REVIEW

by prof. NIKOLAI ELENKOV LAZAROV, MD, PhD, DSc
on the dissertation of Prof. Ludmil Penuv Kirazov, PhD
"*BIOCHEMICAL BASIS OF ALZHEIMER'S DISEASE*"
for awarding the Doctor of Science (DSc) degree

The dissertation of Professor Ludmil Kirazov is written on 270 standard pages, divided into sections as follows: *List of abbreviations* – 2 pages, *Contents* – 5 pages, *Introduction* – 1 page, *Purpose of the research* – 1 page, *Literature review* – 34 pages, *Material and methods* – 27 pages, *Results and Discussion* – 149 pages, *Conclusions* – 3 pages, *References* including 697 titles, all in the Latin alphabet – 45 pages. The work is illustrated with 9 tables and 86 figures, incl. black and white or color photomicrographs, graphs and diagrams. Moreover, some of the figures are single plates with several photomicrographs grouped, so that the actual number of completed photo-documentation is larger. A separate Appendix provides on 3 pages a *Reference on the scientific contributions* and the lists of the *publications and scientific communications* of the author in relation to the dissertation, and their *scientometric indicators* – 7 pages.

The **topic** of the dissertation is adequately chosen. It is dedicated to an extremely topical issue of neurodegenerative diseases, i.e. elucidation of the etiology and biochemical mechanisms of Alzheimer's disease, and development of an effective therapeutic strategy for its treatment. It is well known that this progressive brain disorder is the most common cause of senile dementia and is an important medical problem of great social, demographic and economic importance. The relevance and significance of the problem is obvious from the remarkable exponential growth of such studies and new hypotheses about the genesis of the disease, published in scientific journals in recent years. Despite the fact that the disorder has been recognized for more than a century and has been receiving increasing attention from neurologists and neuropathologists since the late twentieth century, we are still far from fully understanding the causes of the disease and revealing the detailed etiopathogenetic mechanisms of the development and maintenance of illness symptoms in structural, functional and neurochemical aspects. In this regard, the present study is a successful attempt to comprehensively address the problem in all its aspects, with an emphasis on studying the biochemical basis of the disease, clarifying the causes of its incidence

and searching for appropriate experimental models for other (genetic and biochemical) approaches to its treatment.

The **introduction** is short, concisely written and in a synthesized form introduces the reader to the thesis content.

The **literature review** provides extensive information on what is known so far on the morphological and biochemical characteristics of the Alzheimer's disease, the relationship of the established pathological findings in the brain and the severity of the clinical picture, the role of the genetic predisposition, the inflammatory response, the activated microglial reaction and the oxidative stress for the consequent degenerative brain changes. The numerous experimental models applicable in the study of the disease are clearly and consistently described with systematic accuracy and precision. The information on the mixed pathology accompanying Alzheimer's disease has been presented in an appropriate and sufficiently comprehensive manner. Of all the literature sources cited in the references, 204 (>29%) articles are from the last decade, including 12 from 2020, which is another proof of the relevance of the research problem, the completeness of the literature survey and, last but not least, the good literary awareness of the author. The fact that at the end of the literature review in a short concluding paragraph the state of the considered problem is presented in summary and the open and still unresolved issues are outlined makes an excellent impression. This allowed Kirazov to set the exact goal of his research.

In my opinion, here, and not before the literature review, is the place of the **research aim**, which logically follows from the analysis of given statements on the research topic. Regardless of the changing over time study objectives, however, it would be good to formulate them specifically by linking them to the applied methods for their implementation. This is done in the next section.

The **Material and methods** section is comprehensive and well written. To perform the various tasks, materials from several experimental animals, mainly rodents (mice and rats), was examined, but it is difficult for me to determine the exact number of animals used. For better clarity, the numerous experimental material could be presented in an appropriate table, distributed by species, age and sex. I assume that the number of experimental animals is sufficient to obtain statistically reliable results, and that in the course of research the necessary ethical principles for working with experimental animals are met. A large number of different biochemical methods for

protein separation (polyacrylamide gel electrophoresis), electrophoretic mobility shift assay (EMSA), modern highly informative methods such as cell culture of cortical brain sections, *in situ* hybridization, enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry for the detection of amyloid precursor protein (APP) and vascular endothelial growth factor (VEGF), immunoblot analysis with fluorescent detection of β-amyloid peptide on intact and pathological brain material, bioinformatics analysis of metabolic pathways and statistical analysis of the obtained experimental data have been applied to solve the set tasks. Author's modifications of known techniques have been developed for some of the methods used. The experimental models and methods applied have been described in details. The description of the experimental techniques and methods used is given step by step with accuracy and consistency, allowing their reproducibility and replicability by other researchers.

The **Results** section is merged with the subsequent **Discussion** and is set out on 149 pages, which is more than half of the dissertation. It is worth explicitly emphasizing the fact that all the author findings are solidly supported by relevant illustrative material with a detailed description. It is divided into 10 chapters, most of which are further subdivided into subchapters. They consistently and in detail describe the effects of factors affecting the secretion and metabolism of APP, ontogenetic changes in its expression, the influence of amyloid β-peptide on the electrical activity of cultured cortical neurons and the results of comparative analysis of synaptosomal transcriptome in young and aged brain. In the course of the study, it was found that L-glutamate affects the metabolism of APP in the cerebral cortex by acting on the metabotropic glutamate receptor, but its involvement in APP pathological processing and Aβ deposition in senile plaques remains to be elucidated. The effect of protease inhibitors on APP secretion was investigated by immunoblotting and they were reported to increase sAPP by about 14%, which was verified by a Ca^{2+} test. In addition, it has been shown by the superfusion method that sAPP does not affect APP secretion. On the other hand, in cultured brain sections from transgenic mice, VEGF has been shown to affect APP processing in astrocytes, reduce amyloid β-peptide levels, and inhibit Aβ-fibrillogenesis. Following the causal logic that the deposition of β-amyloid fibrils evokes an immune response in the brain that triggers an intracellular signaling cascade leading to neuronal loss and dysfunction, the researcher investigated the effect of inflammatory cytokines such as interleukin-1β (IL-1β) on the level of transcription factors NFκB and AP-1 in the cholinergic

neurons of the cortex and found that the increased expression of IL-1β inhibits muscarinic receptor-mediated signaling receptors via inhibition of these transcription factors. The obtained data allowed Kirazov to propose a potential targeted therapy of Alzheimer's disease by pharmacological regulation of their function. Further, he has revealed the immunohistochemical localization of neuronal growth factor (NGF), its low-affinity receptor, and the levels of the cholinergic markers such as choline acetyltransferase (ChAT) and M1 muscarinic receptor in transplanted NGF-producing fibroblasts on experimental models of cholinergic degeneration in the basal forebrain nuclei in rats. In these experiments, the partial cholinergic immunolesion was shown to reduce ChAT expression in the cortex by about 60%, while transplantation of NGF-producing fibroblasts after degeneration restored the normal ChAT activity in the basal forebrain neurons. The effect of partial cholinergic denervation on the metabolism of APP in the brains of transgenic mice is characterized by its increased expression at both RNA and protein levels, which was established by *in situ* hybridization, immunoblot tests and immunohistochemical staining. The author suggests that the increased amyloid levels in this case are due to changes in APP processing or impaired removal of Aβ. Furthermore, other important pathomorphological manifestations of the disease, such as hippocampal atrophy and loss of synapses, have been identified in this model. Kirazov also uses isolated synaptosomal fractions as a model system for studying APP secretion. Ontogenetic changes in protein level of APP have been observed in homogenate, growth cones, and synaptosomes of developing rat brain and were characterized by low concentrations in the embryonic brain and elevated postnatal levels. During development, changes in the expression and localization of APP transcripts in the embryonic and developing brain and some other organs and skeletal muscles were also observed, and APP695 was found to be the major isoform expressed in them. One important conclusion from these studies is that the reduction in electrical activity of neurons induced by soluble Aβ is a key element in the pathology of the disease and it is not due to oxidative stress, but rather a result of its agonistic effect on inhibitory neurotransmitters. This effect of amyloid peptides is similar to that of other neuroactive substances such as diazepam. In this regard, Aβ monomers are thought to disrupt neural circuits and synaptic communication between cortical neurons, leading to synaptic degeneration and neuronal cell death. The comparative analysis of the sequenced synaptosomal transcript in the brains of young and aged mice allowed the mapping of affected genes, the coding regions (exons) and non-coding sequences (introns) that express unique circular

RNAs. This suggests the effect on dysregulation of protein-coding genes leading to Alzheimer's disease.

The **discussion** is done with skill and understanding, and shows the ability of the author to interpret his own results, comparing them with known facts in the relevant literature, and integrating the available data to draw valuable conclusions and recommendations to clinical practice. Particularly important to practice is the discussion about the possible application of the results in developing an effective therapeutic strategy for the treatment of Alzheimer's disease. In my opinion, the dissertation would acquire a more complete form with a concluding section (or at least a paragraph), in which the concluding remarks on the basic facts established by the author should be summarized and the future research directions should be outlined.

The conclusion section draws the main **conclusions** of the study. They are written concisely and accurately reflect the author's findings. In general, I accept without remarks their wording and scientific value. Only conclusion 6 in my opinion contains both conclusions and author's contribution, and conclusions 9 and 10, although specific, are very circumstantial. In order to better highlight the scientific achievements of the study, most of which are definitely original, they should be presented not in the appendix, but immediately after the conclusions, as done in the dissertation abstract.

The main **contributions** of the dissertation could be summarized as follows:

1. The role of amyloid β-peptide on the electrical activity of neurons and the relationship between secreted amyloid precursor protein and APP secretion is elucidated.
2. The modulatory role of vascular endothelial growth factor in amyloidogenesis has been demonstrated.
3. The participation of the proinflammatory cytokines such as interleukin-1β in the etiology of Alzheimer's disease is postulated and clarified.
4. Experimental models have been developed to study the effect of cholinergic innervation on amyloid precursor protein processing.
5. A comparative characteristic of the expression of amyloid precursor protein in the brain and peripheral organs during ontogenesis.
6. An original model for studying the processing of amyloid precursor protein in the synaptosomal fraction has been proposed, through which the transcriptome in young and

aged mice has been studied and non-coding and circular RNAs have been identified in synaptosomes.

The **documentation** as a whole is of very good quality and clearly illustrates the author's findings. The addition of indicative signs and symbols to the photomicrographs would greatly facilitate the unfamiliar reader in the orientation and evaluation of the author's findings. On the other hand, the statistical data presented in tables and diagrams provide rich and accurate visual information on the established quantifiable metrics.

The dissertation is written in a concise way, the writing style is clear with the appropriate specific terminology. The draft of **the thesis abstract** is prepared according to the requirements. It adequately and sufficiently reflects the state of the problem, the purpose 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.

There is no doubt that the thesis is a **personal work of Prof. Kirazov**. This is proved unequivocally by the list of scientific publications on the topic of the dissertation, as in the majority of them (25 = 80%) he is the first author. The results of the experiments are published in **31 articles**, 8 of which in authoritative international journals such as *Neurobiology of Disease, Neurobiology of Aging, Neurochemistry International, International Journal of Developmental Neuroscience, Neuroscience, European Journal of Neuroscience, Brain Research.* A list of 45 communications in national and international scientific events is presented separately, and 2 of them have been published in prestigious scientific journals such as *Alzheimer's and Dementia* and *Journal of Neurochemistry*. The total **impact factor** of the journals with the published scientific papers included in the dissertation (according to the candidate's data) is **30.23**. The scientific achievements of the author in the present work have found a worthy reflection in the specialized literature. Scientific papers have been cited a total of 425 times in scientific journals, dissertations, science books and patents, incl. 257 citations in Scopus.

In **conclusion**, I found that the dissertation of Prof. Lyudmil Kirazov is factually accurate and makes a significant contribution to fundamental neuroscience, in particular to understanding the mechanisms of neurodegenerative diseases, and has a significant theoretical contribution to their modern pharmacotherapy. It is a well-conceived study, which is precisely methodologically substantiated, conducted accurately with a variety of modern techniques and has been

successfully carried out. The work is appropriately presented, properly illustrated, clearly and adequately discussed. The results are of some practical importance for development of appropriate targeted therapy for Alzheimer's disease. The significance of the scientific contributions of the dissertation could be seen in the table below with the scientometric indicators of the materials presented by the author, which cover the mandatory national requirements and almost twice exceed the minimum quantitative criteria of IEMPAM-BAS for awarding the degree "Doctor of Science":

Group of indicators	Indicator	Mandatory quantitative criteria required for DSc degree (minimum number of points)	Scientometric indicators of *Prof. Ludmil Kirazov* (verified number of points)
A	1. Dissertation for awarding educational and scientific degree "Doctor of Philosophy (PhD)"	**50**	**50**
B	2. Dissertation for the award of the scientific degree "Doctor of Science (DSc)"	**100**	**100**
G	3. Scientific publications that are not used for registration in NACID and have a Q-factor	**100**	**130**
D	4. Citations listed in Scopus that have not been used for registration with NACID	**Minimum number = 100 т.**	**Total number = 372 т.**
Total number		**350**	**652**

All of the above-mentioned reasons convince me to give a positive assessment of the dissertation and as a member of the Scientific Jury of the procedure to support with a positive vote the award of the degree "Doctor of Science" in the professional field 4.3. Biological Sciences, in the scientific specialty "Biochemistry" (code 01.06.10) of Prof. Dr. Lyudmil Penuv Kirazov.

Reviewer:

Sofia, 14.05.2021

(Prof. Nikolai Lazarov, MD, DSc)