

REVIEW

BY PROF. NIKOLAI ELENKOV LAZAROV, MD, PhD, DSc
ON THE DISSERTATION FOR
THE AWARD OF THE DOCTOR OF PHILOSOPHY (PHD) DEGREE
IN SCIENTIFIC SPECIALTY „ANATOMY, HISTOLOGY AND CYTOLOGY“

AUTHOR OF THE DISSERTATION: RADOSLAV HRISTOV SPASOV

**THESIS TITLE: „THE ROLE OF THE TRANSCRIPTION FACTOR PAX6 IN THE
DEVELOPMENT OF THE CEREBELLUM IN A MOUSE“**

SCIENTIFIC SUPERVISOR: PROF. ANTON BOZHIDAROV TONCHEV, MD, DSc

Radoslav Hristov Spasov is an assistant professor in the Department of Anatomy and Cell Biology at the Medical University (MU) "Prof. Dr. Paraskev Stoyanov" – Varna. Since 2021, he holds the rights of a specialist in the scientific specialty "Anatomy, Histology and Cytology". In 2018 Radoslav Spasov was enrolled as a regular PhD student on a full-time basis (Order No. P-109-72/15.01.2018) under the doctoral program "Anatomy, Histology and Cytology" at the same department, where he conducted the main part of the experiments in this dissertation. After completing the training program and successfully passing the exam in scientific specialty, according to the decision of the Faculty Council of the Faculty of Medicine, taken with protocol No. 82/20.02.2023, following Order No. R-109-178/01.03.2023 of the Rector of MU-Varna, he was expelled with the right of a public defense of his thesis before a scientific jury.

The dissertation entitled "*The role of the transcription factor Pax6 in the development of the cerebellum in a mouse*" is written on 144 standard pages, divided into sections as follows: *Title page*, *Contents* – 4 pages, *Introduction* – 2 pages, *Literature review* – 21 pages, *Purpose and objectives of the study* – 1 page, *Material and methods* – 18 pages, *Results* – 74 pages, *Discussion* – 7 pages, *Concluding remarks* – 1 page, *Conclusions* – 1 page, *Contributions* – 1 page, *Bibliography* – 9 pages including 124 titles, all in the Latin alphabet, *Publications and reports related to the thesis* – 1 page, *List of abbreviations* – 3 pages, *Acknowledgements* – 1 page. The results are illustrated with 55 figures, which are plates with a series of mounted photomicrographs and charts. In addition, the literature review includes 8 combined schematic images (with the original source credited) that support the historiographic data, the Material and Methods section contains 7 figures, including schemes, screenshots and heatmaps, as well as a list of antibodies used for immunofluorescence staining given in two tables, and the discussion is illustrated with one

comparative figure and one table with morphometric data for the established phenotypic variations in the developing mouse cerebellum. Thus, the actual number of the overall photographic documentation is considerably greater than that indicated in the Results section.

The topic of the dissertation is adequately chosen. It is dedicated to a socially significant and extremely topical problem of fundamental neuroscience and translational medicine – embryonic and adult neurogenesis. Although the cellular and molecular mechanisms that regulate cerebellar development in rodents have been well studied in recent two decades, relatively little is still known about the role of the transcription factor Pax6 in embryonic cerebellar neurogenesis, as well as its potential involvement in postnatal differentiation and migration of newly generated cerebellar cortical neurons. In this respect, the PhD candidate has set himself the ambitious goal of establishing morphological alterations in cell populations of developing cerebellum under selective Pax6 knockout conditions. In view of the currently known relationship between abnormalities in the proper formation of the cerebellum, due to silenced expression of Pax6, and the etiopathogenesis of some neuropsychiatric developmental disorders with great social significance, such as autism spectrum disorders, elucidating the potential functional significance of this transcription factor for cerebellar neurogenesis and postnatal differentiation of neuronal populations in the cerebellum would contribute to the development of new effective therapeutic approaches for these diseases.

The **introduction** is short, but informative enough to properly introduce the reader to the subject of the dissertation.

The **literature review** is detailed and gives, in thematically separated subsections, extensive information on embryonic development of the cerebellum in mammals and the molecular control of its embryonic neurogenesis, the selective markers of early progenitors in the two germinal zones of the cerebellar primordium, the ventricular zone and the rhombic lip, as well as the cell-specific ones for maturing cells in the developing cerebellar cortex and deep cerebellar nuclei are described in chronological order during embryonic development. The role and effect of the reduced expression of the PAX6 transcription factor in the generation of precursors of progenitor cells in early embryonic development and the potential consequences of its lack for their postnatal differentiation and migration is presented in detail. Convincing arguments have been provided to create a suitable experimental mouse model for exploring this phenomenon postnatally. Bibliographic data are appropriately illustrated with figures and schematic diagrams from the extant literature. Of the

bibliographic citations, almost half were published in the last two decades, incl. one article of 2024, which is another proof of the actuality of the research topic, the completeness of the reference list and, finally, the good literary awareness of the PhD student. In my opinion, it would be appropriate for the literature review to end with a separate section, or at least with a concluding paragraph, in which a short overview of the available information on the topic is concisely summarized. Thus, current state analysis of the problem in one paragraph at the end of the literature review would allow the PhD candidate to develop a good research hypothesis and to better formulate realistic research aims and objectives.

The **purpose** of the study is clearly and precisely defined. The **tasks** are specific and correctly set, and as will be understood from the subsequent discussion, they are feasible by means of the methods used.

The Material and Methods section is relatively short but comprehensive enough. For the study, a transgenic mouse line, produced by crossing of two genetic lines, was generated in accordance with the ethical requirements of the state of Lower Saxony, Germany, from which mutant mouse strains were obtained. The total number of experimental animals is not mentioned, but I accept *a priori* that the tissue samples taken are sufficient to achieve statistically reliable results. The description of the immunohistochemistry/immunofluorescence protocols as well as that of the performed imaging and statistical analysis of the generated digital images for assessment of the obtained morphometric data, are given with accuracy and step-by-step details, thus allowing their reproducibility by other researchers. The necessary information about the primary and secondary antibodies used in immunohistochemical staining, the host species, working dilutions and their manufacturer are given in summary tables. However, the text lacks the mandatory description of the applied stain controls, which are critical to the specificity of an immunohistochemical reaction.

The **Results** section is set out on more than half of the entire text. It is descriptively separated in subsections and presented in a sequence following the chronologically set experimental tasks. The presentation opens with a description of well-known expression patterns of Pax6 in the developing cerebellum, which serve as a basis for comparative assessment of quantitative changes in it upon silencing of its expression. For this purpose, the expression of Pax6 in the cerebellar germinal zones at different time intervals was compared with that of reporter genes from public

databases for gene expression. Using a broad panel of cellular and molecular markers for neurogenesis and gliogenesis, distinguishing individual subpopulations of cerebellar neurons, the distribution patterns, density and phenotype of Pax6-positive neural progenitor cells in the developing mouse cerebellum have been consistently delineated, the phenomenon of overall reduction in area and reduced foliations of the cerebellar cortex in mouse mutants has been described, a decrease in the number of neuronal subpopulations (granular and unipolar brush cells, Purkinje and Golgi cells) in the cerebellar cortex at the postnatal and adult stages of its development has been established due to the silenced expression of Pax6, and the effects of this transcription factor on the postnatal development and migration of cortical cerebellar neurons have been described. Specific quantitative data on selective postnatal dysgenesis in the cerebellum of a Pax6 knock-out mouse model, characterized by reduced cortical area, a thinned granular layer, cerebellar cortical neurons misplaced in the white matter and the presence of ectopic collections of neurons in it in the mutants are provided. At the same time, using statistical analysis software, Spasov has documented the significance of the obtained morphometric data.

Overall, I highly appreciate the fact that all author findings are supported by sufficient illustrative material, including high-quality representative photomicrographs and immunofluorescence microscopy images. On the other hand, statistical data in graphical form incorporated in the panel provided detailed and comprehensive information about the established quantitative indicators in the number and percentage ratio of immunopositive cells in the examined areas and time period.

The **discussion**, although not particularly extensive, is very well-written and shows the author's enviable skill in discussing his own results, comparing them with known facts in the relevant literature and adequately interpreting these to draw important conclusions about the postnatal cerebellar morphology in a Pax6 knock-out mouse model. It highlights the merits of the newly generated mutants, which allowed for the clear demonstration of the postnatal cerebellar phenotype in the selective Pax6 knockout. Probable causes of the misfolding of the cerebellar cortex in case of reduced efficiency of Pax6 function are discussed, including dysregulation of important signaling pathways. By comparing his own data on the reduced migratory and proliferative index of cells in mouse mutants with those obtained on cell cultures, a valuable conclusion was made about the extent of their postnatal differentiation after Pax6 knockout. Of

certain practical interest is also the discussion of the specific relationship between reduced Pax6 levels and cell death in the external granular layer, contributing to the clarification of the mechanisms of deviations from a typical development of the cerebellar cortex. Also noteworthy is the original suggestion of the real involvement of Pax6 in the mechanism of Purkinje cell migration in the postnatal period.

The analysis of data obtained and their in-depth discussion by the PhD candidate served as a good basis for precisely formulating the thesis **conclusions**. They are specific, informative, concise and accurately reflect the author's statements arising from the new data obtained. Generally, I accept their wording, credibility, and scientific value. The scientific achievements of the research are presented in a separate section. The main **contributions** of the dissertation, most of which possessing an original character, could be summarized as follows:

1. The present study is the first detailed examination of the effects of the transcription factor Pax6 on cerebellar development.
2. For the first time, an experimental mouse mutant model with selective Pax6 gene silencing in the cerebellum is demonstrated.
3. Original morphometric data on the effect of Pax6 on cerebellar morphology in the postnatal cerebellar development are provided.
4. The quantitative changes in the morphology and cellular composition of the cerebellum after the completion of its neurogenesis under global Pax6 knockout conditions are of innovative nature.

In the attached documentation a declaration of originality of the thesis work is included which gives me a reason to assume that the current dissertation is a personal work of Dr. Radoslav Spasov. The results of the research are published in three articles in referred scientific journals, one of which is in an impact factor journal positioned in the highest quartile Q1 of the journal ranking. However, in none of these articles is Stoyanov the first author. This also applies to the only scientific communication on the topic of the dissertation presented at an international scientific forum. In view of the topicality of the research and the originality of the data obtained, I would strongly advise the PhD student and his supervisor to continue promoting the results of this study in the international scientific periodicals.

The dissertation is written clearly and comprehensibly, although several spelling and/or

stylistic errors are noticed throughout the text. Only a few specific terms and abbreviations with their English abbreviations are used, which makes it easy for non-familiar with this topic readers to understand it. The **thesis abstract** is prepared according to the requirements. It adequately and sufficiently reflects the current state of the problem, the purpose and objectives of the study, the methods used for its implementation, the results obtained, their analytical description and interpretation of their own data, as well as the author's conclusions and contributions.

In **conclusion**, I found the dissertation of Dr Radoslav Spasov for a complex and in-depth study on a very interesting and topical problem of contemporary neurobiology. It is well thought out and precisely methodically justified, conducted very accurately, and illustrated excellently. The PhD candidate has clearly and thoroughly discussed the obtained data, and they contribute to expanding the available knowledge about cerebellar neurogenesis and its regulatory mechanisms. **Despite some shortcomings of the work mentioned above, the results of the study make an original and significant theoretical and practical contribution elucidating the factors and mechanisms of postnatal cerebellar development and, particularly, the role of the Pax6 gene on cerebellar morphology after the completion of cerebellar neurogenesis.** Without a doubt, a significant contribution to the successful project realization has also been made by the competent scientific guidance of the PhD supervisor, Prof. Anton Tonchev, who is a prominent expert in neurogenesis research. The present work meets the legal requirements for obtaining the scientific and educational PhD degree according to the Law on the Development of Academic Staff in the Republic of Bulgaria, the regulations on its implementation and the criteria in the Regulations on Academic Staff Development at the Medical University of Varna. All the above-mentioned reasons convince me as a reviewer to express a positive opinion for the thesis and as a member of the Scientific Jury to support with a positive vote the award of the educational and scientific degree "*Doctor of Philosophy (PhD)*" in the area of higher education 7. Healthcare and sports, professional field 7.1. Medicine, and in the scientific specialty "Anatomy, Histology and Cytology" to Radoslav Hristov Spasov.

Sofia, 12.05.2025

Reviewer:
(Prof. Nikolai E. Lazarov, MD, PhD, DSc)

Заличено на основание чл. 5,
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