REVIEW

of the dissertation of Prof. Dr. **Ludmil Penov Kirazov** on **"Biochemical bases of Alzheimer's disease" for awarding the scientific degree** "Doctor of Science" in professional field 4.3. Biological sciences, scientific specialty "Biochemistry"

Reviewer: Prof. Dr. Roumyana Mironova - IMB "Acad. Roumen Tsanev "- BAS

Alzheimer's disease (AD) was first diagnosed more than 100 years ago, but is currently incurable. This is due to the complex causes of the disease and its frequent manifestation in the context of a mixed type of pathology together with other degenerative diseases of the brain. At the same time, statistics shows that AD affects more than 50% of the age population over 80 years. Therefore, with demographic changes in favor of the elderly population in recent decades, the disease has become a serious social and economic problem. According to data cited in the dissertation, "... the cost of medical care for Alzheimer's patients worldwide is estimated at more than $ 1 trillion" *per* year. These data emphasize the relevance and significance of the dissertation of Prof. Kirazov, dedicated to the biochemical basis of Alzheimer's disease.

The dissertation is written on 270 pages and contains an introduction, research goal, literature review (34 pages), materials and methods (25 pages), results and discussion (148 pages), conclusions, bibliographic reference and appendices with reference to the contributions and lists of the author's publications and scientific communications on the topic of the dissertation. The documentary materials include 86 figures and 9 tables, and the bibliographic reference - 697 literature sources. This structure speaks of a well-balanced content of the dissertation, which is dominated by the results and their discussion, based on an impressive number of citations. Despite the large volume, the dissertation is easy to read, thanks to the extremely good language and scientific style.

The **literature review** is detailed, in-depth and comprehensive. It examines the existing hypotheses about the etiology of AD, including amyloid and mitochondrial cascade hypotheses and the hypothesis of changes in the blood-brain barrier. Special attention is paid to amyloid β-peptide (Aβ) and its precursor - amyloid precursor protein (APP). The processing of APP and the functions of its products with a focus on the physiological and neurotoxic role of Aβ are considered. For many years it has been thought that senile plaques in the brains of AD patients, where Aβ is deposited in an aggregated form, are the cause of the observed cognitive deficits. In the light of recent research, this notion is increasingly compromised and draws scientists' attention to the neurotoxicity of soluble Aβ. Prof. Kirazov's research aims to "contribute to elucidating the etiology of Alzheimer's disease and to create experimental models to support this." In the literature review, he prepares the reader for an essential part of his experimental work, devoted to the use of synaptosomes and neural networks as a model for studying the metabolism of APP and the toxicity of soluble Aβ.

The dissertation uses a rich arsenal of materials and methods, which are described in detail and fully adequate to the planned research. These include biochemical and molecular biological methods (determination of protein and enzyme activities, LDH and MTT tests, SDS-PAGE, EMSA, *in situ* hybridization, RT-qPCR), immunological (ELISA, immune- precipitation, -blotting, - histochemistry, -fluorescence), cell handling (including primary cell cultures) and experimental animals, including procedures for organ/tissue isolation and cell transplantation. One of the most modern methods for nucleic acid sequencing (NGS) has been used in transcriptome research. A number of software products (Trimmomatic v0.38,

CIRCexplorer2 v2.3.6, GENCODE mm10 vM22, *etc.*) were used in the bioinformatics analysis of the sequencing data, and appropriate methods were used for statistical processing of the results (Student's *t*-test, ANOVA with LSD post hoc test of Fisher, *etc.*), which convince the reader of the reliability of the reported results.

The studies presented in the chapter **Results and discussion** can be thematically referred to four main sections - a) APP metabolism, b) Influence of amyloid β-peptide on the electrical activity of neuronal cells, c) Study of the transcriptome in the synaptosomal fraction and d) Comparison of methods for protein determination by Lowry and Bradford. I will consider them in this sequence, which in my opinion reflects the volume and significance of the research, although in the dissertation they follow a different, rather chronological order. Logically, the greatest place is given to APP metabolism due to the many unknowns related to the functioning of this protein and its processed forms in norm and AD pathology.

APP metabolism

This section examines the effects of various substances and factors on APP secretion (glutamate (Glu), protease inhibitors, calcium (Ca^{2+}) and lead ions (Pb^{2+}), vascular endothelial growth factor (VEGF), cholinergic neurotransmission and aging). Impressive is the wide variety of experimental models used, including synaptosomes, primary cell cultures, experimental animals (rats, mice), including transgenic ones, various organs from experimental animals (brain, skeletal muscle, kidney, liver), parts of brain (hemispheres, cerebellum, forebrain and hindbrain) and brain sections. The aim of all these approaches is to get as accurate and in-depth an idea as possible about the metabolism of APP in native conditions *in vivo* and Prof. Kirazov's research has significant contributions in this direction.

One of the first studies of Prof. Kiratsov (**№8**) filled a gap associated with the lack of experiments with brain sections to assess the effect of the neurotransmitter Glu on APP secretion. His results confirm the observations of other cell culture researchers that activation of glutamate receptors affects APP secretion. Basal secretion of APP has also been established, *i. e.* in the absence of neuronal stimulation. This observation is interesting in the context of the hitherto unexplained physiological functions of APP in the healthy brain. The observations that the APP secreted from the brain sections does not affect its own secretion and undergoes physiological Ca^{2+}-dependent processing after its secretion are original (**№9-11**). These observations were made with Glu-stimulated and basal APP secretion, respectively, and were performed precisely to exclude nonspecific protein degradation as well as nonspecific leakage of Ca^{2+}-dependent proteases from the sections. In a recent study (**№29**), Prof. Kirazov also found that Pb^{2+} inhibited APP secretion in cortex-containing brain structures (hemispheres and cerebellum) of mice. This observation is important because, according to existing data, APP regulates the efflux of iron ions and thus may lead to an increase in their intracellular concentration and to neurotoxicity. The data obtained by Prof. Kirazov support the "metal hypothesis for AB", according to which metal homeostasis is critical for the function of neurons and its violation contributes to the development of AD.

Although brain sections are closer to *in vivo* conditions than cell cultures, Prof. Kirazov's research for the first time attempts to use synaptosomes as a model system for studying APP secretion (**№12, №14, №22**). These experiments are dictated by the fact that synapses are a specific site where neurotransmitter receptors operate and are motivated by the assumption that in synaptosomes the receptors would be more accessible to exposure to different agents and APP would be released directly into the incubation medium without passing through the matrix. The neurotransmitter Glu was again used in these experiments,

but about twice the APP secretion was observed compared to brain sections in stimulated synaptosomes. An even greater secretion of APP from synaptosomes was observed when stimulated directly with phorbol myristate acetate (PMA), which mediates the action of Glu by stimulating protein kinase C. The observation is confirmatory and is thought to be due to a difference in the number receptors associated with Glu and PMA.

Synaptosomes are an experimental model in other studies by Prof. Kirazov, where the focus has shifted to ontogenetic changes in APP expression. The study of APP in synaptosomal fractions is rational, since APP is synthesized in neurons and transported along axons to synapses. The protein has been shown histochemically and in growth cones. For these reasons, Prof. Kirazov subjected to experimental testing the hypothesis that the formation of synapses and their activity are associated with increased secretion of APP. Changes in APP in homogenate, cones, and synaptosomes from rat brain during ontogenesis were studied. These studies, which monitor APP expression at the protein level, confirm the hypothesis by clearly showing that it is necessary both for the formation of neurons and synapses and for the maintenance of synaptic functions (**№13**). The various functions of APP lead the author to suggest that different APP isoforms are most likely involved. This hypothesis was verified and confirmed by messenger RNA (mRNA) assay for the three APP isoforms in embryo and rat brain (№15). The shortest APP isoform (APP695) has been found to play a role in nerve cell differentiation in both the central and peripheral nervous systems, while the two longer isoforms (APP751 and APP770) are expressed primarily in peripheral organs and tissues and much weaker in the brain. This conclusion about the differential expression of APP returns Prof. Kirazov to the study of APP at the protein level in various organs of the rat (brain, skeletal muscle, kidney and liver) in the time course of ontogenesis (**№27, №28**). Important is the fact that the concentration of APP in the brain is many times higher than in peripheral organs. In this regard, I have the following **QUESTION**: **Since there is evidence in the scientific reports for the expression of APP isoforms in the vascular endothelium, I am curious whether in atherosclerotic plaques of blood vessels are found β-amyloid deposits?**

Numerous studies, including those of Prof. Kiratsov, show that the processing of APP is under neurotransmitter control. However, there are no data on whether APP secretion is affected by the activation of muscarinic receptors *in vivo*. Scientific contributions in this direction are the studies of Prof. Kirazov, conducted with rats, in which partial cholinergic degeneration was caused in the basal ganglia of the forebrain (**№30**). The model was created by inducing partial cholinergic immunolesion, followed by transplantation of fibroblasts secreting neuronal growth factor (NGF) to restore cholinergic function. Numerous experiments have been undertaken to validate the model - proving the secretion of NGF, determining the levels of cholinergic markers and their kinetic constants. Only then do researchers allow themselves to move on to study APP metabolism in model animals. The results clearly show that cholinergic hypofunction in the cerebral cortex leads to an increase in membrane-bound APP and a decrease in its secretion. This is a significant scientific contribution, as for the first time *in vivo* results show that APP processing in cortical neurons receiving cholinergic innervation from the outside is under cholinergic control. The contribution is significant as it is well known that in AD patients the cholinergic system in the basal ganglia of the forebrain undergoes severe degeneration. In this regard, it was particularly appropriate to create the same model, but with transgenic Tg2576 mice, which have amyloid pathology, as they contain a human APP gene with a double mutation, described as a familial form of AB. It has been confirmed that in these model animals the

amyloid pathology intensifies and important pathological signs of the disease appear - loss of synapses, neurodegeneration and atrophy of the hippocampus. The results have another important significance - they indicate the feasibility of using cholinergic mimetics for the treatment of AB. The transgenic study was published in the prestigious journal *Neurobiology of Disease* with IF_{2012} 5.624 **(№4)**.

The proven involvement of muscarinic acetylcholine receptors in APP metabolism in the above studies justifies the study undertaken on the role of interleukin-1β (IL-1β) in the signaling cascade of this type of receptors (**№31**). There is evidence in the literature that Aβ-deposits activate a neuroimmune cascade in which IL-1β leads to Glu-mediated degeneration of cholinergic cells and, on the other hand, stimulates amyloidogenic degradation of APP. On this basis, in his research, Prof. Kirazov starts from the hypothesis that IL-1β in the diseased brain can disrupt cholinergic neurotransmission by affecting muscarinic receptors. To test this hypothesis, a cholinergically differentiated neuroblastoma cell line was treated with IL-1β and the cholinergic agonist carbachol, and the effect on the muscarinic receptor signaling cascade was investigated by measuring the hydrolysis of phosphoinositide, acetylcholinesterase-N activity and the DNA-binding acgtivity of transcription factors NFκB and AP-1. The main conclusion of these experiments is that chronic treatment with IL-1β suppresses the signaling pathway of muscarinic receptors, most likely through NFκB and AP-1. This supports the hypothesis that IL-1β is a factor in cholinergic deficiencies in AD and reveals NFκB and AP-1 as potential targets for AD therapy.

Pioneering and original are the studies of Prof. Kirazov (**№1**), which seek an answer to the question of whether VEGF affects the metabolism of APP. Transgenic Tg2576 mice were used as a model in these studies (see above). The model is appropriate because amyloid deposits characteristic of AD are observed in the brain of adult mice. In sections of the brains of these mice, Prof. Kirazov observed increased VEGF-immunoreactivity of vascular endothelial cells, and elevated levels of VEGF were quantified by ELISA. These results are consistent with literature data on elevated levels of VEGF in the brains of AD patients. The observed relationship between Aβ peptides and VEGF is interesting. On the one hand, by correlation analysis, the possibility of VEGF expression being influenced by Aβ was ruled out, and on the other hand, it was found that treatment of sections with VEGF inhibits the formation of soluble Aβ (Aβ1-40 and Aβ1-42), while of the fibrillary forms of the two peptides, only Aβ1-42 is affected (increased), the relative content of which in AD patients is known to increase. All these data suggest a possible involvement of VEGF in β-amyloidogenesis in AD and open the door to new research.

Influence of amyloid β-peptide on the electrical activity of neuronal cells

The interest in APP, to which Prof. Kirazov has devoted much of his research, is due to the fact that protein processing is a potential source of Aβ peptide, which **aggregates** and forms the nucleus of senile plaques. However, more and more data on the neurotoxicity of soluble Aβ are accumulating in the literature and the notion is formed that the main pathological sign of AD are not senile plaques, but the loss of synaptic contacts. The aggregation and deposition of Aβ is considered rather as a protective mechanism against the neurotoxicity of its **soluble** forms. Therefore, Prof. Kirazov focuses his research on the effect of soluble Aβ on the electrical activity of neurons connected in a network through synaptic contacts. For this specific purpose, "neurons grown on microelectrode arrays" derived from the frontal cortex and spinal cord of mice treated with a soluble form of the biologically active Aβ (Aβ25-35) peptide was used for the first time. It has been found to reduce rapidly,

concentration-dependently and reversibly the electrical activity of neural networks derived from the spinal cord, while cortical culture was less affected (**№16, №17**). It is assumed that the observed differences are due to the predominance of different transmitter systems in the two neuronal networks. These results lead to the conclusion that the reduction of the electrical activity of neurons caused by the soluble Aβ25-35, and thus the disruption of the communication between the nerve cells, is a key element in the pathology of AD. In this regard, I have the following **QUESTION: Is there data on the concentration of soluble Aβ in the brain of healthy individuals and AD patients? My question is provoked by the fact that you observe an inhibitory effect of Aβ at concentrations from 25 nM to 77 nM and above 1 μM. Do the concentrations of Aβ in the brains of AD patients fall within these limits?**

There are reports in the literature that AD pathology is associated with oxidative stress caused by Aβ. In experiments conducted with spinal cord neural networks, oxidants ($FeSO_4$, H_2O_2) and antioxidants (vitamin E, propyl gallate), the possibility that the effect of Aβ is due to oxidative stress (**№19, №20**) was ruled out - a conclusion that has confirmatory nature. It seems more logical to assume that Aβ acts through receptors in synapses. To test this assumption, the neural networks were treated with bicuculline and strychnine, which are antagonists of the inhibitory neurotransmitters γ-aminobutyric acid A and glycine (**№18**), respectively. As a result, it was concluded that Aβ most likely acts as an agonist of the receptors for these inhibitory neurotransmitters. This conclusion was confirmed in subsequent studies demonstrating a similar effect of the psychotropic drug diazepam (**№24**).

In other studies, Prof. Kirazov compared the effect of the fragment A□25-35 on the electrical activity of neuronal cells with that of the endogenous forms of the amyloid peptide Aβ1-40 and Aβ1-42. In these studies, the inhibitory effect of Aβ25-35 was found to be the strongest, followed by that of Aβ1-42 and Aβ1-40. The observation is interesting in that the arrangement of the peptides reflects their involvement in the formation of amyloid plaques. It is assumed that the different effect of peptides is due to differences in their mechanism of action or their structure, which requires further research (**№21, №23**). In the experiments conducted so far with the neural networks, soluble forms of the various Aβ peptides were used. As the data on the toxicity of soluble and aggregated Aβ are contradictory, a study was undertaken comparing the effects of monomeric (soluble) and aggregated Aβ1-42 (№26). This peptide was chosen because of its predominance in amyloid plaques. Following a peptide oligomerization procedure, its aggregation was confirmed electrophoretically and targeted studies were undertaken. The definite conclusion is made that Aβ1-42 suppresses the electrical activity of the neural networks in monomeric form, but not in aggregate state. This conclusion supports the original hypothesis of the authors of the study, which is an alternative to the "cascade hypothesis" for the pathogenesis of AD and states that soluble Aβ monomers disrupt synaptic function, leading to loss of communication between neurons.

Examination of the transcriptome in the synaptosomal fraction

In the publications of Prof. Kirazov I have reviewed so far, special attention is paid to the synapses in connection with the above hypothesis of their involvement in the pathogenesis of AB. For a more detailed characterization of the processes taking place in the synapses, a large-scale study of the transcriptome in isolated synaptosomes of mice was undertaken by applying one of the most modern molecular biological methods - next generation sequencing (NGS). The interest is reasonably directed not to the proteome, but to the transcriptome, which more comprehensively reflects the ongoing processes, due to

the regulatory role of non-coding RNAs. The studies are based on the fact that there are no data on changes in the synaptosomal transcriptome during non-pathological aging. Two groups of transcriptome studies on synaptosomal fractions from the cortex of young (2.5 months) and old (23 months) mice were undertaken.

A significant scientific contribution in the first group of studies (**№2**) was the detection of 6,642 differentially expressed genes (DEGs) in young and old mice, not yet annotated in the reference mouse genome, of which almost all (99.9%) are for non-coding RNAs. Interestingly, of these 6 642 transcripts, only 17 were expressed in both groups of animals. Most (4,670) are genes that are expressed only in the adult brain, so they can be regarded as potential molecular markers of aging. As such, 389 long intervening non-coding RNAs could be considered, for which the authors of the study found orthologues in the human genome. Analysis of the gene ontology of the murine genome-encoding protein-encoding DEGs (233 in number) revealed that the genes with reduced expression in aging synaptosomes are mainly related to cell migration and those with increased expression are associated with neurotransmission, immune response and regulation of gene expression. One such gene (Clp1) has attracted the attention of researchers due to its more than 100-fold increased expression in aging synaptosomes. Increased expression of the Clp1 gene has been demonstrated by several independent methods at the level of RNA (RT-qPCR) and protein (immunoblotting, immunohistochemically). Regarding the localization of the CLP1 protein, a partial localization was found in the synaptosomes and a shift to the neuronal outgrowths of hippocampal neurons in the brains of aging mice. The CLP1 protein is a multifunctional kinase involved in the splicing of various types of RNAs, including tRNAs whose splicing is impaired in neurodegenerative diseases. These studies, carried out with unprecedented resolution, represent a significant scientific contribution. They show for the first time age-dependent changes in the expression of protein- and RNA-encoding genes in synaptosomes. The research was published in the prestigious journal *Neurobiology of Aging* (IF_{2017} 4.7).

In the second group of transcriptome studies, the emphasis is on the circular transcriptome in synaptosomes of young and old mice. It is known that the brain is enriched with both long non-coding RNAs and circular RNAs (circRNAs), the latter of which are interesting with their potential for translation. There is evidence that circRNAs are preferentially expressed by genes encoding synaptic proteins and that some circRNAs are found at synapses. However, to date, no data have been published on changes in the circular transcriptome during aging. Prof. Kirazov's research fills this gap by presenting data from bioinformatics analysis of circRNA and experimentally validated circRNA in synaptosomes isolated from the cortex of young and adult mice (**№1**). As in the previous study, it was observed that the total number of circRNAs was higher in the synaptosomes of old (3,778) than in young (2,836) mice, but in this case the proportion of circRNA expressed in both age groups was higher (1,506/6,614). Another significant difference is that only 4 of the common circRNA genes are differentially expressed in young and old mice. All 4 genes (Dgkd, Hdac4, Ptpn4 and Robo2) encode proteins and are less abundant in older mice. The expression of the 4 genes and of a gene expressed only in adult mice (Igfi1) was experimentally validated by RT-qPCR. Analysis of the gene ontology shows that both young and old synaptosomes express predominantly circRNA genes associated with neurogenesis, neuronal protection, and synapse organization, as expected. The importance of the studies on the circular transcriptome is evidenced by the fact that they were published by Elsevier in the journal Neuroscience (IF_{2020} 3.244).

Comparison of Lowry and Bradford protein determination methods

I review these studies at the end of my review because they are related to some methods. This, of course, does not diminish their importance, given that protein concentration has been measured in much of the presented research. The main conclusions made at this point in the dissertation are that the Bradford method measures reduced protein concentrations compared to the Lowry method and that the same protein sample after freezing at -20°C shows lower concentration as measured by the Bradford method (**№5-7**). I am specifically interested in this part of the results in the dissertation, as they confirm our observation related to the measurement of the concentration of purified protein (human interferon-γ) spectrophotometrically and by the method of Bradford (Mironova *et al.*, **J. Biol. Chem.**, 2003, 278 (51): 51068). We also measured a lower (4-fold) concentration with the Bradford method and showed that this was due to non-enzymatic glycosylation (glycation) of the protein during its *in vivo* synthesis. I emphasize this fact because glycation is a process whose role in the aging process is well known. In this context, I found very accurate and far-sighted the assumption made by Prof. Kirazov that the reason for the measured lower concentration of proteins after freezing is their "aging".

The discussion of the results is not presented in a separate section of the dissertation. However, in each section reporting results, the latter are very thoroughly discussed in the context of existing literature and thus enrich the reader's understanding of the intimate mechanisms of the processes involved in AD pathology. I accept all **11 conclusions, 10 original scientific and 2 scientific-applied contributions**, formulated by Prof. Kirazov, as they accurately and correctly reflect the results of his research. I have no remarks regarding the **abstract book**, which represents a short version of the comprehensive dissertation and reflects its essential content.

Scientometric indicators and compliance with the minimum requirements

Prof. Kirazov has published a total of **31 journal articles** on the topic of the dissertation with a total impact factor of **30.23.** In more than half of the articles (18/31) he is the first author, which emphasizes his leading role in the research. In the world-famous database for scientific information Scopus the scientific works of Prof. Kirazov, included in the dissertation, **are cited 257 times** with the total number of citations (including in dissertations, books and patents) being significantly higher - 425. The results of the conducted research were presented at a total of 45 scientific forums at home and abroad. The table below presents the compliance of the activities of Prof. Kirazov with the minimum national requirements (according to the Regulations for the application of LDASRB amended/supplemented SG No. 15/19.02.2019) and those of IEMPAM-BAS for acquiring the scientific degree "Doctor of Sciences", which shows that its activities exceed almost twice the required minimum.

Indicator	Minimum points	Covered
А – PhD Dissertation	50	50
Б – DSc Dissertation	100	100
Г – Publications	100	130
Д – Citations in Scopus	100	372
Total	**350**	**652**

Remarks

I have the following minor remarks:

1. The terms "линеарен" (p. 18), "центрофугация" (p. 51) and "черти" (p. 82) sound better in Bulgarian as „линейна", „центрофугиране" and „ивици" (this latter term refers to the different bands on electrophoretic gels).
2. The designations of some figures (*e. g.* the insets in Fig. 33) are unreadable, probably due to their direct reproduction from the published articles.
3. On page 144, penultimate line, "serial slices (16 mM)" should probably be "serial slices (16 μm)".
4. In Fig. 5, the unit of measurement on the abscissa should be μM instead of mM.

CONCLUSION

Prof. Kirazov presents an in-depth study on the causes of a socially important and currently incurable neurodegenerative disease (Alzheimer's disease). With the proposed new models, hypotheses and their verification, this study contributes significantly to the understanding of the biochemical basis of the disease and its pathogenesis at the molecular level. The results of these studies have been published in prestigious journals and have found an adequate response among the international scientific community. Prof. Kirazov's scientometric indicators fully meet and exceed the minimum national requirements and those of IEMPAM-BAS for obtaining the scientific degree "Doctor of Science". All this gives me a reason to strongly recommend to the members of the Scientific Jury to vote **FOR** the award of the Scientific Degree "Doctor of Science" to Prof. Dr. Ludmil Penov Kirazov.

25/05/2021

Reviewer:

/Prof. R. Mironova/