# R E V I E W

**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“**
**PROFESSIONAL FIELD 7.1 MEDICINE**

**AUTHOR OF THE DISSERTATION: MARTIN NIKOLAEV IVANOV**
**THESIS TITLE: *„PROLIFERATION AND DIFFERENTIATION OF PROGENITOR CELLS IN THE SUBVENTICULAR ZONE OF ADULT PRIMATE FOREBRAIN“***
**SCIENTIFIC SUPERVISOR: ASSOC. PROF. STOYAN PAVLOV PAVLOV, MD, PHD**

Martin Ivanov is an assistant professor in the Department of Anatomy and Cell Biology at the Medical University (MU) "Prof. Dr. Paraskev Stoyanov " – Varna. From 01.06.2023 he holds the rights of a specialist in the scientific specialty "Anatomy, Histology and Cytology", as evident from the attached specialty certificate. In 2019 he was enrolled (with order No. P-109-81/01.02.2019) as a regular PhD student on a full-time basis 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 exams in scientific specialty and a foreign language, according to the decision of the Faculty Council of the Faculty of Medicine, taken with Protocol No. 11/23.10.2023, by order No. P-109-469/09.11.2023 of the Rector of MU-Varna, he was expelled with the right of a public defense of his thesis before a scientific jury, appointed by the same order.

The dissertation entitled "*Proliferation and differentiation of progenitor cells in the subventricular zone of adult primate forebrain*" is written on 147 standard pages, divided into sections as follows: *Title page, Contents* – 3 pages, *Dedication* – 1 page, *List of abbreviations* – 3 pages, *Introduction* – 2 pages, *Literature review* – 18 pages, *Purpose and objectives of the research* – 2 page, *Material and methods* – 29 pages, *Results* – 51 pages, *Discussion* – 15 pages, *Shortcomings of the study* – 2 pages, *Conclusions* – 1 page, *Contributions* – 1 page, *Publications and scholarly communications on the topic* – 2 pages, *References* – 14 pages including 179 titles, one in Cyrillic and 178 in the Latin alphabet, *Acknowledgements* – 1 page. The work is illustrated with 46 figures, most of which are plates with a series of

mounted photomicrographs and charts, so the photographic documentation is considerably greater. In addition, in the literature survey and the Material and Methods section, 13 figures (with the original source credited) and 3 tables are included, which support the historical data and clearly illustrate the experimental procedure, the methods applied, the genes studied, the antibodies and markers used.

The **topic** of the dissertation is adequately chosen. It is dedicated to an extremely topical problem of fundamental neuroscience and translational medicine – adult neurogenesis. Although the cell proliferation, migration and differentiation in the germinal zones of the rodent hindbrain have been well studied in recent decades, relatively little is still known about the fate of neural stem and progenitor cells, as well as the detailed mechanisms of ischemia-induced neurogenesis in primate and human brains. In this respect, the PhD candidate has set himself the ambitious goal of selecting and phenotyping candidate genes with potential expression in the primate neurogenic niche, examining and comparing their expression levels in one of its best studied regions – the subventricular zone of the lateral ventricle in the adult hindbrain of untreated Japanese macaques (*Macaca fuscata*) and monkeys with global brain ischemia.

The **literature review** is detailed and gives, in thematically separated subsections, extensive information on neurogenesis and neural stem cells, provides data on the composition of the subventricular zone and its morphological features in primates, on the role of brain ischemia in activating neurogenesis and the altered transcriptome in it, on the methods and experimental models for the study of neurogenesis. Literature data are appropriately illustrated with figures and schematic diagrams from the extant bibliography. Of the bibliographic citations, half were published in the last decade, incl. 19 articles from the last two years, which is another proof of the actuality of the research topic, the completeness of the reference list and, last but not least, the competency of the doctoral 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 made in a condensed form. Analysis data on the current state of the problem in one paragraph at the end of the literature review will 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 not very clearly defined. Instead, it is extensively described on more than one page, even containg information relevant to the Introduction and Literature review sections. The **tasks** are specific and correctly set. Perhaps, as a separate task, performing an image and statistical analysis of the obtained data could also be included.

The **Material and Methods** section is comprehensive and well written. In the course of the research, brain material obtained from six adult Japanese macaques and post mortem human brain tissue from three cadavers was used. Certainly, this number is insufficient for global conclusions at the population level, but given the ethical considerations in primate research, it is quite sufficient to achieve statistically significant results in a single study. The experimental procedures were approved by the ethics committee of the University of Kanazawa, Japan, where the global cerebral ischemia model was performed. Brain tissue for the *in situ* hybridization experiments was provided by the German Primate Center in Göttingen, Germany from macaques treated according to the national ethical guidelines for the use of animals in research. The use of human cadaveric tissue for the comparative studies was approved by the Committee on Ethics of Scientific Research (KENI) at MU-Varna. The description of all experimental protocols, incl. tissue preparation and processing, the surgical, immunohistochemical and *in situ* hybridization procedures performed, as well as the preparation of cDNA probes and template synthesis, the phenotypic analysis for identification of genes, acquiring and analysis of digital microscopic images, and the subsequent statistical data analysis are given with accuracy and step-by-step sequence, thus allowing their reproducibility by other researchers. The necessary and complete information about the reagents, primary and secondary antibodies used in the immunohistochemical experiments, the manufacturers and suppliers, the host and their working dilutions, as well as a list of the examined genes and the primers used for their visualization are given in overview tables. The text also presents a detailed description with illustrations of the applied controls for the specificity of the *in situ* hybridization and immunohistochemical reactions, incl. negative and positive controls, but no information is provided about the relevant antibody specificity tests.

The section **Results** covers one third of the entire text. Is was descriptively separated in subsections. However, in my opinion their wording is not particularly appropriate, since in

this section the specific results related to the expression, the gradient in the expression levels and the phenotypic characterization of the examined genes are described, while the analysis of the obtained data is the subject of the discussion. Also, in subsection 6.1. the method and criteria for selecting the studied genes are described, and this has already been done in the previous section 5.1. Using non-radioactive *in situ* hybridization with digoxigenin-labeled probes, the expression of Tenascin-C (TNC), Apelin Receptor (APLNR), Gap Junction Alpha-1 protein (GJA1) and CD (Cluster of Determination)38 in the subventricular zone of control and ischemic monkeys at the mRNA level, and by immunostaining with specific markers of neurogenesis, their expression was determined at the protein level. In ischemic animals, the observed hybridization signal was quantified with CellDetekt software as stronger in cells located in or beneath the ependymal layer as well as perivascularly, but with no apparent dorso-ventral gradient of gene expression. However, a distinct, but different among species, rostro-caudal gradient was found, and in the most rostral levels of the lateral ventricle, expression levels of TNC, APLNR, and GJA1 in the subventricular zone were comparable to those in the striatum, while that of CD38 was highest in caudal levels of the subventricular zone, but is relatively low in the striatum. Using immunohistochemical markers of proliferation, the PhD candidate has described the cell density of Ki67-immunopositive neurons along the entire length of the ventricle and found its marked decrease in the caudal direction. Comparative, incl. statistical data on the presence of significant rostro-caudal differences in the expression of the examined genes in rodents and monkeys were presented. By fluorescence in situ hybridization (FISH) and immunohistochemistry, a successful attempt has been made to phenotypically characterize subpopulations of adult stem/progenitor cells which express specific markers. Thus, it was found that in intact monkeys the TNC gene was expressed in a subpopulation of stem cells, whereas APLNR, GJA1 and CD38 were probably expressed by dormant neural stem cells, but during differentiation their expression decreased. In humans without concomitant pathology, the apelin ligand was present in glial and/or neuronal progenitors in the subventricular zone, and APLNR was expressed at all stages of their differentiation.

All author findings were supported by sufficient illustrative material including Venn diagrams and high-quality photomicrographs. On the other hand, statistical data in graphical form provided detailed and comprehensive information about the established quantitative indicators in the number and percentage ratio of labeled cells.

The **discussion** section is not particularly lengthy, but it is well written and shows the author's enviable skill in discussing his own results, comparing them with known facts in the relevant literature and interpreting them adequately to draw important conclusions about the proliferative, differentiation capacities and phenotype of stem/progenitor cells in the cerebral cortex under ischemic conditions. The discussion regarding the differential expression of the studied genes along the rostro-caudal axis of the lateral ventricle is also of certain practical interest. In the aspect of neurogenesis, the analysis of verified by *in situ* hybridization and immunohistochemistry subpopulations of cells in the one of the major neurogenic niche in mammals is particularly valuable, since it is quite possible that ischemia-induced gene expression in neural stem cells is an indicator of the level of their differentiation. Certainly, these data need to be confirmed by transcriptomic studies on isolated neural stem cells in primate brain. Future studies on the mechanisms of involvement of the examined genes at the molecular level, suggested in the concluding remarks of the dissertation, would clarify the possible application of the obtained data in the development of replacement cell therapies for neurodegenerative diseases.

Regardless of some objective shortcomings of the present study, correctly mentioned and discussed at the end of the text, the obtained data 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 study of gene expression in the largest neurogenic niche of the primate brain under conditions of global cerebral ischemia.

2. The expression of Tenascin-C, Apelin Receptor, Gap Junction Alpha-1 and CD38 proteins at the mRNA/protein level in the subventricular zone of the lateral ventricle in adult primates was experimentally demonstrated for the first time.
3. Original data on a rostro-caudal gradient and species differences in the gene expression in rodent and monkey brains are presented.
4. The thesis is a scholarly piece of work with a pioneering character, since the phenotypic characterization of neural stem cells in the subventricular zone of non-human primates and humans could serve to develop an effective replacement cell therapy in certain neurodegenerative and psychiatric diseases.

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. Martin Ivanov. An additional proof for my assumption are his scientific publications and communications on the topic of the dissertation. The results of the research are published in two articles in refereed scientific journals, one of which *Genes*, an impact factor journal positioned in the second quartile Q2 of the journal ranking. The Scopus reference shows that this article has received two citations in the international periodicals so far, most likely due to the fact that it was published recently. In one of the above-cited articles, Ivanov is the first co-author, and in the other one he is the second co-author. Nine communications at national and international scientific events are also presented, but the author names of these reports are not listed, which makes it impossible to specifically determine the personal contribution of M. Ivanov in them.

The dissertation is written clearly and comprehensibly, despite the difficult subject matter for a non-specialist to understand. Only single spelling and/or stylistic errors are noticed throughout the text but a large number of specific terms and abbreviations with their English abbreviations are used, which makes it somewhat difficult for the lay reader to read 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 Martin Ivanov for a complex and in-depth study on an interesting and topical problem of contemporary neurobiology. It is well thought out and precisely methodically justified, conducted very accurately and illustrated excellently. The obtained data have been clearly and thoroughly discussed and they contribute to expanding the available knowledge about adult neurogenesis. The results of the study make an original and significant theoretical and practical contribution elucidating the factors and mechanisms of proliferation and differentiation of neural stem cells in the cerebral cortex of primates and humans, the gene expression of certain genes and the relationship of their altered expression levels after ischemic damage in the subventricular zone with the process of neurogenesis activation. 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 MU-Varna. All of 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 Martin Nikolaev Ivanov.

Sofia, 19.12.2023 Reviewer:
(Prof. Nikolai E. Lazarov, MD, PhD, DSc)