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OPINION

From: Prof. Dr. Anton Bozhidarov Tonchev, MD, PhD – Department of Anatomy and Cell Biology, Medical University "Prof. Dr. Paraskev Stoyanov" - Varna, on a dissertation for awarding the educational and scientific degree **"Doctor"** in the field of higher education 7. Health and Sports, professional field 7.1. Medicine, **PhD Programme in Anatomy, Histology and Cytology.**

Author: Radoslav Hristov Spasov, PhD student at the Department of Anatomy and Cell Biology, Faculty of Medicine, Medical University "Prof. Dr. Paraskev Stoyanov" - Varna

Title: Role of Pax6 transcription factor in mouse cerebellar development

Scientific supervisor:

Prof. Dr. Anton Bozhidarov Tonchev, MD, PhD – Dept. Anatomy and Cell Biology, Faculty of Medicine, Medical University "Prof. Dr. Paraskev Stoyanov" - Varna.

1. Subject of review

By Order No P-109-182/08.04.2025 of the Rector of the Medical University 'Prof. Dr. Paraskev Stoyanov' – Varna, I was appointed as a member of the Scientific Jury for the procedure for the defence of a dissertation entitled *"Role of Pax6 transcription factor in mouse cerebellar development"* for obtaining the educational and scientific degree 'Doctor'/PhD in the field of higher education 7. Health and Sports, Professional field 7.1. Medicine, **Doctoral Programme "Anatomy, Histology and Cytology".** The author of the dissertation is Dr Radoslav Hristov Spasov, Anatomy and Cell Biology Department, Faculty of Medicine, Medical University 'Prof. Dr. Paraskev Stoyanov', Varna.

The presented set of materials on paper / electronic media is in accordance with the Procedure for acquiring the PhD degree at the Medical University - Varna and includes the following documents:

1. Dissertation.
2. Autoreferate.
3. Statement to the Rector of MU-Varna.
4. Autobiography.
5. A copy of a higher education diploma.
6. Order for enrollment of the doctoral student in the doctoral program at MU-Varna signed by the Rector of MU-Varna.
7. Meeting minutes of the Departmental Council for a doctoral minimum.
8. Meeting minutes of the Departmental Council with the right of PhD defence.
9. Order by the Rector of MU-Varna for assigning the doctoral student to the doctoral program at MU-Varna.
10. Declaration of originality of the documents.
11. List of scientific publications on the topic of the dissertation.
12. The scientific publications on the topic of the dissertation.
13. Statement of assurance of the attached documents.
14. Declaration of active profiles in the scientific database.
15. Similarity report prepared by the Publishing Department at MU-Varna.
16. Academic transcript of active scientific profiles in Google Scholar and ORCID published by the library at MU-Varna.

The PhD applicant has also applied 3 publications and 1 congress participation related to the dissertation.

The submitted documents **meet the requirements of the Law on the Development of Academic Staff in the Republic of Bulgaria and the Rules of the Medical University "Prof. Dr. Paraskev Stoyanov" - Varna for admission to the defense of the academic and educational degree "Doctor".**

Technical Data on the Text of Dissertations

The dissertation contains 144 pages, including 71 figures (most of which are multi-panel), 33 diagrams and 3 tables. Of the figures, 8 are in the Literature review section, 7 in the Materials and methods, 55 in Results and 1 in the Discussion. In total, 3 tables are presented: 2 in Materials and Methods, 1 - Discussion. The work contains 124 references.

It is structured in the following sections:

1. Introduction - 2 pages.
2. Literary Review - 21 pages.
3. Objective and tasks - 1 page.
4. Materials and Methods - 23 pages.
5. Results - 74 pages.
6. Discussion - 7 pages.
7. Conclusion - 1 page.
8. Summary - 1 page.
9. References – 9 pages.
10. Publications and meeting presentations - 1 page.

This structure and volume of the dissertation are in line with the requirements for awarding a PhD degree.

2. Short biographical data of the applicant

Radoslav Spasov was born in Kyustendil in 1988. He graduated from the High School of Mathematics 'Prof. Emanuil Ivanov' in Kyustendil in 2007, and from the Medical University 'Prof. Dr. Paraskev Stoyanov' in Varna, specialty 'Medicine' in 2014. From 2014 to 2015 he worked in the Intensive Care Unit of the University Hospital 'St. Marina EAD. Since September 2015, he has been a part-time assistant professor at the Department of Anatomy and Cell Biology at the Medical University of Varna, and since March 2016 he has been appointed as a full-time assistant professor at the same department. He was enrolled in the full-time doctoral programme at the Medical University of Varna in January 2018. Since May 2021 he passed a national examination for specialist in "Anatomy, Histology and Cytology". In 2022, he was awarded as the Best professor by the medical students at MU - Varna. He is fluent in English. Dr. Spasov's scientific interests are focused in the field of neurobiology.

Dr. Spasov is a Member of the Bulgarian Medical Association and the Bulgarian Anatomical Society.

3. Relevance of the topics and objectives of the PhD to the current scientific field

The cerebellum is an important region in the central nervous system that is involved in motor control. In recent years, there has been evidence of the involvement of the cerebellum in processes such as some types of memory and emotions. It is the brain region that contains over 70% of all neurons. There is

evidence that neurodevelopmental disorders (such as autism spectrum disorders) have been found to impair cerebellar development. Disturbances in the mechanisms of proliferation of cerebellar granular neurons are the basis for the development of medulloblastoma, the most common brain tumour in children. The above listed substantiates the need to study the mechanisms of cerebellum development, where transgenic mouse models represent a powerful tool for accumulating new scientific data. I therefore evaluate the topic of the dissertation as up-to-date and relevant to the modern knowledge in the field of morphological neuroscience.

The goals and objectives are clearly defined. **In the light of the methodologies presented, I consider the objective to be achievable.**

4. Knowledge on the scientific problem

The introduction clearly motivates the need for research in the field of dissertation work. In summary, the main structural elements of the cerebellar cortex are considered. The literature review, spread over 21 standard pages, shows the applicant's good awareness of the available literature sources on the topic of cerebellum structure and mechanisms of its formation, including chains of transcription factors that affect development. A special role of these transcription factors is played by Pax6, and the applicant has justified why Pax *6* is the target gene in this dissertation. I evaluate the applicant's handling of the large number of publications on the topic of mechanisms of small brain development, given the over 120 bibliographic sources presented.

5. Methodology of the study

For the dissertation were used tissues from experimental animals created and bred in Germany following clearance by a local ethics committee in the province of Lower

Saxony, observing the principles of minimizing animal suffering. The innovative Cre/LoxP technology for in vivo animal genetic modification has been used, in which the Cre recombinase enzyme, located behind a regulatory element of the *Pax6* gene so as to ensure tissue selectivity of expression in the cerebellum, recognizes specific (flox) sequences around the target *Pax6* gene. In this way, a specific gene silencing in the cerebellum is carried out, allowing the animals to experience the perinatal lethality characteristic of a *Pax6* global knockout. Overcoming perinatal lethality is key, as the neurogenesis of granular cerebellar neurons, the most numerous cell type in the cerebellum and throughout the nervous system, takes place after birth. For this reason, the *Pax6* global knockout leading to death at birth (due to causes associated with dysfunction in other organs) is not applicable in the study of granular cerebellar neurogenesis. Successfully achieving *Pax6* inactivation in the cerebellar cortex allows for the first time to observe in vivo the results of reduced *Pax6* function for the cerebellum.

The Materials and Methods section presents in detail histological (histochemical and immunohistochemical) methods of processing, visualization of samples and statistical methods of analysis.

Conclusion: The materials and methods are described in detail in a way that allows experiments to be repeated by independent researchers.

6. Characteristics and assessment of the dissertation

The results span 70 standard pages, which contain 55 figures and 2 tables. The figures are of very good quality and clearly visualize the thesis of the dissertation project. Many of the figures are composed by several panels, so the total number of figures is greater than 55. The results thus presented support the theses of the dissertation.

The results of the study provide data on the selection of Cre-recombinase action in the context of the particular experiment. Use of a reporter-line demonstrates that Cre has a predominant, almost exclusively selective, expression in the cerebellum. This proves that the inactivation of Pax6 is achieved in the target brain region of interest, in the zone of physiological expression of Pax6 in the external granule cell stream. The thesis then investigates the morphological changes in the cerebellum when *Pax6* function is disabled. The cortex area (grey and white matter) and the individual layers were measured in detail and the mutant reduction was calculated in relation to the control individuals. Quantitative characteristics of cerebellar neuronal types (glutamatergic and GABAergic) have been identified in mutants. Ectopic clusters of neurons in white matter in mutants have been described, suggesting migraine deficiencies not only of glutamatergic but also of GABAergic precursors flowing an inactivation of *Pax6*. The dissertation also shows some of the likely mechanisms by which the deficiency of Pax6 leads to the cerebellar malformations that are observed.

Conclusion: The results presented are of high quality and support the claims of the dissertation.

The discussion once again demonstrates the applicants' good literary awareness and analytical thinking. Own results are convincingly summarized and commented in the light of a thorough synthesis of familiar data in literature. Molecular mechanisms have been convincingly proposed by which *Pax6* influences cerebellar morphology.

7. Scientific contributions of the thesis

This dissertation is original in the study of the role of Pax6 in postnatal neurogenesis in the cerebellum. Since most cerebellum neurons are formed after birth, to date it has not been possible to determine the cerebellar deficits under reduced Pax6 function, because mice with a global *Pax6* gene knockout die at birth. This paper describes for the first time in the world the processes of reduced Pax6 function that occur after birth in a mammalian brain.

Conclusion: I believe that the contributions are sufficient to acquire the degree "Doctor"/PhD.

8. Assessment of the publications on the dissertation

The list of publications attached to the procedure for the acquisition of NSA Doctor includes 3 publications, supplemented by 1 participation in a scientific forum, on the topic of neuroscience. The dissertant should prepare a publication in a journal, indexed in Web of Science with the main results of the thesis.

9. Personal involvement of the author

As a scientific supervisor, I can testify that the esertant's participationin the study of morphological changes in the cerebellum after inactivation ofPax6 is **central,** and that the formulated contributions are **the personal merit** of Radoslav Spasov. The creation of transgenic mice was carried out at the Max Planck Institute in Göttingen, Germany.

10. Autoreferate

Andthe second abstract,presented together with the dissertation, ismade according to the requirements and correctly reflects the main results achieved in the dissertation.

CONCLUSION

The dissertation of Radoslav Hristov Spasov from the Department of Anatomy and Cell Biology of the Medical University 'Prof. Dr. Paraskev Stoyanov' in Varna is devoted to a problem that is extremely interesting from a medical and biological point of view. **The dissertant has presented a number of original results prepared as a result of the application of morphological and genetic methods. I believe that the dissertation fully complies with the ZRAS of the Republic of Bulgaria and the Regulations of the Medical University of Varna for the acquisition of the degree "Doctor"/PhD. I shall therefore vote <u>in favour</u> and allow myself to recommend the same to the other members of the honourable Scientific Jury created by Order No P-109-182/08.04.2025 of the Rector of the Medical University 'Prof. Dr Paraskev Stoyanov' – Varna.**

Author of Opinion:

Заличено на основание чл. 5, §1, б. „В“ от Регламент (ЕС) 2016/679

Prof. Dr. Anton B. Tonchev, MD, PhD

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Varna, 10.05.2025