to characterize the circadian dynamics of one of the identified and prominent transcription factors in epilepsy – the Zbtb14 in brain structures of significant importance in epilepsy (hippocampus and somatosensory cortex) in a preclinical model of epilepsy.

The next chapter is the materials and methods section. I must admit that I have not encountered such a way of presenting a list of materials used as a very detailed list, sometimes overly detailed. Some of the reagents are standard, and in my opinion, do not require such detailed listing. Nevertheless, the way of presenting the materials used, although somewhat surprising, is correct and can serve as a good instruction for people wanting to use the methodology used in their own research. The methods are described properly, in detail, allowing for the replication of the research, although some information is missing. In the materials section, the number of animals used in the research should be provided, as well as a brief justification, why the particular model was chosen. Additionally, I suggest using international names of substances used in experiments instead of trade names: for example, instead of "Morbital," it is better to write "pentobarbital." The research methodology is adequate to the adopted objectives. The choice of research methods, as well as the way the experiments were conducted, does not raise significant objections.

The results of the research were divided into 3 main subchapters: antibody optimization, immunoprecipitation validation, and a section discussing circadian rhythm experiments. The first two chapters could basically be included in the methods section due to the scope of the described actions (optimization of methods), which do not actually refer to the conducted research but rather to the proper selection of methods for the main analysis. However, the main section, referring to the research aim and hypothesis, should only be section 3. Corrections are needed in the numbering of subchapters in section 4.1.6. The subsequent subsections have numbers "3.1.6.x" and should have "4.1.6.x". After section 4.1.9.2, there is section 3.1.9.3. Detailed descriptions of the optimization of the antibody set and procedures should end with a summary of which ones were used for further research. At present, such information appears when describing individual antibodies, which somewhat complicates navigation through the material.

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As a result of the conducted research, it was shown that: a) Zbtb14 protein is expressed exclusively in neurons; b) the Zbtb14 protein oscillates through a circadian cycle in the ventral hippocampus only but not in the dorsal hippocampus nor in the somatosensory cortex; c) the oscillation of the Zbtb14 protein occurs in both the cytoplasm and nucleus but in a different pattern; d) the genes that are downregulated in the in vitro model of epileptiform discharges have a ZF5 motif in their promoters, these downregulated genes that are potentially regulated by the Zbtb14 transcription factor mostly play a role in synaptic plasticity and transmission, e) the circadian dynamics of the Zbtb14 protein are changed by epilepsy pathology in an in vivo model of epilepsy.

The results are presented clearly, however, some doubts arise. Analyses were performed in the left or right hippocampus depending on the designations. Are there data that the expression of individual labelled molecules is not lateralized? If not, it is worth noting this in the thesis to avoid doubts.

Referring to the research questions, the PhD student decided to indicate which cells express the Zbtb14 in the brain, but the analysis concerned hippocampi and somatosensory cortex, not various regions of the whole brain. Therefore, it is more appropriate to specify that the expression was determined in cells in the examined regions of the brain, i.e., hippocampus and somatosensory cortex. In the description of the expression of Zbtb14, the text does not specify all the time points at which the evaluation was conducted. On the graphs, however, the time points are clearly indicated. It is worth supplementing the descriptions in the text so as not to raise doubts. In the final stage, the expression of Zbtb14 protein was analyzed in the temporal lobe epilepsy model. A different response in Zbtb14 expression was observed in epileptic animals in comparison to control, in the dark phase (11 PM).

Analyzing the obtained data raises some the questions. Why was the pilocarpine model used as the sole in vivo epilepsy model? Were spontaneous seizures observed in animals classified as "epileptic"? There is a risk that the assessment was made in animals at a different time from the seizure. Only 2 time points were selected, which was the reason for choosing these points. For a more comprehensive assessment, it would be useful to examine the Zbtb14 at several time points. Additionally, the expression value in the control group at 11 PM was almost twice as low as previously assessed in the nuclear fraction. What were the likely reasons for this phenomenon? It also seems to me that to complete the picture of changes, an assessment of the expression of the Zbtb14 protein in acute seizure model is needed, which would better correspond to the in vitro results as well as in other models of chronic seizures. The pilocarpine

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model itself is a very valuable model, but due to the fact that seizures are a consequence of the status epilepticus, it is associated with very large molecular changes.

The thesis concludes with a discussion, divided into 5 chapters, in which the Doctoral Student confronts the obtained results with data from current literature. The discussion is conducted competently. However, in some fragments there are alternative explanations to the provided hypotheses for the observed changes in the level of Zbtb14. For example, the lack of identification of a moment with zero expression of Zbtb14 in nucleus does not necessarily have to result from bidirectional transport, but for example, from a lack of more frequent sampling of Zbtb14 level, which is currently every 4 hours. The subchapter analyzing differences in the expression of Zbtb14 protein in the ventral and dorsal hippocampus focuses on anatomical and functional differences and relationships of these two regions, but does not discuss likely reasons why these differences in the expression of Zbtb14 occur in these regions and what functional significance it may have? The next subchapter of the discussion addresses the significance of changes in Zbtb14 expression in the epilepsy model, which ends with a rather controversial statement regarding the comorbidity of epilepsy, depression, and anxiety disorders. The pathogenesis of anxiety and depression disorders and epilepsy is much more complex than resulting from a simple translation of the hippocampus's importance in emotional disorders. Especially since, simply put, it may have a reactive nature to chronic disease, which we see in the case of many other chronic conditions, e.g., in diabetes. Finally, I missed a clear indication of what significance the increase in Zbtb14 expression in the hippocampus has for seizures?

The final chapter of the thesis is the section "conclusion and limitations" divided into two subchapters: "conclusion" and "limitations." In both subchapters, the Doctoral Student described a summary of the obtained results and limitations associated with the interpretation and scope of the obtained data. As mentioned above, the conclusions section should rather briefly indicate in a few points the most important achievements related to the conducted research, to which I strongly encourage.

In the limitations section, the PhD Student points out the most important area, which is the ventral hippocampus as a result of Zbtb14 action, however, the research was not planned to analyze many other structures critically important in epilepsy, hence I suggest caution in indicating the hippocampus as the most important place where circadian regulation occurs. It is better to indicate that this is one of the places that may be important. Referring to gender issues is undoubtedly important. Nevertheless, experiments were aimed at the TLE model, where the importance of gender is not as critical (at least from a clinical point of view) as in the case of

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other types of seizures, closely related to gender. In summary, it must be stated that some imperfections identified in the work do not diminish its value. The methodology of the research applied deserves special recognition.

PhD dissertation meets the requirements specified in Article 187 of the Act of July 20, 2018, Law on Higher Education and Science (Journal of Laws of 2023, item 742, as amended). Therefore, I request the Scientific Council of the Institute of Experimental Biology to admit Ilke Guntan to further stages of the procedure for obtaining the doctoral degree.

Rozprawa doktorska spełnia warunki określone w art. 187 Ustawy z dnia 20 lipca 2018 r. Prawo o szkolnictwie wyższym i nauce (Dz. U. z 2023 r. poz.742 z późniejszymi zmianami). W związku z powyższym, wnioskuję do Rady Naukowej Instytutu Biologii Doświadczalnej o dopuszczenie Ilke Guntan do dalszych etapów postępowania w sprawie nadania stopnia doktora.

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