



Faculty of Medicine and Health Sciences

**Nanoparticulate Brain-Derived Neurotrophic Factor (BDNF) as a
Potential Antidepressant via Neuroendocrine Mechanisms in
Experimental Model of Depression**

Nur Amirah Diyana Binti Razlan

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Nanoparticulate Brain-Derived Neurotrophic Factor (BDNF) as a
Potential Antidepressant via Neuroendocrine Mechanisms in
Experimental Model of Depression

Nur Amirah Diyana Binti Razlan

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.


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Signature

Name: Nur Amirah Diyana Binti Razlan

Matric No.: 20020376

Faculty of Medicine and Health Sciences

Universiti Malaysia Sarawak

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ABSTRACT

The hypothalamo-hypophyseal-adrenal axis (HHAA) links neurogenesis, neurotransmission, and immunoendocrine factors in depression. Stress-induced HHAA activation lowers brain-derived neurotrophic factor (BDNF) in the hippocampus, causing atrophic changes and depressive symptoms. Traditional antidepressants increase BDNF, but BDNF cannot cross the blood-brain barrier. Nanocarriers aid delivery, yet nano-BDNF's potential remains underexplored. This study examines nano-BDNF's antidepressant effects in mice. Thirty C57BL/6 mice were divided into five groups: normal control, depression model, negative control, fluoxetine-treated, and nano-BDNF-treated. Behavioral tests, ELISA, and histological analysis assessed reserpine-induced depression. Results showed nano-BDNF and fluoxetine reversed depression effects, but nano-BDNF was superior in restoring HHAA function and immune balance. Histology confirmed nano-BDNF reversed adrenal and thymic changes linked to depression-induced immunosuppression. This study identifies nano-BDNF with poloxamer 188 as a promising antidepressant, comparable to fluoxetine in behavior recovery but superior in restoring HHAA balance.

Keywords: Hypothalamo-hypophyseal-adrenal axis, depression, brain-derived neurotrophic factor, nanoparticulate, poloxamer 188

Faktor Neurotropik Berasal dari Otak (BDNF) sebagai Potensi Antidepresan melalui Mekanisme Neuroendokrin dalam Model Eksperimen Depresi

ABSTRAK

Paksi Hipotalamus-Hipofisis-Adrenal (HHAA) menghubungkan neurogenesis, neurotransmisi, serta faktor imun dan endokrin dalam kemurungan. Pengaktifan HHAA akibat tekanan menurunkan Faktor Neurotropik Berasal dari Otak (BDNF) dalam hipokampus, menyebabkan atrofi dan gejala kemurungan. Antidepresan tradisional meningkatkan BDNF, tetapi BDNF tidak boleh menembusi penghalang darah-otak. Nano-BDNF berpotensi sebagai rawatan baharu, tetapi masih kurang dikaji. Kajian ini menyelidik kesan nano-BDNF sebagai antidepresan dalam tikus makmal. Tiga puluh ekor tikus C57BL/6 dibahagikan kepada lima kumpulan: kawalan normal, model kemurungan, kawalan negatif, dirawat dengan fluoxetine, dan dirawat dengan nano-BDNF. Ujian tingkah laku, ELISA, dan analisis histologi digunakan untuk menilai kemurungan akibat reserpine. Keputusan menunjukkan fluoxetine dan nano-BDNF membalikkan kesan kemurungan, tetapi nano-BDNF lebih berkesan dalam memulihkan keseimbangan HHAA dan imuniti. Histologi mengesahkan nano-BDNF dapat memulihkan perubahan adrenal dan timus yang berkaitan dengan kemurungan. Kajian ini mengenal pasti nano-BDNF dengan poloxamer 188 sebagai antidepresan berpotensi, setanding dengan fluoxetine dalam pemulihan tingkah laku tetapi lebih berkesan dalam menormalkan HHAA.

Kata kunci: *Paksi Hipotalamus-Hipofisis-Adrenal, kemurungan, faktor neurotropik yang berasal dari otak, zarah nano, poloxamer 188*

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LIST OF ABBREVIATIONS

BDNF	Brain-derived neurotrophic factor
HHAA	Hypothalamo-hypophyseal-adrenal axis
CNS	Central nervous system
ELISA	Enzyme-linked immunosorbent assay
PLGA	Poly(lactic-co-glycolic) acid
HPAA	Hypothalamo-pituitary-adrenal axis
CRH	Corticotropin-releasing hormone
ATCH	Adrenocorticotrophic hormone
NP	Nanoparticles
TrK	Tyrosine kinase
GABA	Gamma-aminobutyric acid
SNPs	Single nucleotide polymorphisms
MWCO	Molecular weight cut-off
µg	microgram
°C	Celcius
W	Watt
kHz	kilohertz
rpm	revolutions per minute
mL	millilitre
i.p.	intraperitoneal
i.v.	intravenous
g	gram

nm	nanometer
kg	kilogram
mg	milligram
FST	Force swimming test
cm	centimeter
IACUC	Institutional Animal Care and Use Committee
ng	nanogram
ZF	Zona fasciculata
CMR	Cortico-medullary ratio
TBM	Tingible body macrophages
CYP17A1	Cytochrome P450 17A1

CHAPTER 1

INTRODUCTION

1.1 Study Background

Depression is the most common mental problem worldwide (WHO, 2024), and its fast spread is threatening. The number of incident cases of depression worldwide increased from 172 million in 1990 to 258 million in 2017, comprising an increase of 49.86% (Liu et al., 2020). The emergence of the COVID-19 pandemic has even more exacerbated certain determinants of depressive disorders. According to WHO, based on the data obtained from the Institute of Health Metrics and Evaluation, Global Health Data Exchange (GHDx) (WHO, 2024) by March 4, 2023, the overall prevalence of depression reached 3.8%; while in adults it comprised 5%. Currently, over 280 million people suffer from depression (Collaborators C1MD, 2021, Zhu et al., 2023). So far the effective treatment of depression remains an unresolved problem, as at least 30% of people suffer from its treatment-resistant form, which is a tremendous individual and societal burden (Kverno & Mangano, 2021; Toloumis, 2021; Baig-Ward et al., 2023; Gavzan et al., 2023; Li, 2023; McIntyre et al., 2023). Better prevention and treatment strategies are required to control the spread of this disorder, but this task is impossible without a better understanding of its pathogenesis.

1.2 Problem Statement

In the pathophysiology of depression, the hypothalamic-hypophyseal-adrenal axis (HHAA) is recognized as a major neurobiological link between its inducing factors, such as dysfunctional neurogenesis and neurotransmission, immunological and endocrine factors (Jesulola et al., 2018; Mikulska et al., 2021). The condition of the HHAA is critical for the maintenance of mental and physical health. Experimental research showed that in

modelled stress, activation of HHAA results in the development of depression. Clinical investigations demonstrated that dysregulation of the HHAA has been linked to several mental and physical disorders (Jesulola et al., 2018; Leistner and Menke, 2020) Activation of the HHAA is considered one of the links to the pathogenesis of depression in humans, as confirmed by the autopsy findings of hyperplasia of the zona fasciculata of the adrenal cortex in patients with heavy depression (Busch et al., 2020).

In stress, glucocorticoid signaling reduces the level of neurotrophic factors, such as brain-derived neurotrophic factor (BDNF) in the hippocampus, resulting in its atrophic changes and development of depression, while effective antidepressants increase the level of BDNF in the brain, promote differentiation of hippocampal progenitors and alter monoaminergic synaptic transmission (Jesulola et al., 2018; Shen et al., 2022; Gulyaeva, 2023; Meng et al., 2024), but it remains unclear how depression itself affects HHAA, as depression is not necessarily associated with the past stress exposure.

In addition to that, despite the vast amount of fundamental research data showing the regulatory action of BDNF on the HHAA (Noushad et al., 2021), and the subsequent cascade in the development of depression, the effect of therapeutic modulations on the neuroendocrine axes as antidepressants has never been explored.

Limited permeability of the blood-brain barrier firmly controls the exchange between blood and brain, therefore penetration of large molecules, such as BDNF is hampered, making pharmacotherapy of CNS diseases using these large molecules not effective. The searches of the successful carriers of BDNF and other neurotrophic factors through the blood-brain barrier are of primary importance for modern pharmacotherapy (Tosi et al., 2020; Han and Jiang, 2021).

Taking into consideration a huge medical and social significance of the problem of depression, insufficient understanding of its mechanisms, absence of the optimal medications for its treatment which would be free from side effects, delayed onsets of the effects and individual unresponsiveness to treatment, we decided to undertake this study aimed investigation of the antidepressant potential of nanoparticulate BDNF with a special insight into its possible mechanism of action through the cross-talk with the HHAA activity.

1.3 Objectives of the study

The general objective of the study is to evaluate the effect of nanoparticulate BDNF on modelled depression in mice

- i. To assess the effect of modelled depression not associated with stress on the HHAA and immune organs of the body.
- ii. To evaluate the interplay between the efficacy of BDNF treatment of depression and activity of the HHAA in an experimental model of depression.
- iii. To identify the most suitable surfactant for effective sorption of the nanoparticles on BDNF molecules.

To implement these objectives, we undertook an animal study using C57BL/6 mice, which are known for their susceptibility to depression modelling. We used a standard reserpine model of depression, as it does not involve any stress exposure of experimental animals, which was very important for us, because our hypothesis states the effect of depression on the HHAA, while the reversed mechanism (the effect of excessive activation of HHAA on the development of depression) is a well-known fact. The first series of

experiments included behavioural tests (open field and forced swimming), ELISA corticosterone test and histological/morphometric evaluation of the depression-related changes in the endocrine glands and immune organs. The second series of experiments included assessment of the changes in the behaviour, levels of activation of the HHAA, hyperplasia of the adrenal cortex and immunosuppressive changes in the lymphoid organs on the background of treatment with fluoxetine, which is considered as one of the most effective traditional antidepressant in comparison with a developed by us formulation of nanoparticulate BDNF with a poloxamer 188 surfactant for treatment of experimental depression. Statistical evaluation of all described parameters in experimental and control animals before and after depression modelling, and after the specified treatment enabled us to demonstrate the validity of the selected model of depression to our objectives, the proper choice of the parameters to be controlled at all stages of the experiments and the comparative efficacy of nanoparticulate BDNF with a surfactant vs. traditional antidepressant for normalization of behavioural reactions, elimination of overactivity of the HHAA and associated immunosuppression. It also allowed us to recommend toxicology studies of the PLGA-BDNF with poloxamer 188 surfactant as the next step before the clinical trial of the proposed medication.

1.4 Chapter summary

This chapter has provided the general introduction into the medical and social problem of depression and the main challenges of its pharmacotherapy. It describes the recent achievements of nanopharmacology of the diseases of the central nervous system and its difficulties in view of low permeability of the blood-brain barrier. It formulates and justifies the main and specific objectives of the study and summarizes its main findings and recommendations.

CHAPTER 2

LITERATURE REVIEW

2.1 Depression as a major health threat of modern life

Depressive disorders are a big problem of modern healthcare worldwide, which is due to the constant increase in morbidity, severe personal sufferings of patients, the risk of suicide, and a significant financial burden on the society caused by long-term disability of patients. Today, about 280 million people suffer from this disease, which makes it the most common neurological disorder and one of the most common causes of death. Unfortunately, not uncommon that depression becomes fatal. According to WHO, by 2030, depressive disorders will rise from the 3rd place as a disease burden, which they occupied in 2008, to the first place. COVID-19 pandemic increased prevalence of depression by 27.6% all over the world. Treatment of depression remains a significant problem of modern medicine since almost 70% of patients are refractory to drug treatment (Beurel, et al, 2020; COVID-19 Mental Disorders Collaborators, 2021; Dodd et al., 2021; Monroe & Harkness, 2022; Kondo, 2023; Alizadeh Pahlavani et al., 2024; Stecher et al., 2024). Depression is not only a medical problem, but also a social problem as well, as it has a tremendous growth rate among young people (Wang et al., 2021; Shorey et al., 2022).

Scientists around the world are actively engaged in research on depression. It was calculated that 3.8% people in the world suffer from depression. The rate for the adults is higher than the overall prevalence (5%) and even higher in old people (5.7%) with total number of 280 million people with depression in the world (Institute of Health Metrics and Evaluation, 2023). Numerous research is being dedicated to investigation of the triggering factors for development of depression, but in spite of that pathophysiology of depression is

not fully understood and, as consequence, its treatment remains a big challenge for the modern medicine, as proper treatment may not be found without a clear understanding of the pathophysiology of the disease. Because depression arises from a complex mixture of factors, including somatic, neurobiological, epigenetic influences, and adverse life events, patients frequently encounter recurring depressive episodes. Hence, it is of utmost important to understand holistically the pathophysiologic basis of the disease to achieve effective treatment and successful outcome of this suffering (Jesulola et al., 2018; Lichter et al., 2023).

2.2 Pathophysiology of depression

Among numerous theories of depression, the most recognized are theories of monoamines imbalance, dysregulation of the hypothalamic-pituitary-adrenal axis (HPAA) with associated immune disorders, and hampered neurogenesis in the structures of hippocampus, mostly through the neurotrophic pathways (Markov et al., 2023). In spite of the development of quite a few theories which are currently recognized, none of them is considered comprehensive (Sun et al., 2023). There are studies identifying cytokines and markers of oxidative stress as causative agents of depression. According to one of the recent theories, inflammation is one of its important links. Dysregulation of the adaptive and innate immune response promotes depression, increases its susceptibility and makes it resistant to the antidepressant treatment. Some studies suggest that anti-inflammatory treatment may be effective in management of depression, which is triggered by microbiota alteration, hereditary predisposition or even stress exposure (Beurel et al., 2020; Medina-Rodriguez et al., 2020). Numerous cytokines, such as interleukin-1 beta, interleukin-6, interferon-gamma, transforming growth factor beta, produce strong reaction to different pathogens and stressors, which results in the extended inflammatory reaction, dysregulation

of hormonal axes and depression (Liu et al., 2020; Michopoulos et al., 2020; Harsanyi et al., 2022). Association of cytokines with immune system as a result of intervention of external and internal factors leads to inflammation which in turn triggers depression and hampers the effect of antidepressants used for its treatment (Liu et al., 2020; Worthen et al., 2020). An interesting aspect of cytokine involvement in depression is presented in one of the recent studies (Yuan et al., 2024). Microglia activation, after chronic stress exposure, results in secreting of cytokines and growth factors. Effective antidepressant treatment inhibits activation of microglia, reverses hypertrophy of glial macrophages and reduces the number of their processes, which results in increased BDNF synthesis and activation of BDNF-mediated neurogenesis.

This research demonstrates the role of microglia and its cytokines in the neuroinflammatory pathways of depression development. Stress exposure promotes stimulation of glial macrophages acting on the blood vasculature. Other researchers support this concept, indicating it as a potential natural compound for depression treatment. Among the possible links in the pathophysiological cascade of microglia-stimulated depression, the possible mechanisms of how microglia regulates adult hippocampal neurogenesis in depression, the researchers list toll-like receptors, microglial polarization, fractalkine-C-X3-C motif chemokine receptor 1, as well as previously mentioned HHAA, cytokines, BDNF, and the microbiota-gut-brain axis (Jia et al., 2021; Troubat et al., 2021; Bai et al., 2022; Wang et al., 2022, Fang et al., 2023).

Other than cytokine and inflammation hypothesis are neurotransmitter/receptor, and neuroplasticity hypothesis. Though they have evidence-based confirmation, still they do not provide full understanding of the pathophysiology of depression and do not explain

why in many cases it remains drug-resistant. That is why more and more studies are being conducted on the interaction of several organs and systems, which trigger development of depression (Cui et al., 2024). Most of the studies support the concept of the role of HHAA in development of depression, and we also emphasize its significance for emergence of this suffering. Not only mechanisms of depression, but also mechanisms underlying the effects of its pharmacological treatments are complicated and unclear, that is why more research is required both on the pathophysiology of depression and mechanisms of the new medications which might be potentially used for its treatment (Cui et al., 2024).

2.3 Hypothalamic-Hypophyseal-Adrenal Axis and Depression

There are classical and new theories of depression. Neuroendocrine theory is one of the oldest but it does not lose its popularity. It is well known that endocrine disorders have a long-term effect on the mood (Markov et al., 2023). HHAA is a neuroendocrine feedback pathway. If it is dysfunctional, this may affect neuropeptide and neurotransmitter synthesis in the CNS, resulting in disruption of not only neuroendocrine, but also behavioral responses which may lead to development of depression (Sheng et al., 2021). Activation of HHAA starts with corticotropin- releasing hormone activation cascade outside of the hypothalamus in stress response, which stimulates release of adrenocorticotrophic hormone from the adenohypophysis, which in turn stimulates suprarenal glands to secrete glucocorticoids. Glucocorticoids control homeostasis and stress reactions. Their negative feedback suppresses activation of the HHAA. Type 1 CRH receptors are responsible for depression and stress response, while type 2 receptors control coping with stress. It was earlier described that dysfunctional relations between HHAA and thyroid axes result in development of depression, but this pattern is disputed by the other researchers (Hirtz et al., 2021; Gizzi et al., 2022).

Characterising HHAA activation and identifying the potential biomarkers of stress, the researchers put cortisol in the first place, followed by ACTH and BDNF (Noushad, et al., 2021).

Considering neuroinflammation as a trigger of depression, some researchers believe that dysregulation of HHAA is one of the three key factors which “backups” depression, another two being depletion of brain serotonin, and defective neurogenesis in the dentate gyrus of the hippocampus. This triple cascade is reinforced by chronic inflammation with circulating inflammatory markers which may cross blood-brain barrier and activate microglia (Troubat et al., 2021)

In many cases depression is associated with an exposure to severe stress, which activates HHAA, causing endocrine disturbances, immunosuppression and alterations in the brain, including hampered neurogenesis and neurotransmission (Biltz et al., 2022; Corrigan et al., 2023). At the same time some people develop depression without stress exposure, obviously in this scenario pathophysiology of depression is different from the one emerging as a result of stress, but information on this pathway is limited.

2.4 Modern trends in pharmacotherapy of depression treatment and antidepressants drug delivery to the brain

Modern antidepressants are not the ideal medications. Even the most effective ones show poor efficacy in certain cases together with unnecessary side effects. That is why research has shifted to searches of the new classes of medications, especially the natural products or natural components with no adverse effects as therapeutic alternatives. These new potential medications need to have a neuroprotective power and include the effects on the pathophysiology of depression, and the underlying mechanisms of these effects (Wang

et al., 2024). Since conventional antidepressants are not sufficiently effective, there is an urgent need to develop new antidepressant medications (Lundberg et al., 2023; Markov et al., 2023). New approaches suggest application of the medications which control BDNF level in the brain.

Melanocortins, including peripherally administered non-corticotropic agonists, regulate HPA axis activity, exhibit anti-inflammatory effects, stimulate the levels of neurotrophic factors, and enhance hippocampal neurogenesis and neurotransmission. Therefore, endogenous melanocortins and their analogs are able to complexly affect the functioning of those body's systems that are closely related to depression and the effects of antidepressants, thereby demonstrating a promising antidepressant potential (Markov et al., 2023).

Another possible alternative to the existing antidepressants could be neurotrophins themselves, such as BDNF, the deficit of which was shown to cause depression. So far neither BDNF nor other neurotrophins were ever used for depression treatment, the reason being, despite the promising therapeutic applications, the physico-chemical properties of BDNF have rendered it difficult to be delivered effectively into the central nervous system from the systemic circulation, as its relatively large molecular weight, short half-life, and relative impermeability to cross the blood-brain barrier remain the main hurdle of drug delivery to the structures in the brain which are involved in the pathological process (Khalin et al., 2016; Kapitonova et al., 2018; Kamarudin et al., 2020; Tosi et al., 2020).

The blood-brain barrier is a highly specialized barrier, which is formed by the endothelial cells which comprise the internal lining of the microcirculatory blood vessels, interconnected by tight junctions and reinforced by the astrocyte foot processes (Han &

Jiang, 2021). Because of the low permeability and high selectivity of the blood-brain barrier, only small molecules with a molecular weight of less than 400-600 Dalton can pass through it, provided they are highly hydrophobic or have good affinity for endogenous transporter molecules. In addition to that, the blood-brain barrier is intact in many chronic disorders affecting the central nervous system, which further limits the transport of large molecules. Currently, several types of carriers are used for drug delivery through the blood-brain barrier, such as liposomes, micelles, viral vectors, receptor-bound proteins, and exosomes. Nanoparticles (polymer, membrane-coated, and inorganic) which are colloidal carriers with a size varying from 1 to 1000 nm, remain one of the most successful carriers to deliver the medications to the brain through the blood-brain barrier. Among polymer NP, polylactic-co-glycolic acid (PLGA) nanoparticle are known as successful degradable carriers of the large protein molecules, such as neurotrophins, to the lesion site in the brain (Khalin, 2016; Kapitonova et al., 2018; Chen et al., 2022, Li et al., 2021; Wu et al., 2023).

2.5 Brain-derived neurotrophic factor in depression

Numerous studies on depression demonstrate the role of BDNF deficit in it. BDNF is a member of the family of neurotrophins, which are the proteins that regulate neuronal differentiation, survival, synaptic transmission, and plasticity in the central and peripheral nervous systems. Four neurotrophins are described so far: nerve growth factor, BDNF, neurotrophin-3 and neurotrophin-4. Only zebrafish has neurotrophin-6 and neurotrophin-7, which are not found in humans (Xue et al., 2021). Neurotrophins have two types of receptors: neurotrophin receptor p75 and tropomyosin receptor kinase (Trk). P75 is the receptor which is shared by the four known neurotrophins. For Trk receptors, nerve growth factor binds TrkA; BDNF and neurotrophin-4 bind TrkB; while neurotrophin-3 primarily binds TrkC. When neurotrophins were first described, they were found only in the central

nervous system, but later on it was proven that they are expressed by the cells of all other tissues: epithelial (endocrine cells), connective (macrophages and other immunocytes), and muscle tissue (smooth muscle cells and skeletal muscle fibers) (Brigadski & Leßmann, 2020; Cefis et al., 2020, Miranda-Lourenço et al., 2020; Xue et al., 2021; Ahuja et al., 2022; Chan et al., 2024; Fukuyama et al., 2024). According to some researchers, not only neurogenesis, survival, and regeneration of neurons, their synaptic plasticity, but also myelination of nerve fibers is associated with BDNF, which binds to the TrkB receptors (Brigadski & Leßmann, 2020; Cefis et al., 2020, Miranda-Lourenço et al., 2020; Xue et al., 2021; Fukuyama et al., 2024).

BDNF is involved in learning, memory, pain, and stress responses (Colucci-D'Amato et al., 2020; Sun et al., 2024; Lozano-Ureña et al. 2023; Antolasic et al., 2024). So far it is the best studied neurotrophin, it was shown to manage numerous cellular processes involved in the development and maintenance of normal brain function by binding to and activating TrkB. In the brain, BDNF is expressed by glutamatergic neurons, glial cells, and microglia. During prenatal development, BDNF–TrkB signaling maintains differentiation of progenitor cells of the cerebral cortex with subsequent maturation them into multipolar cortical neurons. Recent studies demonstrated that neurogenesis continues after birth, particularly in the hippocampus; it may involve dentate gyrus and subventricular zone (Sun et al., 2023; Lozano-Ureña et al. 2023; Zhang et al., 2023). It is believed that neurotrophins, the most important of which is BDNF, play a major role in the development of depression and may be used as a predictor of its treatment's response. (Rana et al., 2021; Pathak et al., 2022; Zelada et al., 2023; Talaee et al., 2024).

One of the recent studies described the glucocorticoid model of depression in

rodents, which has demonstrated reduction of the BDNF expression in the dentate gyrus together with the inhibition of nerve stem cells, progenitor cells and neuroblasts cell division and translocation, and stimulation of their apoptosis. Besides, it promotes autophagy of the neurocytes in the hippocampus due to increased autophagy protein 5 expression and excessive degradation of neuronal BDNF. The researchers believe that if triggered autophagy of the neurons is overcome, it might have an antidepressant effect in modelled depression (Zhang et al., 2023). This study contributes to the neurotrophic theory of depression, supported by other researchers, according to which environmental stressors decrease BDNF synthesis and disrupt BDNF/TrkB signaling in the brain regions involved in the cognitive function and mood regulation, resulting in deficits in neurogenesis and neuroplasticity, reduced synaptic plasticity, impaired synaptic transmission, and increased neuronal degeneration. These impairments result in structural changes in the brain, particularly atrophy of the prefrontal cortex and hippocampus, which underlie depressive disorders (Yang et al., 2020; Miyanishi et al., 2021; Madsen et al., 2024, Ye et al., 2024, Zagrebelsky & Korte, 2024; Zarza-Rebollo et al., 2024; Zhao et al., 2024)

At the same time, there is evidence that selective serotonin reuptake inhibitors, such as fluoxetine, do not increase BDNF levels in the blood even if the clinical effect is achieved, and therefore it cannot be used as an indicator of the severity of the disease and the effectiveness of treatment. Besides, even if antidepressants increase the serum level of BDNF in patients with depression, neither initial level of BDNF, nor its dynamics in the process of treatment with antidepressants, correspond to the severity of depression and the outcome of its treatment (Madsen et al., 2024, Talaei et al., 2024). This discrepancy may be explained by BDNF expression found in endotheliocytes, lymphocytes, and the other blood cells, as well as in smooth myocytes (Brigadski & Leßmann, 2020; Cefis et al., 2020)

BDNF is able to form and preserve dopaminergic, serotonergic, GABAergic, and cholinergic neurons. BDNF is also involved in neuroinflammation neuroprotection. The independent influence of each BDNF/BDNF-AS gene variation, as well as the interplay between SNPs and their linkage disequilibrium, environmental factors, including early-life experiences, and interactions with other genes, lead to alterations in brain architecture and function, shaping vulnerability to mental health disorders. The potential translational applications of BDNF/BDNF-AS polymorphism knowledge can revolutionize personalized medicine, predict disease susceptibility, treatment outcomes, and guide the selection of interventions tailored to individual patients (Shkundin et al., 2023).

2.6 Chapter summary

This chapter describes the recent research achievements in the area of pathophysiology and treatment of depression. It demonstrates growing prevalence of depression and the tremendous medical and social consequences of this growth. It discusses different theories of depression and emphasizes its most popular neurotrophin and endocrine theories. It introduces recent information on the neurotrophins and describes their role in development of depression. It narrates on the role of HHAA in emergence of depression and shows association between HHAA activity and the level of BDNF, the most studied neurotrophin related to depression. It analyses the most recent literature (the last five years) and provides evidence that so far there is no information on application of BDNF as a medication for treatment of depression. It shows the ways for the BDNF as a potential drug to penetrate through the blood-brain barrier. It also provides evidence that the relation between depression not associated with stress and activity of HHAA were not described before.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

This section lists the chemicals used in our experiments, describes the preparation of formulations and gives characteristics of the animals involved in the study.

3.1.1 Chemicals

The following chemicals were used in the study.

- i. **For modelling and treatment of depression in mice:** Reserpine, crystallized, >99.0% (HPLC), (#83580 Sigma Aldrich, USA); 0.9% Sodium chloride (#S0817, Sigma Aldrich, USA); Fluoxetine hydrochloride (#F132, Sigma-Aldrich, USA); Recombinant Human BDNF Protein (#AB206642, Abcam; UK); 100 nm PLGA nanoparticles (PLGA NP) (#805092, Sigma Aldrich, USA); 10% poloxamer 188 solution (#P5556, Sigma Aldrich, USA).
- ii. **For sorption test of BDNF:** Vivaspin 6 MWCO 30 000 (#28932317, Cytiva, USA); Phosphate buffered saline, 10x concentrate, (#P5493 Sigma-Aldrich, USA); Total BDNF Immunoassay; Quantikine ELISA, (#GZ-DBD00, R&D Systems Inc, USA), polysorbate 80 (#63161, Sigma Aldrich; USA).
- iii. **For corticosterone test:** Corticosterone ELISA kit (#AB108821, Abcam, UK).
- iv. **For histological analysis:** Formaldehyde solution 37% GR for analysis stabilized with about 10% methanol (#1040032500, Merck, Germany); Ethanol absolute, denatured (#C0314, HMBG); Hematoxylin (#7231Thermo Scientific, USA), Eosin (#7111, Thermo Scientific, USA), Bluing reagent (#7341, Thermo

Scientific, USA); Xylene (isomeric mixture) for histology (#1082984000, Merck, Germany); Histosec Pastilles, Solidification 56-58 °C (#1116092500R, Merck, Germany).

3.1.2 Preparation of drug formulation for the sorption test

This section describes the procedure of preparation of the solution of BDNF, suspension of PLGA NP, sorption of BDNF on the nanoparticle with different surfactants.

3.1.2.1 Preparation of BDNF solution

A stock solution of BDNF was prepared at the concentration of 0.003 µg/mL. BDNF was dissolved in normal saline. Similarly other concentrations of BDNF were prepared (0.002 µg/mL, 0.0008 µg/mL, 0.0004 µg/mL and 0.0002 µg/mL).

3.1.2.2 Preparation of PLGA suspension

Preparation of 100 nm PLGA NP at the concentration of 2.4 µg/mL was obtained from lyophilized PLGA powder which was dispersed in an isotonic solution. The solution was gently mixed using a pipette without causing any bubbles. Once the suspension had been thoroughly mixed, it was placed in the ultrasonic bath (23°C) for 20 minutes (GT-Sonic D2, China) at the power of 50 W with frequency of 40 kHz and subsequently placed on the orbital shaker for 3 hours at 300 rpm.

3.1.2.3 Preparation of BDNF-PLGA-surfactant suspension

For preparation of 0.003 µg/mL PLGA-BDNF, the prepared suspension of BDNF was added to 2.4 µg/mL of PLGA suspension and mixed by gently tapping on the side of the vial. The BDNF-PLGA suspension was incubated at low temperature (4°C) for 3 hours and continued to be shaken on orbital shaker for 3 hours at 300 rpm. The suspension was

added to 0.1 mL of 10% poloxamer 88 and continued to be mixed on the orbital shaker for another 2 hours at 300 rpm. BDNF and PLGA suspension were prepared in 0.45 mL volume each to maintain the end volume of 1 mL of PLGA-BDNF-surfactant suspension preparation. Similarly, the PLGA-BDNF-surfactant preparation was done for all remaining concentrations of BDNF (0.002 µg/mL, 0.0008 µg/mL, 0.0004 µg/mL & 0.0002 µg/mL) and repeated with another surfactant, Polysorbate 80.

3.1.2.4 Preparation of suspension for filtration in vertical membrane

Filtration in vertical membrane was done using the Vivaspin 6 centrifugal concentrator. The suspension was pipetted inside the Vivaspin 6 vial separately for each respective concentrations tested. The vials were placed inside the swing bucket centrifuge (KUBOTA, Japan) for 30 minutes at 4000 g. The centrifugation process filtered the suspension and the supernatant remained at the bottom of the vials once filtration is completed. The filtration process only filtered molecules that were less than 30,000 MWCO. In this study, only remaining BDNF molecules that were not successfully sorbed on the NP could be found in the supernatant. The remaining supernatant of each concentration including control were used for the ELISA test.

3.1.2.5 Determination of BDNF loading by ELISA test

The evaluation of BDNF loading into the NP was done to determine the optimum uptake of BDNF molecule by the 100 nm PLGA NP with the aid of surfactant. Surfactant acts as a reducer of the surface tension during the delivery of BDNF through the blood-brain barrier. For the quantitative evaluation of the BDNF adsorption study, ELISA test was done to detect the remnants of BDNF molecule by targeting it at the absorbance of 450 nm using microplate reader (SpectraMax iD3, USA).

The summary of the procedure of preparation of nanoparticulate BDNF suspension with a surfactant is shown in Figure 3.1.

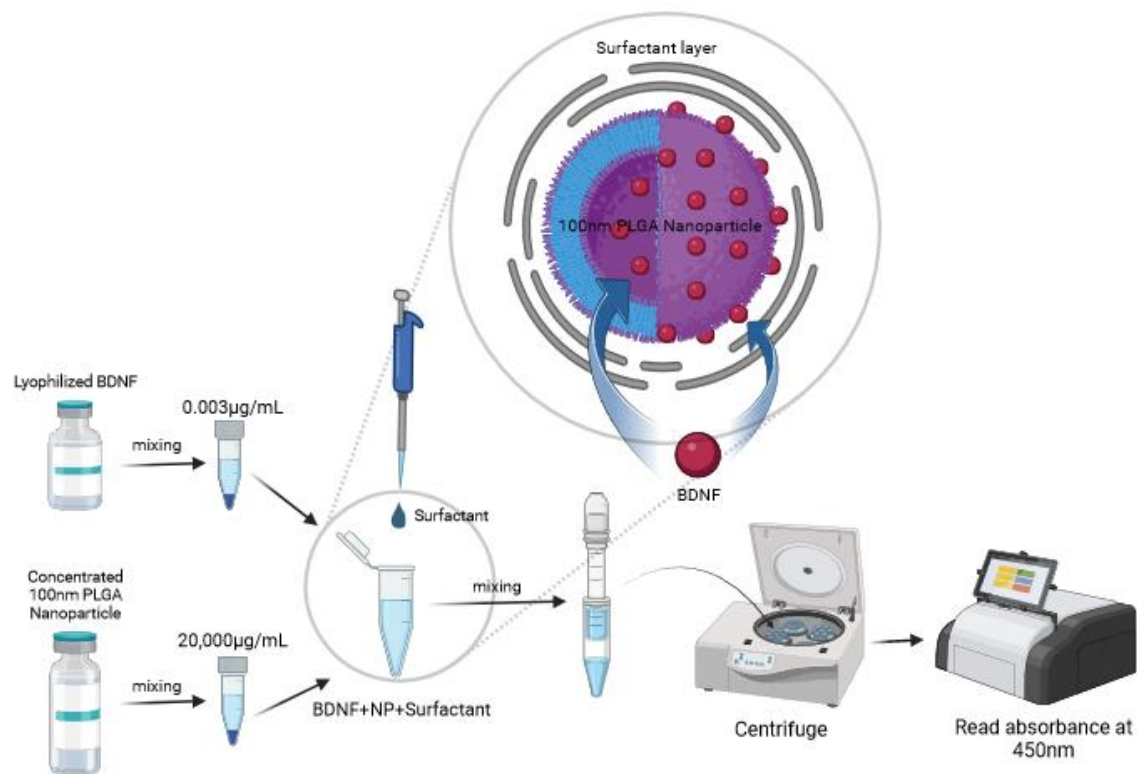


Figure 3.1: Algorithm of preparing BDNF-loaded NP with surfactants

3.2 Animals

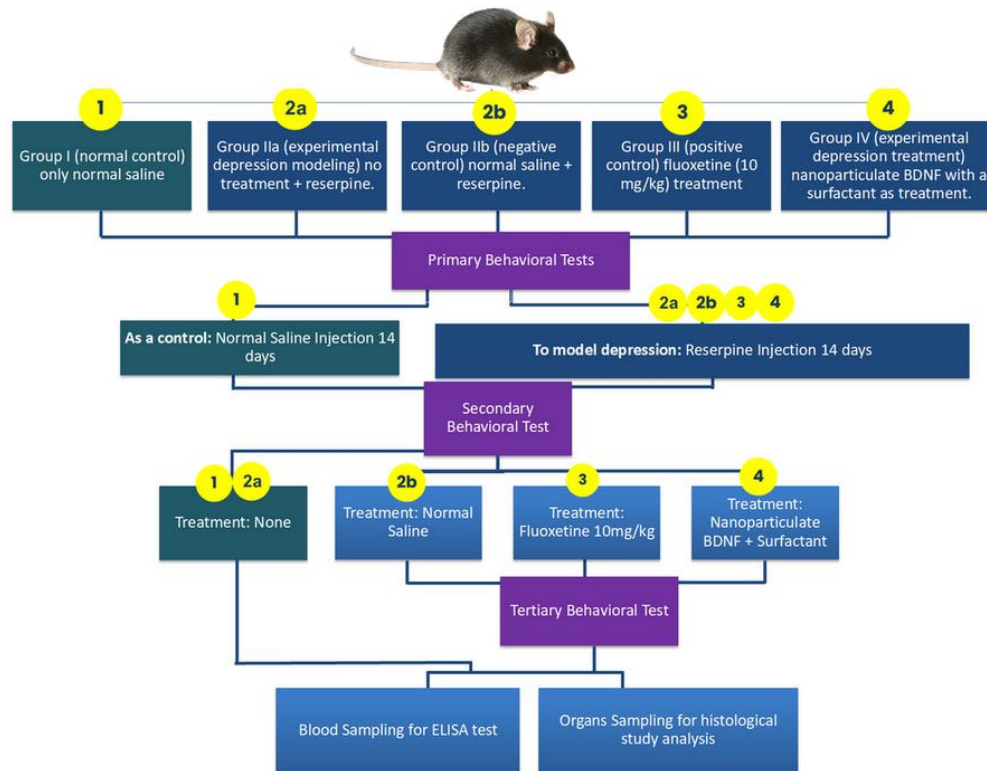


Figure 3.2: Methodologies overview of the study

In this study, 30 male C57BL/6 mice (25-30 g) were involved. The animals were housed 3-4 mice per cage with 12-hour light and dark cycles (lights on at 0600 hours) and at a set room temperature. Standard tap water and standard rodent chow was available *ad libitum* for at least seven days before starting the experiment for habituation. Animals were tested in light phase. The mice were habituated to various handling procedures, prior to the experiment, in order to annul the psychological effect of the new environment. All mice were weighed at day zero and at the last day before sacrificing. The experiments were conducted in accordance with the Malaysian Code of Practice for the Care and Use of Animals for Scientific Purposes (MyCode). All protocols for experimentation were reviewed by the UNIMAS Animal Ethics Committee (UNIMAS-AEC), the approval

protocol code is UNIMAS/AEC/R/F07/060. The animals were randomly divided into five groups. Group I (normal control) mice only received normal saline intraperitoneally (i.p.); Groups IIa, IIb, III and IV mice were used for modelling of reserpine-induced depression without stress exposure: Group IIa – experimental (depression modelling), its animals did not receive any treatment of depression and were sacrificed one day after the last injection of reserpine together with Group I animals; Group IIb - negative control, its mice were treated from depression with intravenous (i.v.) injections of 0.2 mL of normal saline; Group III – positive control, its animals were treated with a traditional antidepressant fluoxetine at a dose of 10 mg/kg continuously; Group IV – experimental (depression treatment), its mice were treated with i.v. injections of nanoparticulate BDNF with a surfactant.

3.3 Formulation

3.3.1 Preparation of the formulation

Reserpine model of depression was used to induce depression in C57BL/6 mice of Groups IIa, IIb, III and IV (n=24). Powder of reserpine was dissolved in 50 mL normal saline and injected in a dose of 0.5 mg/kg with 0.1% of lidocaine i.p. (de Freitas et al., 2015).

3.3.2 Application of the formulation for depression modelling

A single daily intraperitoneal dose was given for a period of 14 days to model depression. Normal control mice (Group I, n=6) were injected with 0.1 mL of normal saline instead of reserpine.

3.4 Treatment of depression

3.4.1 Modes of depression treatment

On day 15, after the last reserpine injection, the mice were further treated with intravenous injections of normal saline (Group IIb) or traditional antidepressant fluoxetine at a dose of 10 mg/kg (Group III) or nanoparticulate BDNF with surfactant poloxamer 188 (Group IV) (Kapitonova et al., 2018; El-Marasy et al., 2021).

3.4.2 Preparation of Suspension NP

The lyophilized powder of PLGA (20 mg) was suspended in 1 mL of normal saline. It formed a lacteous white color homogenous suspension. After sonification of the suspension for 15 min at a power of 60 W it was placed on the magnetic stirrer for stirring at the speed of 300 rpm for 3 hours.

3.4.3 Preparation of BDNF

In preparation of BDNF stock, 50 µg of lyophilized BDNF powder were dissolved in 1 mL of normal saline.

3.4.4 Preparation of Nanoparticulate BDNF

The solution of BDNF was added to the suspension of nanoparticles, incubated for 3 hours at a low temperature of 0-4 °C for 3 hours and stirred using a magnetic stirrer at the speed of 300 rpm for about 3 hours.

3.4.5 Preparation of the suspension of NP with BDNF and surfactant

In preparation of the suspension of NP together with BDNF and surfactant, the initial suspension of nanoparticulate BDNF stock was added to 0.2 mL of 10% poloxamer 188 and stirred using magnetic stirrer. The final formulation was a 2 mL preparation

containing 1% suspension of NP with poloxamer and 50 µg of BDNF. Only 0.9 mL of normal saline were used to dissolve the NP and BDNF to make the total volume of 2 mL of the preparation. Each animal was injected with 0.2 mL of preparation (Kapitonova et al., 2018).

3.5 Methods

In our study we used behavioral methods for assessment of the level of depression, ELISA test for corticosterone which is the main glucocorticoid hormone in mice, histological methods of tissue processing, microscopic method for assessment of the changes in the endocrine glands and immune organs, and image analysis for the quantitative assessment of HHAA activation and immunosuppressive changes in the body of experimental and control animals.

3.5.1 Behavioral Tests

Open field and forced swimming tests (Park et al. 2020) were conducted three times to evaluate severity of depression and effectiveness of its treatment: before modelling of depression (primary test), one day after the last i.p. injection of reserpine (Groups IIa, IIb, III, and IV) or normal saline (Group I) (secondary test) and 7 days after treatment of depression with normal saline (Group IIb), fluoxetine (Group III) and nanoparticulate BDNF with a surfactant (Group IV) (tertiary test).

3.5.1.1 Open Field Test

In the open field [45 (W) x 45 (D) x 40 (H) cm] test (Panlab Harvard with USB digital camera and SMART 3.0 video tracking software, Barcelona, Spain) individual rodents were placed in an open containment chamber (Figure 3.3) which was large enough for the animals to move around, which was divided into central and peripheral

compartments.



Figure 3.3: Open Field Test with an experimental mouse in the peripheral compartment

Activity mapping was recorded by the digital camera summarized using the SMART 3.0 software in the 2D and 3D mode (Figures 3.4 and 3.5).

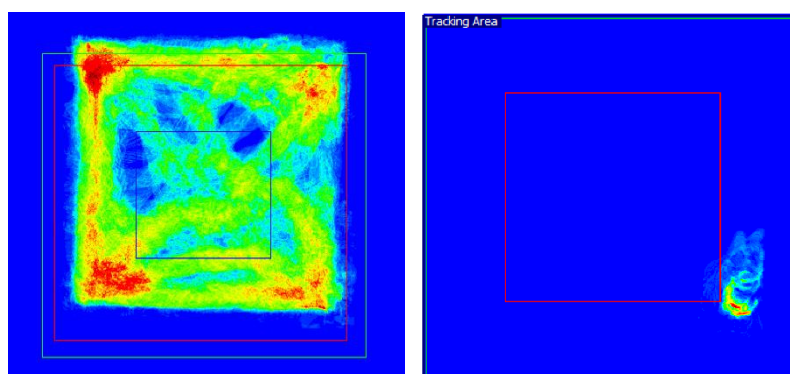


Figure 3.4: Activity map detecting mouse body heat in 2D motion: left – normal control mouse, right –negative control mouse after depression modelling

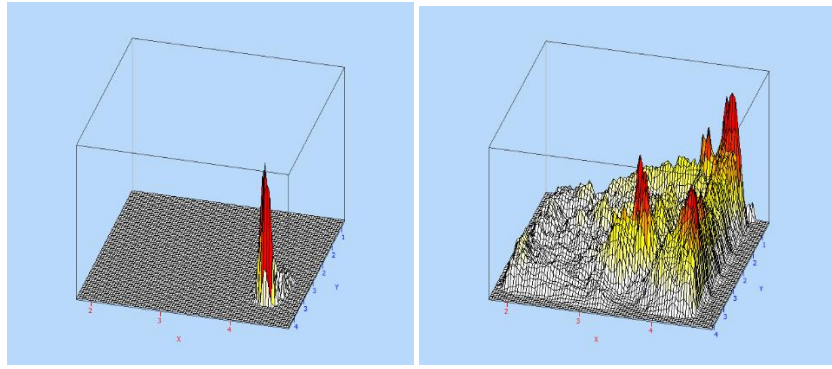


Figure 3.5: Activity map detecting mouse body heat in 3D motion: left – negative control mouse, right – experimental mouse after BDNF treatment of depression

Walking distance, rearing and grooming frequencies, defecation and urination activities were assessed in each mouse (Figure 3.6).

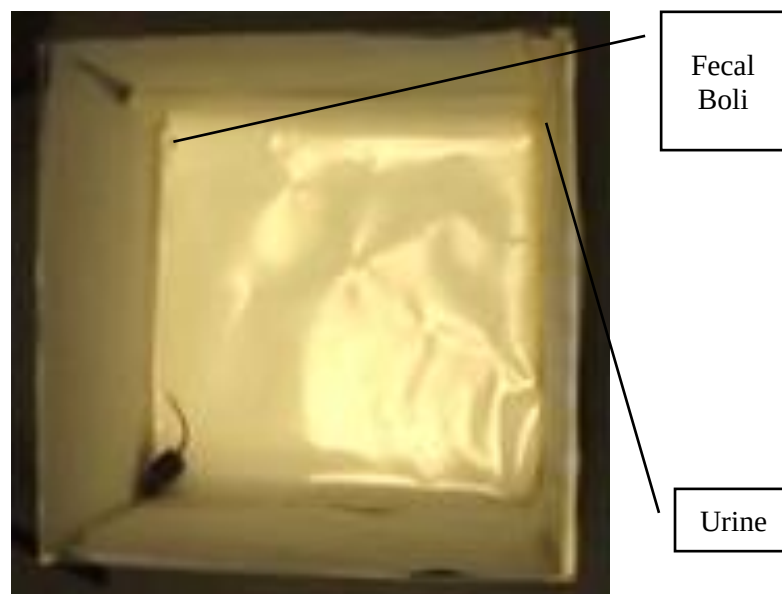


Figure 3.6: Defecation and urination assessed manually during the recording of open field test

The behavior of the mice was recorded for 10 min and analysed using video tracking software (Figure 3.7).

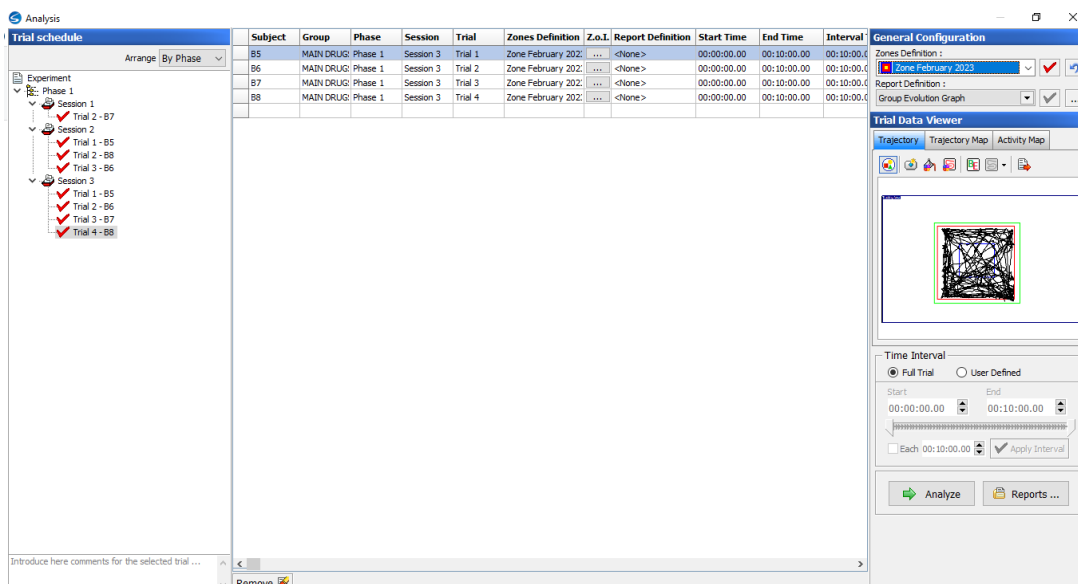


Figure 3.7: Analysis of the recorded videos with SMART software.

After each individual rodent testing, the entire field was wiped clean with 95% ethanol and let dry to eliminate the rodent scent for the next individual testing.

3.5.1.2 Forced Swimming Test

The forced swimming test (FST) was based on the method of Porsolt (Park et al., 2020).

For this type of testing each mouse was placed in a cylinder (height: 40 cm; diameter: 25 cm) containing tap water ($25 \pm 2^\circ\text{C}$; depth: 20 cm) which is inescapable, and the animal undertakes certain physical activities to stay afloat for a 6 minutes trial (Figure 3.8).



Figure 3.8: Forced swimming test for the negative control mouse

Total immobility time was measured during the last 4 minutes of the trial; during the first two minutes mice are usually very active in this test, and this high activity may mask the potential effect of medications as well as the background behavior of the experimental animals. Water was changed between subjects. The assessed behavioral parameters included: immobility duration (time spent floating), swimming, and climbing (struggling) time. The duration of each mode of behavior was recorded and comparison between the groups of animals was done.

3.5.2 Blood Sampling

Upon completion of the last open field test, blood was directly sampled from the heart by cardiac puncture technique and placed into cryovials.

The sample was centrifuged, and the supernatant was tested by enzyme-linked immunosorbent assay (ELISA) kit for corticosterone in accordance with manufacturer's protocol (Figure 3.9).

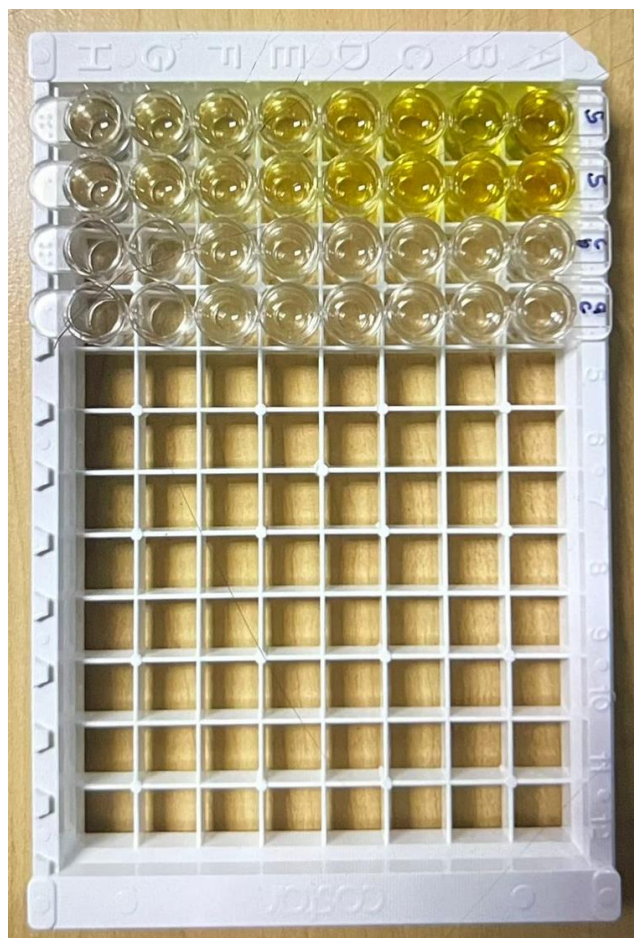


Figure 3.9: Preparation for the corticosterone test using ELISA

3.5.3 ELISA Assay Protocol

ELISA assay is widely used to detect the presence of an antigen in a sample. The antigen-specific antibody was capturing the antigen which was immobilized to the well. An antigen-antibody complex would form if the detection antibody connected with the antigen, provided the latter was present. A spectrophotometer was used to measure the amount of the respective antigen. In this study, ELISA kit was used for detection of corticosterone. We followed the procedure described by the AVIVA Systems Biology (AVIVA systems, 2024). The absorbance of each well was read with a microplate reader at optical density of 450 nm (SpectraMax iD3, USA).

3.5.4 Tissue Sampling

After all tests were completed at the end of the experiment, the animals were sacrificed by decapitation under chloroform inhalation anesthesia in accordance with the protocol of the Institutional Animal Care and Use Committee (IACUC).

Adrenal glands and thymus of the mice were sampled as shown in the cross section of Figure 3.10 and fixed in 10% neutral formalin.

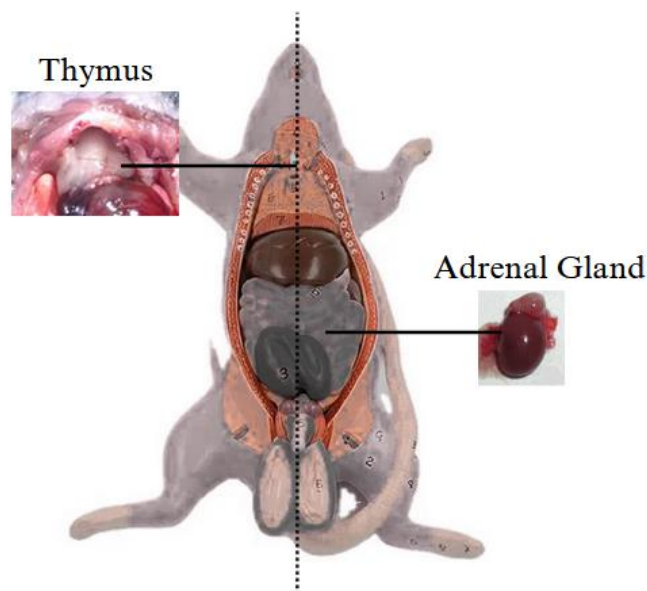


Figure 3.10: Tissue sampling in experimental mouse

3.5.4 Tissue processing

Automatic tissue processing using Excelsior As tissue processor (Thermo Scientific, USA) included dehydration in a series of graded alcohols, clearance in xylene and embedding in paraffin using paraffin wax dispenser (Thermo Scientific, USA). Three-

micron thick histological slides were sectioned using semi-automated HM340E rotary microtome (Thermo Scientific, USA). The procedure of histological methods for tissue processing of the organs are shown in Figure 3.11.

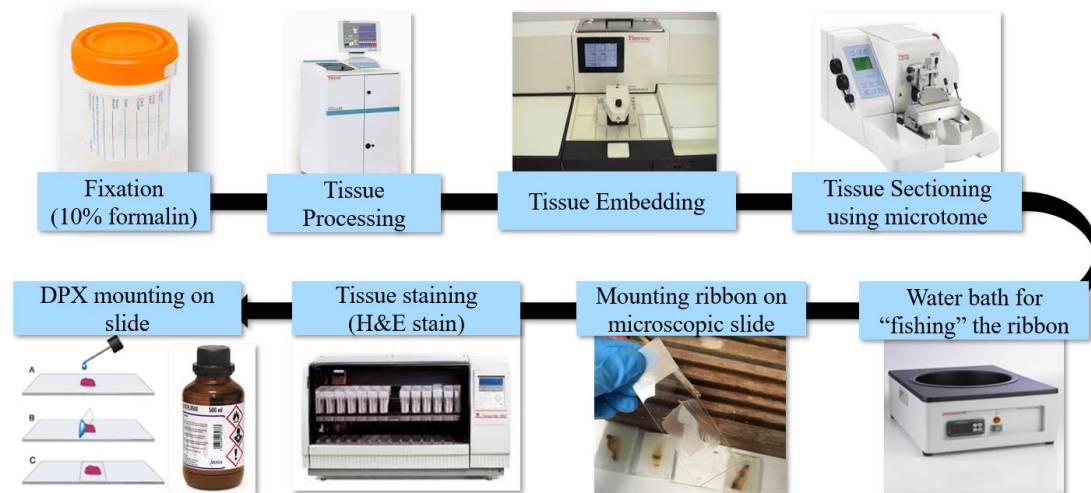


Figure 3.11: Procedure of Tissue Processing for Histological Analysis

The slides were dewaxed in xylene, then xylene was removed by absolute alcohol, and the slides were hydrated in the series of graded alcohols until water and stained with hematoxylin-eosin staining using a manual 12-bowl stainer (Diapath, Italy). A standard protocol by Leica biosystems was used for staining of the histological slides (Leicabyosystems, 2024). The histological slides were stained with hematoxylin-eosin using Mayer's hematoxylin, differentiated, blued, stained with eosin Y, dehydrated in a series of graded alcohols, cleared in xylene and coverslipped. After drying the histological slides were viewed under Zeiss Primostar 3 microscope (Jena, Germany). On the qualitative level in the adrenal cortex, attention was paid to the behavioral changes to the width of the adrenal cortex and its zones, the number of cellular layers in the zona glomerulosa and zona reticularis, the length of the cords in the zona fasciculata and the number of cell columns in them, the demarcation between zona fasciculata and zona

reticularis, the size, shape and tinctorial features of adenocytes, the distribution blood vessels and the degree of plethora in them.

On the qualitative level in the thymus we assessed the prominence of the cortico-medullary border, relative width of the cortex, the density of its cellularity and presence of the tingible-body macrophages containing apoptotic bodies, presence of the Hassall's corpuscles in the medulla.

3.5.5 Image Analysis

For image analysis 10 randomly taken photos of the histological slides of the adrenal glands and thymus using objective x10 and x40 were saved as digital images and further processed using Image Pro+ 7.0 software (Media Cybernetics, USA). For digital morphometry left adrenal gland and left lobe of thymus of every mouse were chosen. The measurements were performed on the sections from the middle portion of adrenal glands and from the coronal sections of the thymus. The following parameters were selected to characterize the changes in the organs of experimental and control animals. For adrenal glands: cortico-medullary ratio, volume density of the zones of the adrenal cortex, mean area of the respective cells and their nuclei. These parameters allow to evaluate the degree of adrenocortical hyperplasia and the contribution to it of the zonae and particular adenocytes. In the thymus the selected parameters indicating its functional condition were its cortico-medullary ratio and volume density of the tingible-body macrophages with apoptotic bodies which indicate the presence (if any) of immunosuppressive changes in the organ.

3.5.6 Statistical Analysis

Data collected from behavioral tests, ELISA and digital morphometry of the

histological slides were statistically processed and analysed using Excel (Microsoft, USA) and one-way ANOVA (IBM SPSS statistics 26) with calculation of mean, standard deviation, mean error by Tukey's post-hocs test. Shapiro-Wilk test was used for normality of distributions. Differences were considered significant if $p < 0.05$.

3.6 Chapter summary

This chapter summarizes the materials (chemicals and animals, mice) and the methods used in the study: modelling of depression, treatment of depression, behavioural methods, ELISA, histological examination, digital morphometry and statistical methods. Behavioral methods (open field, forced swimming) were used to determine the level of depression in animals treated with a new medication proposed by us (PLGA-BDNF with poloxamer 188) and a traditional antidepressant fluoxetine in comparison with normal and negative controls. In the open field we assessed the horizontal and vertical activity, urination, defecation and grooming. In the forced swimming test, we evaluated the duration of the active phase (swimming, struggling) and passive phase (floating). ELISA was used in the sorption test to determine the best surfactant for our medication and in the corticosterone test to determine the level of the hormone in blood. The histological methods were used to assess the microscopic changes in the endocrine glands and immune organs. Digital morphometry was used to receive quantitative data in the microstructural changes in the adrenal glands (volume density of the zona fasciculata, area of spongiocytes and their nuclei) to evaluate the degree of adrenocortical hyperplasia) and in the thymus (cortico-medullary ratio, volume density of the tingible-body macrophages with apoptotic bodies). Methods of parametric statistics (ANOVA, Tukey test) were used to determine statistical differences between the experimental and control groups.

Thus, combination of different methods: physiological (open field, forced swimming test), biochemical (ELISA), histological (microscopy of the tissues) and image analysis (digital morphometry) allowed us to obtain meaningful results.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Selection of the surfactant for optimal sorption of the BDNF on the NP

The evaluation of BDNF loading into the NP was done to determine the optimum uptake of BDNF molecule on the 100 nm PLGA NP with the aid of the surfactant. Surfactant acted as a reducer of the surface tension during transportation of BDNF through the blood-brain barrier. For the quantitative evaluation of the BDNF adsorption, ELISA test was done to detect the remnants of BDNF molecule by targeting it at the absorbance of 450 nm using microplate reader (SpectraMax iD3, USA).

4.1.1 A radius of selected BDNF concentrations

The primary aim was to determine the optimal concentration with the combinations of surfactant that effectively enhance the delivery of BDNF by the nanoparticles as a potential antidepressant. In achieving this, a diverse range of BDNF concentrations were considered according to the past studies that successfully delivered BDNF to the targeted area (Kamarudin et al., 2020; Han et al., 2021). Quantikine ELISA kit of Human Free BDNF Immunoassay was used in detecting the remaining BDNF left in the supernatant after centrifugation using Vivaspin separator vial. Based on ELISA kit detection range, the standard reading was from 0 µg/mL to 0.004 µg/mL, and the minimum BDNF detection was 0.0002 µg/mL. Hence, in finding the reading suitability, 0.003 µg/mL BDNF concentration was selected as a maximum concentration in comparison to an actual past study concentration, which was 25 µg/mL. According to the mentioned studies, 25 µg/mL BDNF concentration was dispersed in 20,000 µg/mL of PLGA NP suspension with the ratio 1:800. The PLGA concentration remained the same for all concentrations of BDNF.

Therefore, 2.4 µg/mL PLGA concentration was used in dispersing the whole BDNF range of concentrations in this assay.

4.1.2 Comparative analyses of sorption efficacy in different concentrations of BDNF in the absence and presence of poloxamer 188 as a surfactant

The comparison was done by preparing two types of BDNF suspension with same range of concentrations (C1; concentration 1 (0.003 µg/mL), C2; concentration 2 (0.002 µg/mL), C3; concentration 3 (0.0008 µg/mL), C4; concentration (0.0004 µg/mL) and C5; concentration (0.0002 µg/mL). The first type (Type 1-A) of BDNF suspension was prepared with poloxamer 188 as a surfactant and the second type (Type 2) was without poloxamer 188.

Within the Type 1 and Type 2 of the trend lines in the graph (Fig. 2), a significant decrement of BDNF percentage in the Type 2 of suspension was discovered. The range of concentrations in Type 2 group showed significant differences in the values of percentage compared to the concentrations with Type 1-A. C1 (Type 2) had 98.3% success rate of BDNF loading on the nanoparticles compared to C1 (Type 1-A) which was 99.9%. C2 (Type 2) had 95.6% success rate of BDNF loading on the nanoparticles compared to C2 (Type 1-A) which was 99.9%. C3 (Type 2) had 91.1% success rate of BDNF loading on the PLGA NP compared to C3 (Type 1-A) which was 99.9. C4 (Type 2) had 80.8% success rate of BDNF loading in the PLGA NP compared to C4 (Type 1-A) which was 99.9%. C5 (type 2) had 60.3% when compared to C5 (Type 1-A) which was 99.8%. Type 1-A revealed a high level of BDNF loading on the nanoparticles in the sorption process (Figure 4.1).

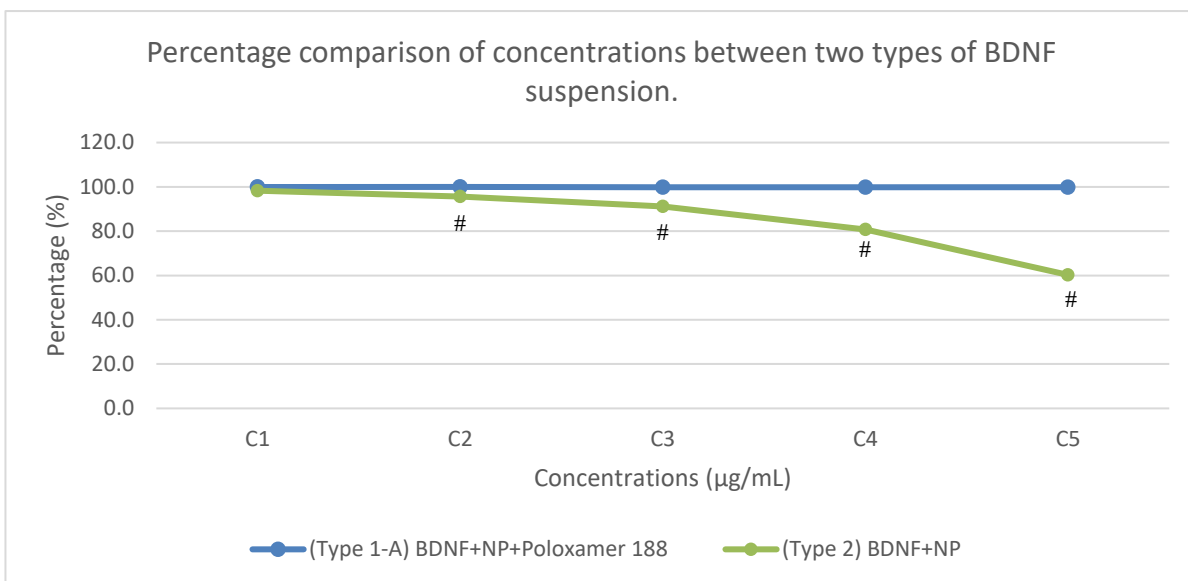


Figure 4.1: The line graph indicating the trends of percentage comparison between two types of BDNF suspension. The first type (Type 1-A) is in presence of surfactant (BDNF+NP+poloxamer 188) and the second type (Type 2) is in absence of surfactant (BDNF+NP). #- $p < 0.05$ when second type compared to the first type of BDNF suspension

4.1.3 Comparative analyses of sorption efficacy in different concentrations of BDNF in the absence and presence of Polysorbate 80 as a surfactant

This assay was performed in using the second type of surfactant for analysing the different impact on success rate of BDNF loading on the PLGA NP. The preparation consisted of polysorbate 80 in the type 1-B suspension (BDNF+NP+Polysorbate 80) to be compared with type 2 suspension (BDNF+NP). All concentrations in the preparation of type 1-B and type 2 suspension remained the same according to the specified range (0.003 µg/mL, 0.002 µg/mL, 0.0008 µg/mL, 0.0004 µg/mL & 0.0002 µg/mL). The distinct percentage distributions in Type 2 group can be seen when compared to the Type 1-B. C1 (Type 1-B) had a 99.9% success rate compared to C1 (Type 2). C2 (Type 1-B) had a 98.0% success rate compared to C2 (Type 2). C3 (Type 1-B) had a 95.6% success rate compared to C3 (Type 2). C4 (Type 1-B) had a 90.4% success rate compared to C4 (Type

2). C5 (Type 1-B) had an 80.0% success rate compared to C5 (Type 2). Type 1-B appeared to have a higher success rate in sorption of BDNF on the nanoparticle compared to Type 2 (Figure 4.2).

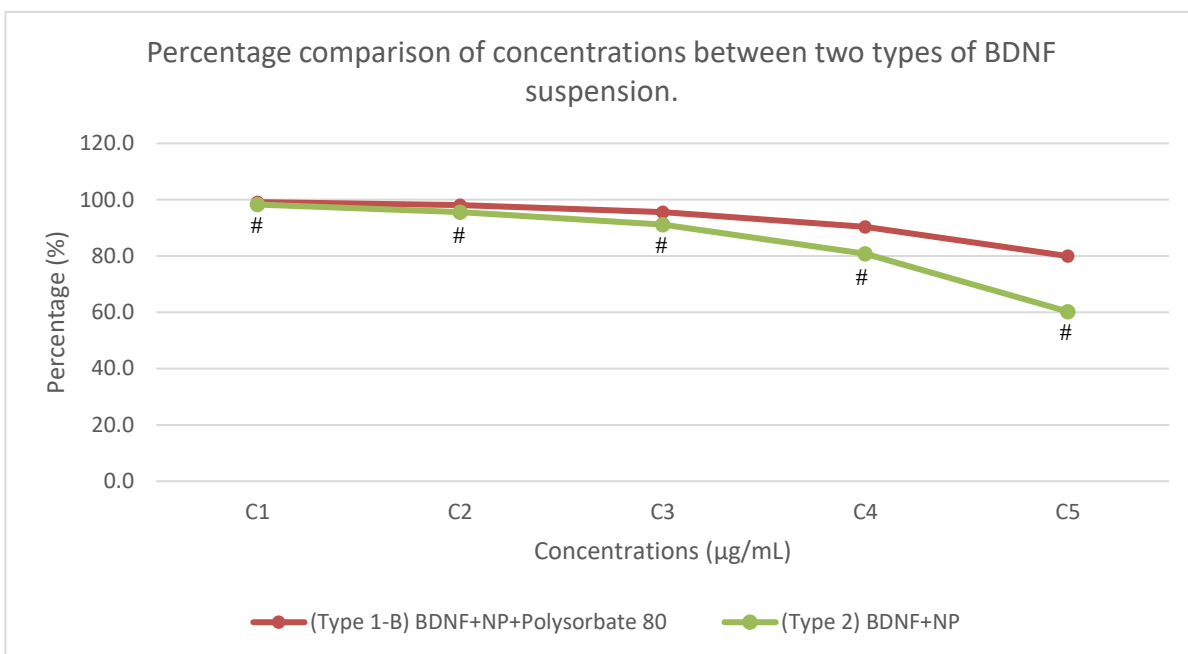


Figure 4.2: The line graph indicating the trends of percentage comparison between two types of BDNF suspension. The first type (Type 1-B) is in presence of surfactant (BDNF+NP+Polysorbate 80) and the second type (Type 2) is in absence of surfactant (BDNF+NP). #- $p < 0.05$ when second type compared to the first type of BDNF suspension

4.1.4 Comparative analyses of sorption efficiency in different concentrations of BDNF loaded on the nanoparticles with conventional surfactants

The sorption analyses (Figures 4.1 and 4.2) showed that the absence of surfactants resulted in the reduced efficacy of sorption. Hence, it was statistically proven that the presence of surfactants enhanced the chances of BDNF to be fully utilized as a potential medication for treatment of depression, as it may more effectively penetrate through the blood-brain barrier without a need to increase the concentration of nanoparticles. In refining the efficiency of the surfactants' presence in BDNF-loaded nanoparticles,

comparative statistical analysis was done between the two conventional surfactants, poloxamer 188 and polysorbate 80.

In the line graph (Figure 4.3), it indicated that type 1-B has an apparent decrement in trend lines compared to the type 1-A. C1 (Type 1-B) had a success rate of 99.1% compared to the C1 (Type 1-A) which is 99.9%. C2 (Type 1-B) had a success rate of 98.0% compared to the C2 (Type 1-A) which is 99.9%. C3 (Type 1-B) had a success rate of 95.6% compared to the C3 (Type 1-A) which is 99.9%. C4 (Type 1-B) had a success rate of 90.4% compared to the C4 (Type 1-A) which is also 99.9%. While C5 (Type 1-B) had a success rate of 80.0% compared to the C5 (Type 1-A) which is 99.8%. Type 1-A appeared to have higher success rate of BDNF loaded on nanoparticles in the presence of poloxamer 188 as a surfactant compared to type 2-B (Polysorbate 80).

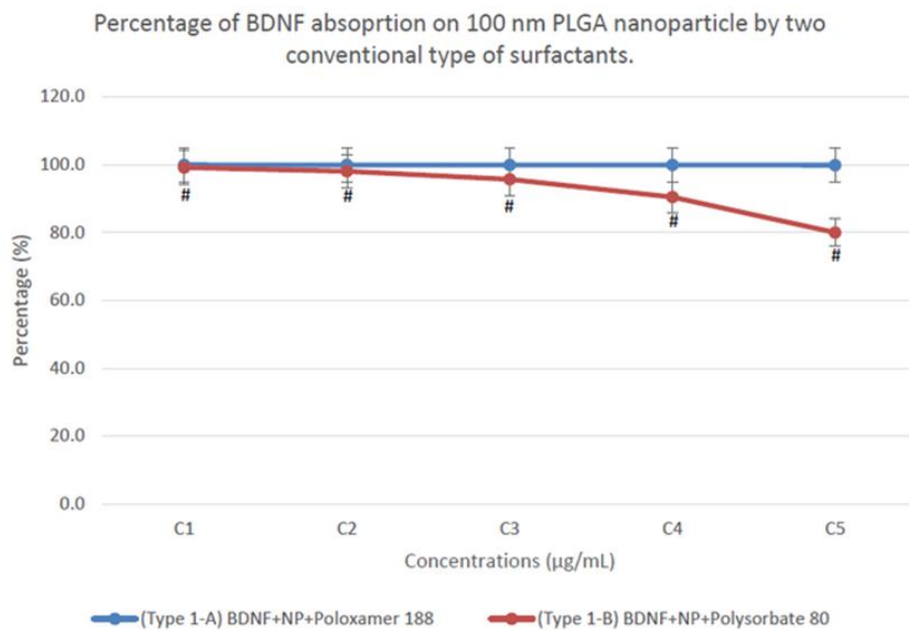


Figure 4.3: Line graphs of BDNF sorption percentage of the two different types of conventional surfactants. #- $p < 0.05$ compared to the type 1-A

4.2 Outcomes of depression modelling (Part I)

The response to modelled depression in the mice of the normal control (Group I) and experimental (for depression modelling, Group IIa) groups was demonstrated by the behavioural tests.

4.2.1 Behavioral test results

Behavioral tests applied in our research included open field and forced swimming. These tests are standard trials to measure the depth of depression in rodents. Open field test allows to assess different manifestations of the mode of behavior, such as a walked distance, frequency of rearing, grooming, urination and defecation; while forced swimming test permits to evaluate the readiness of the rodents to fight for survival, as the animals are choosing the type of physical activity (floating, struggling or swimming) in water, which is not their common environment, depending on their mood. In depressed animals the share of the time of floating is increasing, while the time of the active behavior in the water, such as swimming or struggling is getting reduced. Both tests are considered to be sensitive and specific for the assessment of the level of depression in mice.

4.2.1.1 Open field test

Analysis of the horizontal activity of the mice in the primary open field test showed that animals of both groups (Groups I and IIa) were freely moving around; but in the secondary open field test horizontal activity of the animals of Group IIa was confined to the peripheral part of the field, while animals of the normal control group (Group I) were still freely moving around, investigating the new environment. An example of the itinerary travelled by the Group I (normal control) and Group IIa mice (experimental for depression modelling) in the secondary test is shown in **Figure 4.4**.

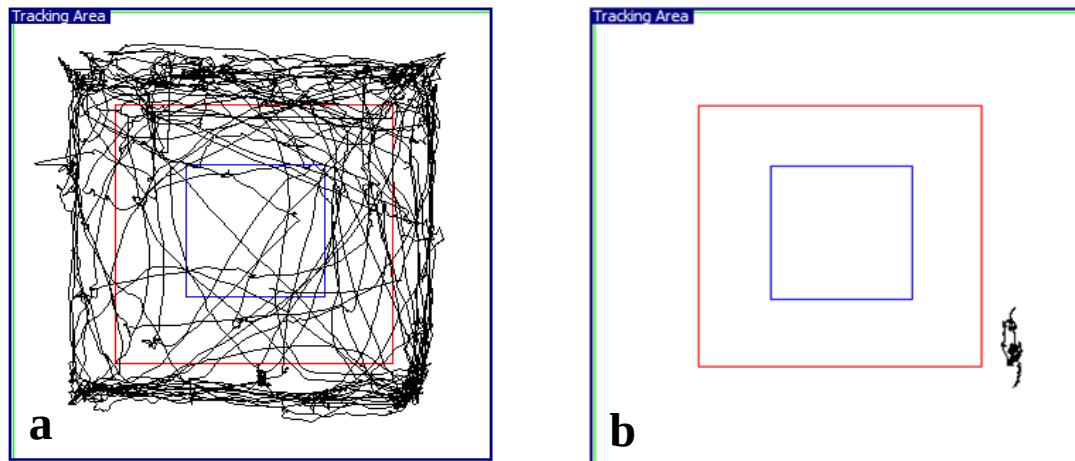


Figure 4.4: Horizontal activity of the normal control (a) and experimental (for depression modelling) (b) animals in the secondary open field test

The results of the open field test are shown in Figures 4.5 – 4.9.

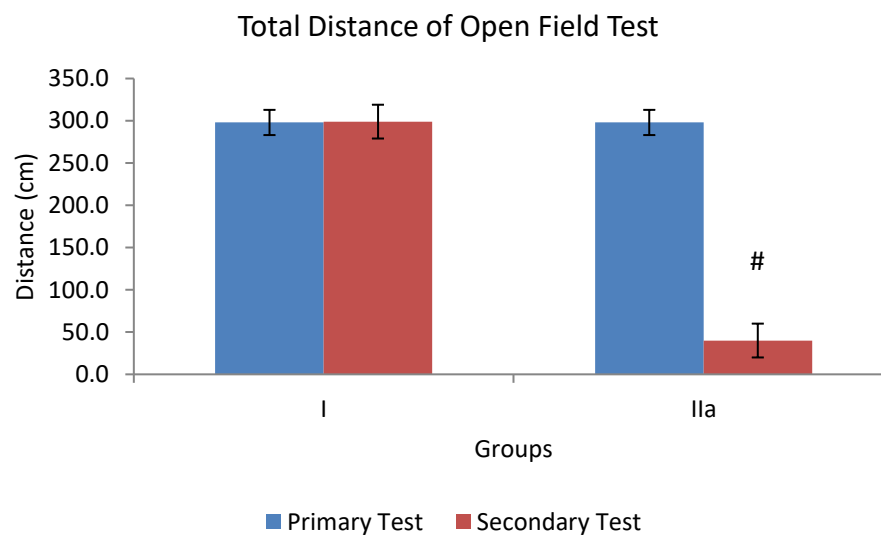


Figure 4.5: Distance walked by the experimental (for depression modelling) and normal control animals (cm, $M \pm S$); # - $p < 0.01$

As follows from Figure 4.5, the animals of Group IIa (experimental for depression modelling) significantly reduced the walked distance in secondary test, while the animals of the normal control group (Group I) sustained it at the same high level as in the primary test.

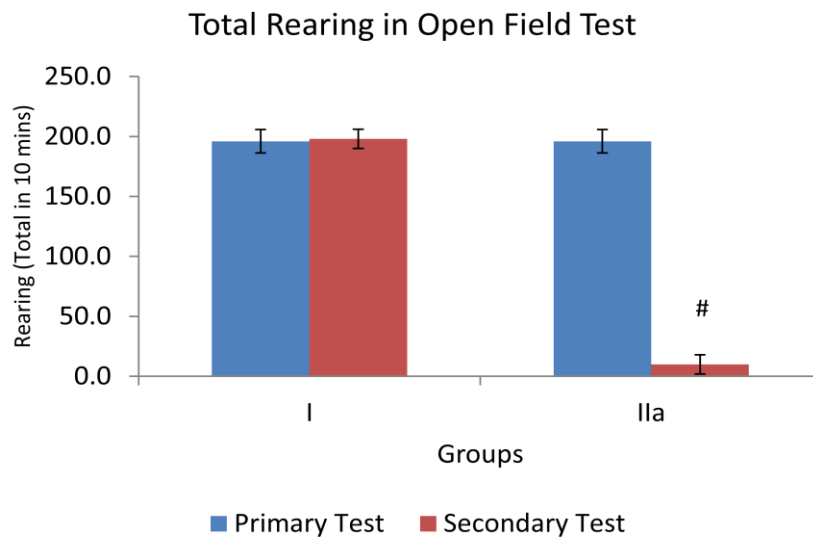


Figure 4.6: Rearing frequency in the experimental (for depression modelling) and normal control animals (M±S), # - $p < 0.01$

Similarly, as follows from Figure 4.6, the animals of Group IIa significantly reduced the number of rearings after depression modelling, while the animals of the normal control group (Group I) sustained it at the same high level.

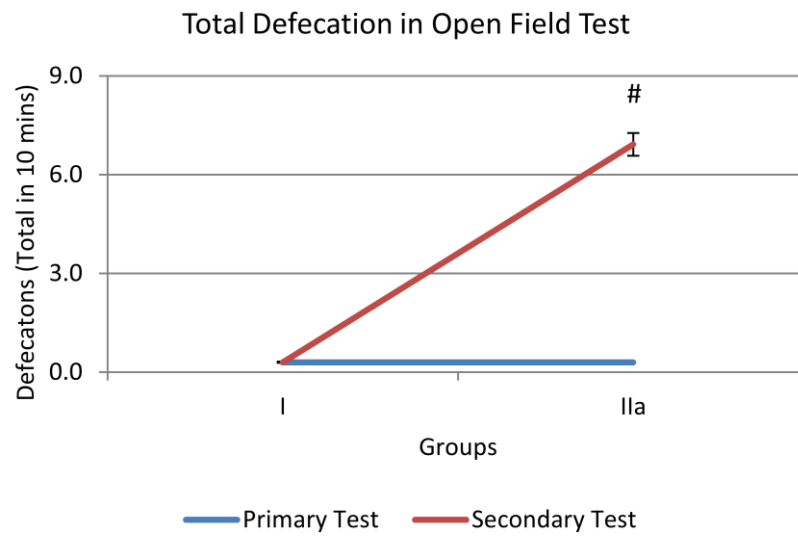


Figure 4.7: Frequency of defecation in the experimental (for depression modelling) and normal control animals ($M \pm S$), # - $p < 0.05$

As follows from Figure 4.7, the animals of Group IIa significantly increased the number of defecations after depression modelling, while the animals of the normal control group (Group I) sustained it at the same low level.

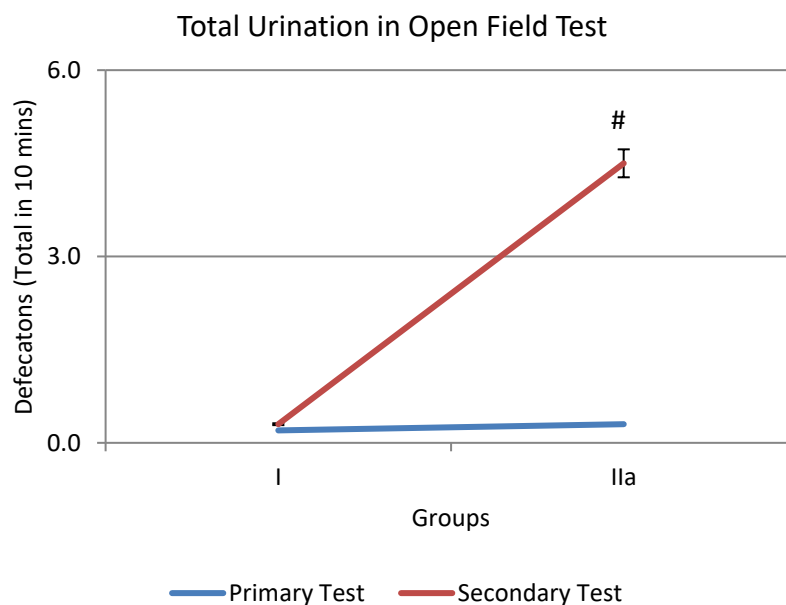


Figure 4.8: Frequency of urination in the experimental (for depression modelling) and normal control animals ($M \pm S$), # - $p < 0.05$

Regarding urine boluses, their number was slightly different between the Group I and Group IIa animals in the primary test (before depression modelling), while in the secondary test (after depression modelling) the difference between the groups became significant (Figure 4.8). The overall the dynamics of urination in the groups did not differ from the defecation pattern (Figure 4.7).

The number of grooming cycles in the open field test is shown in Figure 4.9.

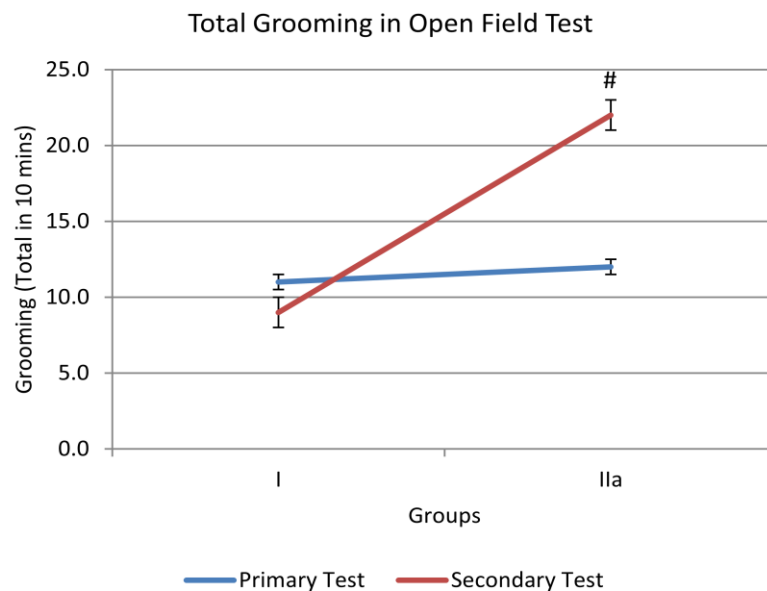


Figure 4.9: Grooming frequency in the experimental (for depression modelling) and normal control animals ($M \pm S$), # - $p < 0.05$

As follows from Figure 4.9, after depression modelling the grooming rate in Group IIa mice was significantly higher than before modelling and compared to the normal control group (Group I) which remained at the same level.

Regarding such parameter as grooming, we need to comment that rodents' grooming may be of two types. One type is a so called “complete cycle of grooming”

which is comprised of a sequence of certain movements (head, neck and body self-grooming, including genitalia) indicating that the animal is healthy, quiet and relaxed. Another type is a so called “incomplete cycle of grooming” which indicates that the animal is frustrated; in this case certain movements, such as neck and genitalia strokes, are skipped. In the animals of the IIa group the frequency of grooming was increased at the expense of the “incomplete” grooming cycles which are typical for the frustrating type of behavior. The animals of Group I demonstrated the “complete” grooming cycles throughout the experiment.

4.2.1.2 Forced swimming test results of depression modelling in the control and experimental mice

Forced swimming test results are shown in Figure 4.10.

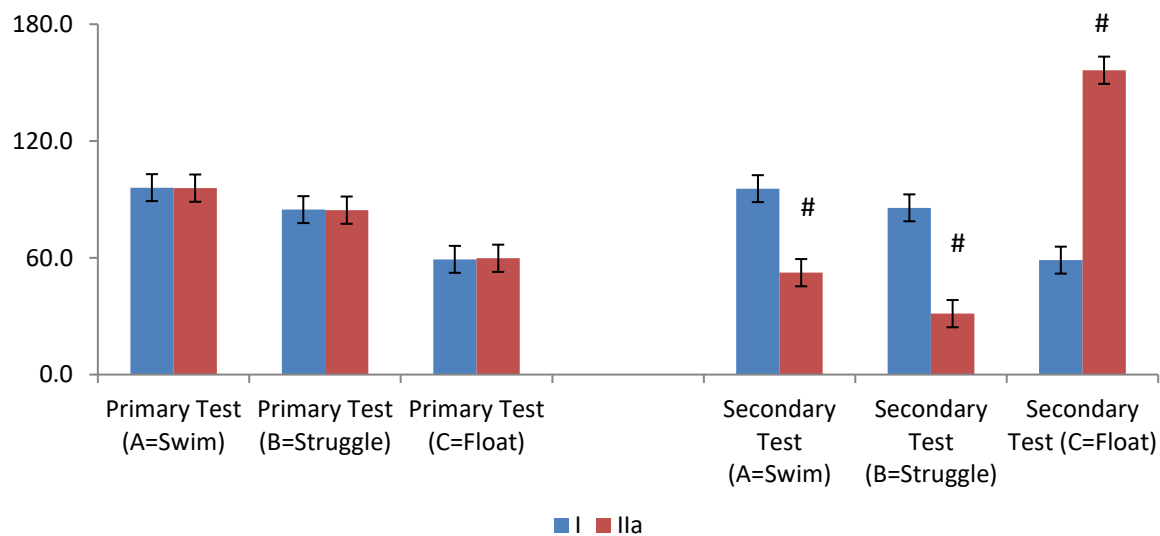


Figure 4.10: Duration of swimming activities of the experimental (for depression modelling) and normal control animals in the forced swimming test (sec, M±S).
- $p < 0.01$ compared to Group I

As follows from the Figure 4.10, before depression modelling (primary test) most of the test time the animals of both groups were swimming or, slightly less commonly,

struggling. Floating time was shortest among other activity types' of duration. In the secondary test normal control animals (Group I) sustained the same pattern of swimming behaviour, while animals of Group IIa with reserpine-induced depression significantly increased time of floating, while struggling period became the shortest. Thus, the results of the primary behavioural tests (open field, forced swimming) did not show any difference between the two groups (normal control and experimental for depression modelling), while secondary tests (after depression modelling) showed that in the animals of Group IIa (experimental for depression modelling) there were statistically significant changes in all behavioural parameters compared to the normal control group (Group I): reduced walking distance and rearing ($p>0.01$); increased incomplete grooming, urination and defecation rates in the open field ($p<0.05$); reduced active phase (swimming, struggling) and extended passive phase (floating) in the forced swimming test ($p<0.01$) indicating development of depression in Group IIa mice after repeated i.p. injections of reserpine.

4.2.2 Corticosterone test

The results of the corticosterone test are shown in the Figure 4.11.

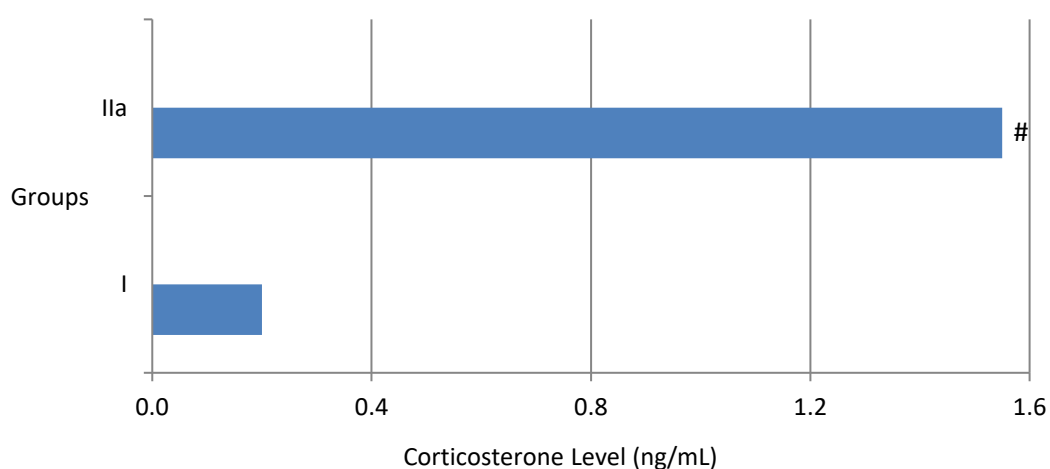
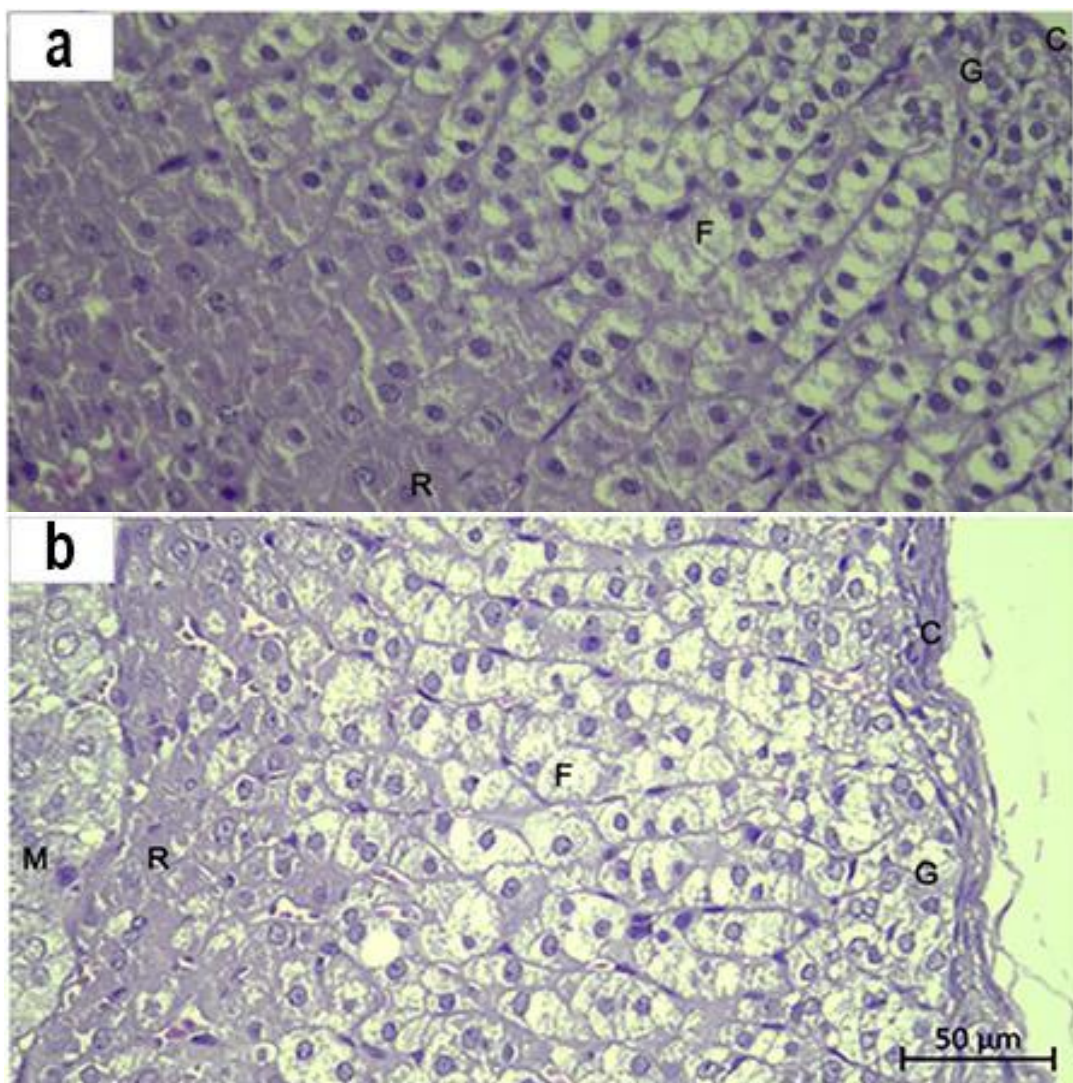


Figure 4.11: The results of the corticosterone test in the normal control and experimental (for modelling depression) animals, $p<0.01$

Statistically, the average control group corticosterone level was 0.2 ng/mL and the experimental group - 1.55 ng/mL with a $p < 0.01$, showing significant differences between two groups. This indicates the activation of the HHAA in animals with modelled depression without any stress exposure.

4.2.3 Histological investigation of the endocrine glands and immune organs

The animals of Group I and IIa were sacrificed one day after the last i.p. injection of normal saline or reserpine respectively. Figure 4.12 demonstrates the histological images of the adrenal glands and the thymuses of Group I and Group IIa mice.



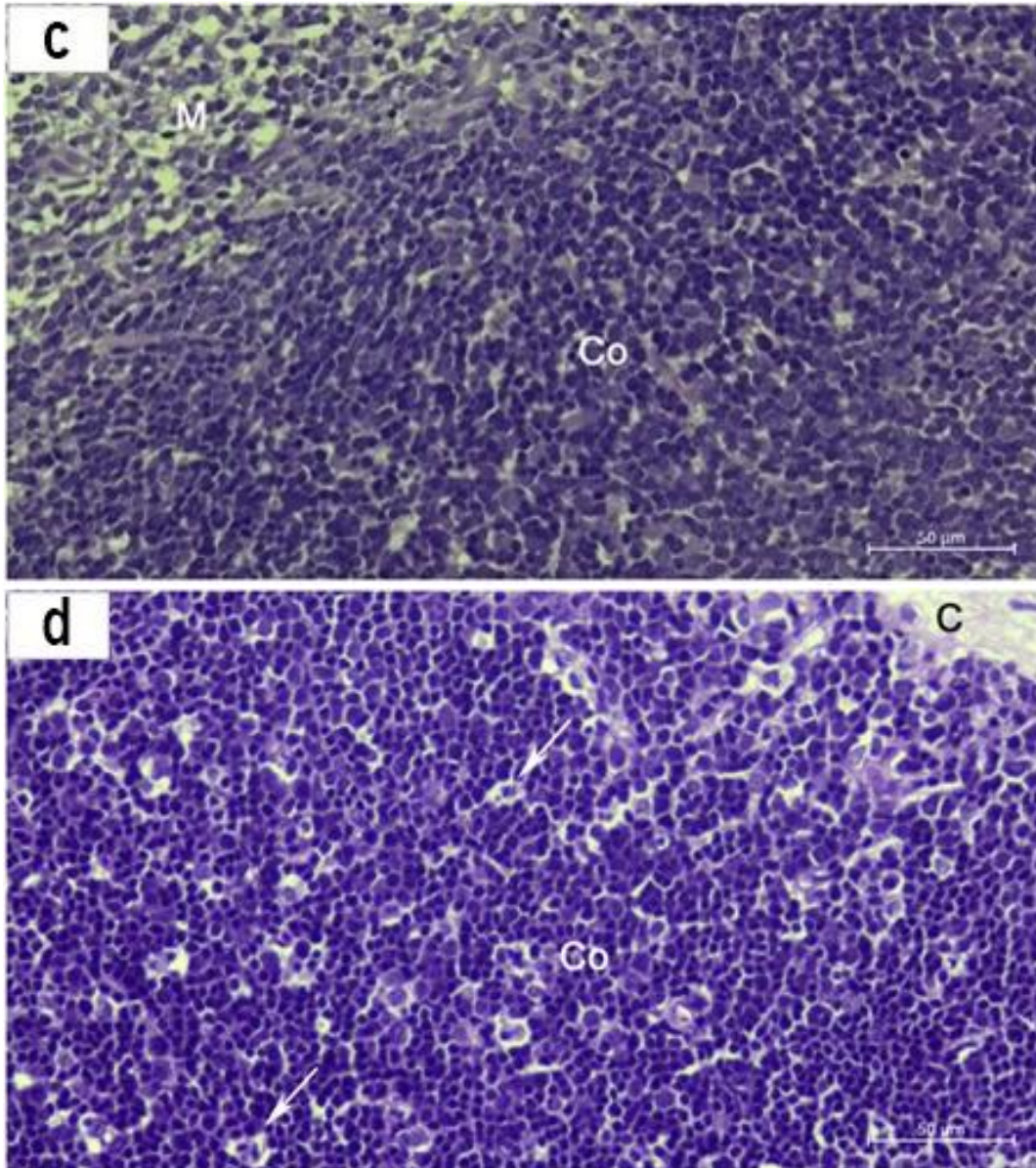


Figure 4.12: Adrenal gland (a,b) and thymus (c,d) of the normal control (a,c) and experimental (for depression modelling) (b,d) animals. Micrographs. Hematoxylin-eosin staining. Objective x40. C – capsule, Co – cortex, M – medulla, G – zona glomerulosa, F – zona fasciculata, R – zona reticularis, arrow – tangible body macrophage

On the histological slides of the adrenal gland of the normal control animals, three zones were clearly defined in the cortex: zonae glomerulosa, fasciculata and reticularis

(Figure 4.12a). The zona glomerulosa was the thinnest out of the three; its relatively small cells of a cylindrical or pyramidal shape formed ovoid clusters. Round or oval nuclei usually contained nucleoli. The zona fasciculata was the widest in the adrenal cortex; its cells contained light foamy cytoplasm and formed the cords which were one or two cell rows thick. The nuclei of spongiocytes were large, light, and contained one or two nucleoli. Though dominated by the cells with light cytoplasm, zona fasciculata displayed a small number of cells with dark cytoplasm. Cells of the zona reticularis were smaller in size and had a light eosinophilic cytoplasm, which sharply contrasted with weakly basophilic cytoplasm of the cells in the adrenal medulla.

In animals of the experimental (for depression modelling) group, the microscopic picture of zonae glomerulosa and reticularis did not differ significantly from the control group, while the zona fasciculata was hyperplastic, its cells were larger in size, the cytoplasm was foamier, and adenocytes with dark cytoplasm were scarce (Figure 4.12b).

In the histological slides of the thymus in the normal control group, the cortical layer in the lobules distinctly dominated over the medulla with prominent cortico-medullary demarcation (Figure 4.12c). The cortex was densely packed with thymocytes, between them the epithelial cells were identified near the capsule (Type I), deep in the cortex (Type II) and at the border with the medulla (Type III). The tingible body macrophages were scarce throughout the cortex. A few Hassall's corpuscles were detected in the medulla (Type VI of epithelial cells); in general, its cellularity was lower than in the cortex; its epithelial cells were identified at the border with the cortex (Type IV) and deeper in the medulla (Type V). The micromorphology of the thymus of experimental (for depression modelling) animals revealed significant changes compared to the normal

control mice (Figure 4.12d): the thickness of the cortex was reduced, cellularity of the cortex was decreased and it obtained a “starry-sky” appearance due to the increased number of macrophages with ingested apoptotic bodies. The border between the cortex and the medulla in the lobules looked less prominent, and the number of Hassall’s corpuscles increased.

Thus, the histological picture of the adrenal glands of the Group IIa mice showed hyperplasia of the adrenal cortex at the expense of the zona fasciculata indicating activation of the HHAA and aligning with the results of the corticosterone test described in part 4.2.2, while the micromorphology of the thymus of the Group IIa mice corresponds to the histological picture which is typical for immunosuppression developing on the background of a stress-related surge of corticosterone. In this connection we would like to emphasize that in our experiments, depression modelling did not include any stress exposure, which means that stress-free depression alone is capable of activating the HHAA, as evidenced by ELISA and histological data in Group IIa animals. Though qualitative assessment of the histological slides clearly showed the presence of the adrenal hyperplasia and thymic involution in all animals with modelled depression, to confirm the qualitative observation results we undertook the image analysis of the histological slides for the quantitative data on the micromorphological changes in the adrenal glands and the thymus of the animals involved in the project.

4.2.4 Image analysis of the histological changes of the endocrine glands and immune organs

The results of the image analysis of histological slides of the adrenal gland and the thymus are shown in Figures 4.13 and 4.14.

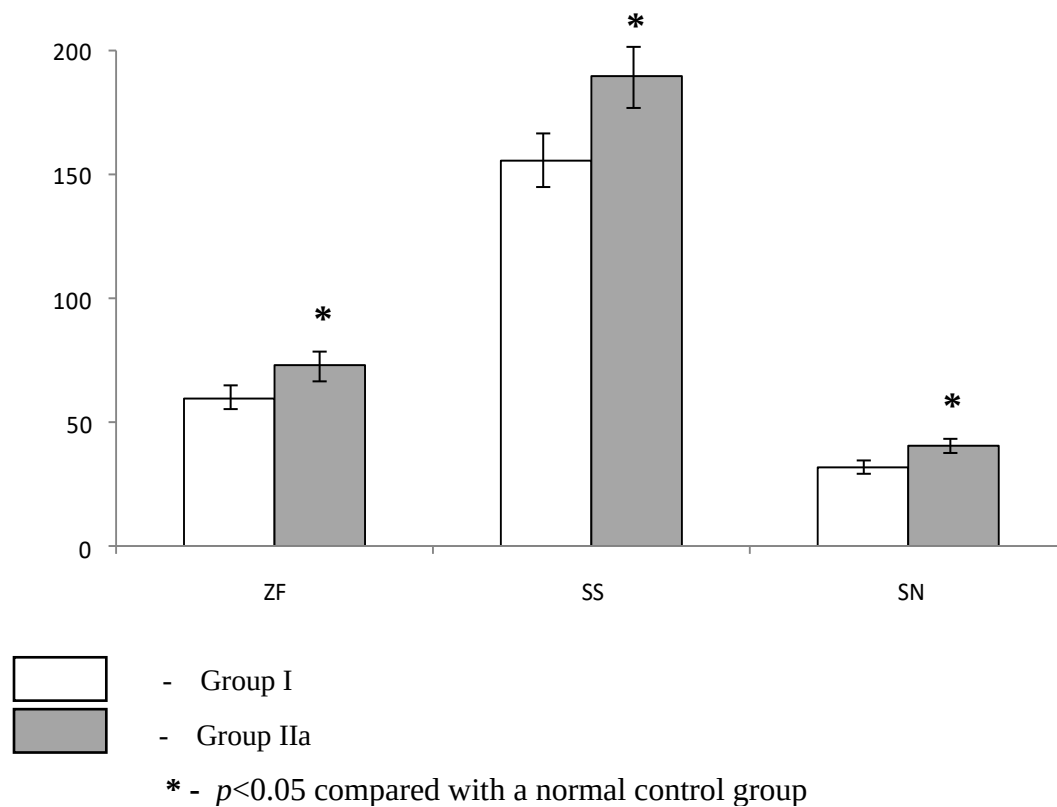


Figure 4.13: Volume density of the zona fasciculata (ZF, %), area of spongiocytes (SS, mcm²), and their nuclei (SN, mcm²) in the adrenal cortex of the experimental and control animals, M+/-m

As follows from Figure 4.13, the animals of the experimental group (for depression modelling) had a significantly larger volume density of the zona fasciculata in the adrenal cortex ($p < 0.05$), as well as the area of spongiocytes and their nuclei ($p < 0.05$), compared with the normal control group.

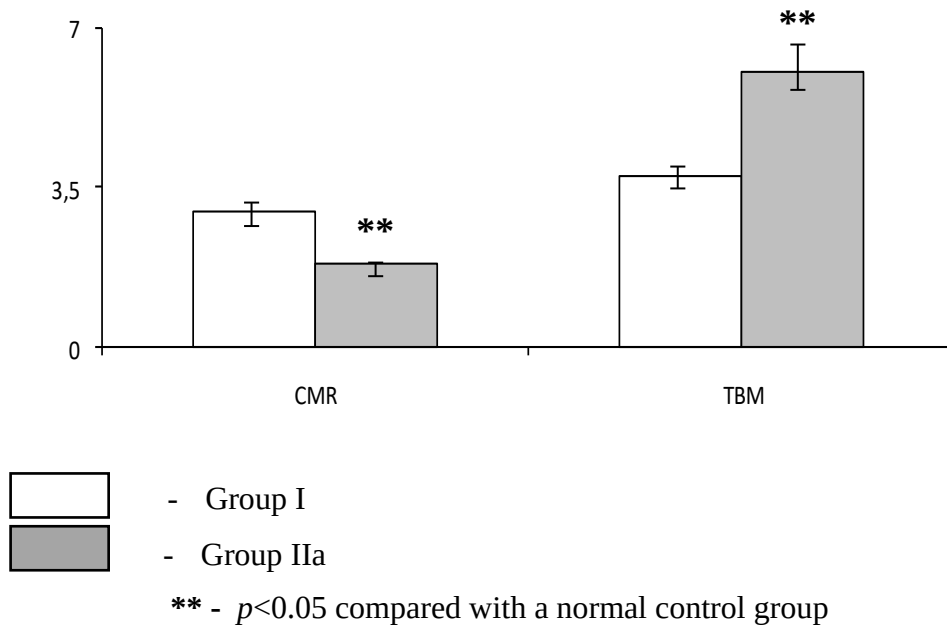


Figure 4.14: Cortico-medullary ratio (CMR) and volume density (%) of tingible body macrophages (TBM) in the thymus of the experimental and control animals, M+/-m

As shown in Figure 4.14, the thymus of experimental animals (for depression modelling) had a significantly lower cortico-medullary ratio and a larger volume density of tingible body macrophages compared with the normal control group ($p < 0.05$). Thus, image analysis provided quantitative confirmation of the qualitative observations in the adrenal glands and thymuses of the Group IIa mice during histological investigation and confirmed the fact of overactivation of their HHAA and associated immunosuppression which are commonly seen after severe chronic stress. However, as we mentioned earlier, our model of depression was free from any stress exposure. Besides, in the normal control mice neither behavioral tests, nor the serum corticosterone test values/image analysis data of adrenal glands and thymus demonstrated any changes pertinent to stress exposure. These results enable us to conclude that reserpine-induced depression in mice, modelled without any stress exposure, caused overactivation of the HHAA and the subsequent immunosuppression in the body of experimental (for depression modelling) mice, as

evidenced by the corticosterone test, histological examination and image analysis of their adrenal glands and thymuses.

These results were important for the second part of our experimental work aimed at the comparison of the efficacy of treatment of reserpine-induced depression using nanoparticulate BDNF with surfactant proposed by us as a potential antidepressant vs. traditional antidepressant fluoxetine.

4.3 Outcome of depression treatment (Part II)

The remaining three groups of animals were used in this part of our research. Group IIb – negative control group, which received normal saline as a “treatment” of depression, Group III – positive control group treated with fluoxetine and Group IV – experimental (for depression treatment) group which received i.v. injections of PLGA-BDNF with a surfactant. The animals of these groups underwent the same set of behavioral trials, corticosterone test and histological examination of the adrenal glands and thymus complemented by image analysis. On the contrary to the animals of Groups I and IIa, they underwent three sets of tests: primary tests before depression modelling, secondary tests after depression modelling and tertiary tests after depression treatment.

4.3.1 Open field tests

The results of primary, secondary and tertiary behavioral tests are presented in Figures 4.15 – 4.19.

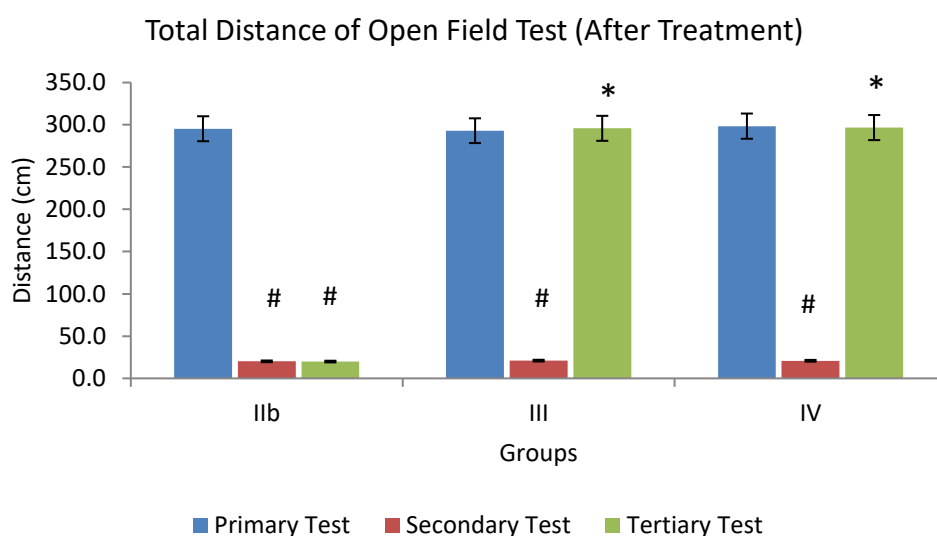


Figure 4.15: Distance walked by the negative and positive control, and experimental (for depression treatment) animals (cm, $M \pm S$), # - $p < 0.01$ compared to the primary test, * - $p < 0.01$ compared to the Group IIb tertiary test

As follows from Figure 4.15, treatment of depression by fluoxetine (Group III) and PLGA-BDNF with poloxamer 188 (Group IV) almost completely reverted the effect of depression on this parameter, as the distance walked was significantly increased in the tertiary test in Group III and Group IV compared to Group IIb ($p < 0.01$). Moreover, it was also significantly increased in Groups III and IV in the tertiary test compared to the respective secondary test results ($p < 0.01$). There was no difference in this parameter between Group III and Group IV in the tertiary test ($p > 0.05$), which indicates that fluoxetine and nanoparticulate BDNF with surfactant were equally effective in reverting the inhibition of the walking activity by the depression.

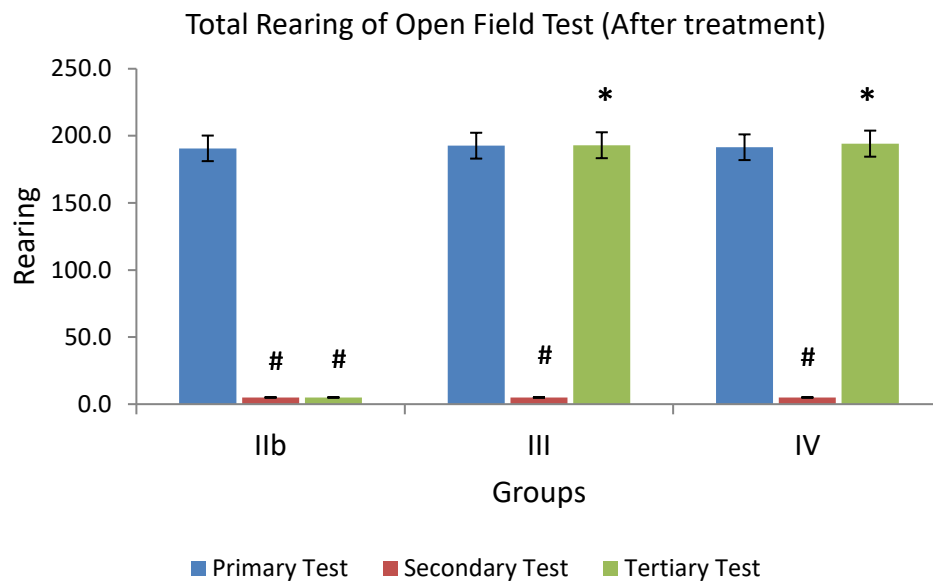


Figure 4.16: Rearing frequency in the negative and positive control, and the experimental (for depression modelling) animals ($M \pm S$); # - $p < 0.01$ compared to the primary test, * - $p < 0.01$ compared to the Group IIb in the tertiary test

As follows from Figure 4.16, the animals of all three groups significantly reduced the rearing frequency after depression modelling. After treatment, this parameter remained at the same level in Group IIb, while in Groups III and IV it increased almost to the pre-depression level and became significantly higher compared to the Group IIb without significant differences between Groups III and IV. This result indicated that similarly to the walking distance, both fluoxetine and nanoparticulate BDNF were equally effective in reverting the reduced rearing activity of the mice after modelling of depression.

Total defecation rate of the mice involved in the second part of our experiments is shown in Figure 4.17.

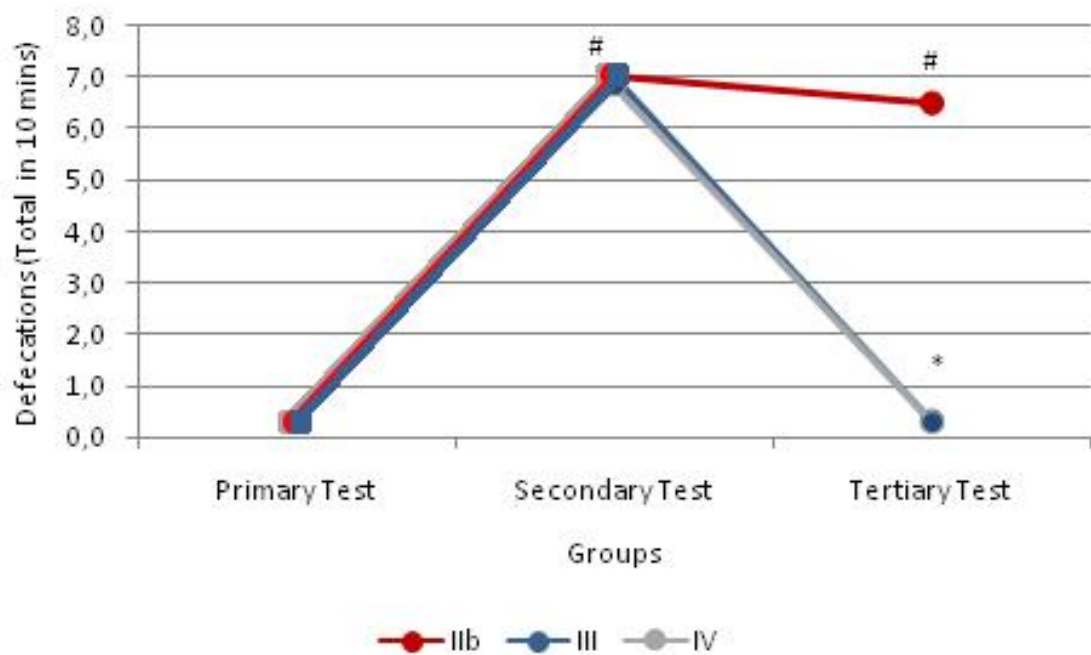


Figure 4.17: Open field defecation rate by the negative and positive control and experimental (for depression treatment) mice ($M \pm S$); # - $p < 0.05$, compared to the primary test, * - $p < 0.05$ compared to the IIb group in the tertiary test

As follows from Figure 4.17, treatment of depression with fluoxetine (Group III) and PLGA-BDNF with poloxamer 188 (Group IV) almost completely reverted the effect of depression on this parameter, as it was significantly decreased in Group III and Group IV compared to Group IIb ($p < 0.05$) in the tertiary test. Moreover, it was also highly significantly decreased in Groups III and IV in the tertiary test compared to their respective secondary test results. There was no difference in this parameter between Group III and Group IV tertiary test ($p > 0.05$).

Total urination rate is shown in Figure 4.18.

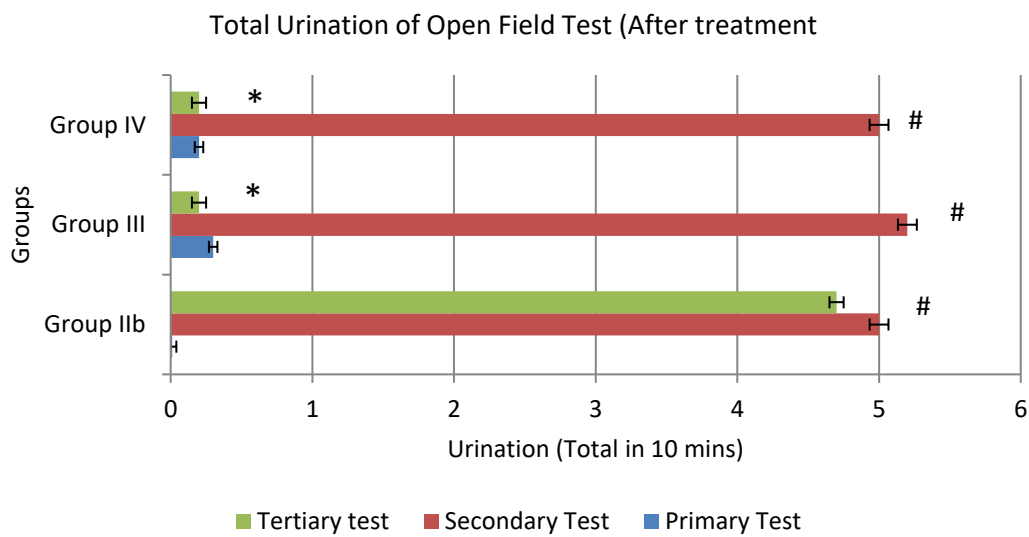
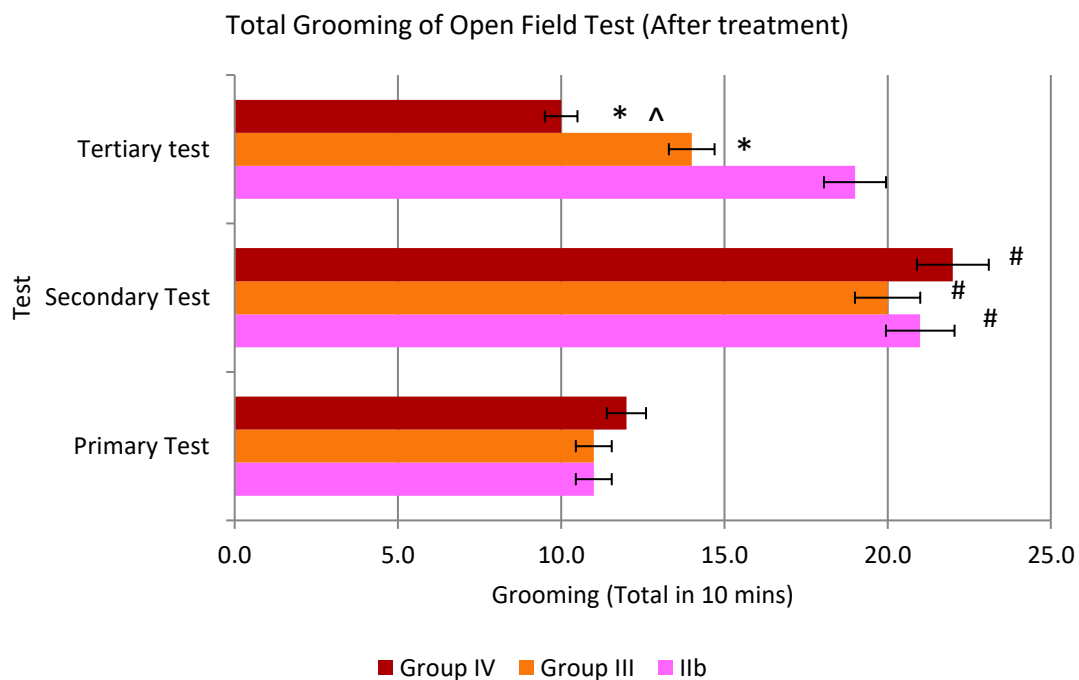


Figure 4.18: Open field urination rate by the negative and positive control and experimental (for depression treatment) mice ($M \pm S$), # - $p < 0.05$ compared to the primary test, * - $p < 0.05$ compared to the secondary test

As follows from Figure 4.18, treatment of depression by fluoxetine (Group III) and PLGA-BDNF with poloxamer 188 (Group IV) almost completely reverted the effect of depression, as this parameter was significantly decreased in Group III and Group IV compared to Group IIb in the tertiary test ($p < 0.05$). Moreover, it was also significantly decreased in Groups III and IV in the tertiary test compared to their respective secondary test results. There was no difference in this parameter between Group III and Group IV tertiary test results ($p > 0.05$).

Total grooming rate is shown in Figure 4.19.



- $p < 0.01$ compared to the respective primary test

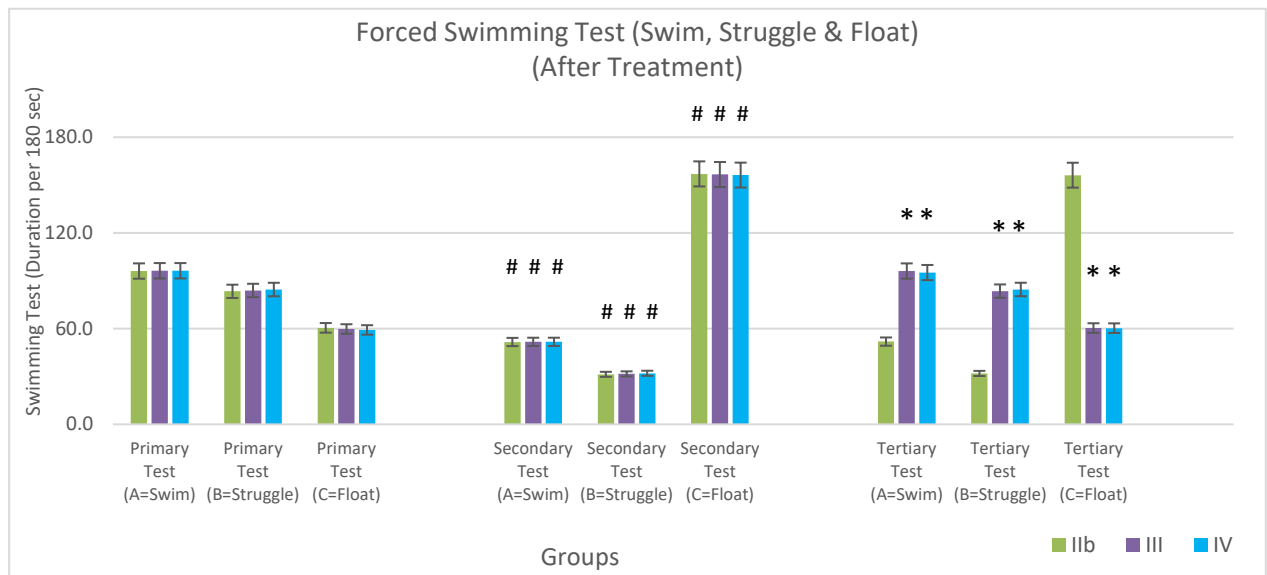
*- $p < 0.01$ compared to the respective secondary test

^- $p < 0.01$ compared to the Group III in the tertiary test

Figure 4.19: Open field grooming rate in the control and experimental mice ($M \pm S$)

As follows from the Figure 4.19, treatment of depression by fluoxetine (Group III) and PLGA-BDNF with poloxamer 188 (Group IV) almost completely reverted the effect of depression on this parameter, as self-grooming was significantly decreased in the tertiary test in Group III and Group IV compared to Group IIb ($p < 0.01$). On the contrary to the previously described behavioral tests, this parameter was significantly lower in Group IV than in Group III in the tertiary test ($p < 0.05$), indicating higher efficacy of BDNF treatment of depression compared to the traditional antidepressant fluoxetine as evidence by this test. Once again, we would like to emphasize that elevation of this parameter in the secondary test in all the groups of mice and its reduction in the tertiary test in Groups III and IV was at the expense of the “incomplete” cycles of self-grooming which are typical for the frustrational mood.

4.3.2 Forced swimming test results



- $p < 0.01$ compared to the respective primary test

* - $p < 0.01$ compared to the respective secondary test

Figure 4.20: Forced swimming test results in experimental (for depression treatment), positive control and negative control groups, (sec, $M \pm S$)

As follows from the Figure 4.20, before depression modelling (primary test) most of the test time the animals of all groups were swimming or, slightly less commonly, struggling. Floating time was the shortest among other activity types' of duration. After depression modelling in all three groups floating became the dominating type of activity. After treatment Group IIb kept all parameters on the pre-treatment level, while Groups III and IV returned to the pre-depression level for all three types of activity. As a result, in Groups III and IV floating time became significantly lower than in Group IIb, while swimming and struggling – significantly higher than in the Group IIb mice. Between Groups III and IV there were no significant differences in the tertiary test for all three types of activity ($p > 0.05$).

Thus, the behavioural tests (open field, forced swimming), showed that treatment with fluoxetine and nanoparticulate BDNF with a surfactant significantly improved their results: depression-related reduction of walking distance and rearing was increased to the pre-depression level; depression-related increase of the frequency of grooming, urination and defecation returned to the pre-depression low level; depression-related reduction of the active phase (swimming, struggling) and extension of the passive phase (floating) in the forced swimming test was also reverted to the pre-depression level. For all these parameters (except one) BDNF and fluoxetine treatment showed comparable dynamics, only grooming rate demonstrated higher efficacy of BDNF compared to fluoxetine.

4.3.3 Corticosterone test in animals of experimental (for depression treatment), and negative and positive control groups after treatment of depression

It was important to assess how the activity of HHAA manifested by the level of corticosterone in blood of experimental and control animals after BDNF treatment aligned with the respective behavioural changes and histological findings in the endocrine glands and immune organs of mice.

The results of the corticosterone test are shown in Figure 4.21.

Comparison of corticosterone level of the control and experimental groups.

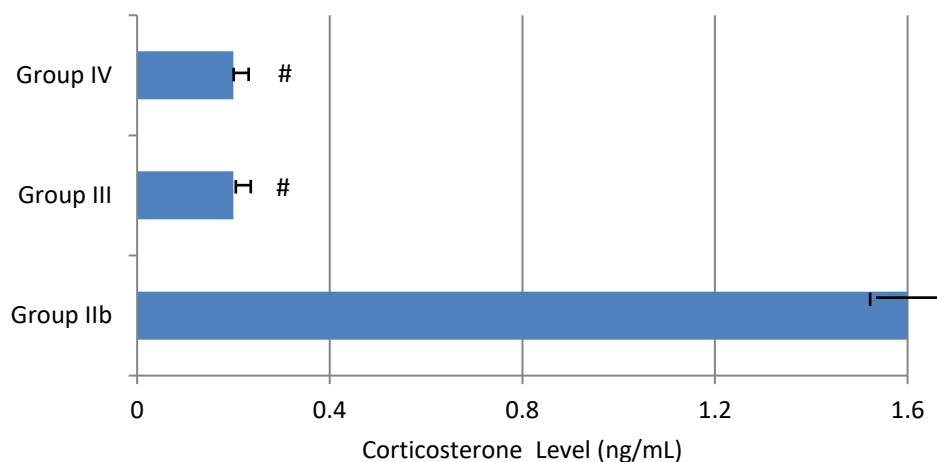
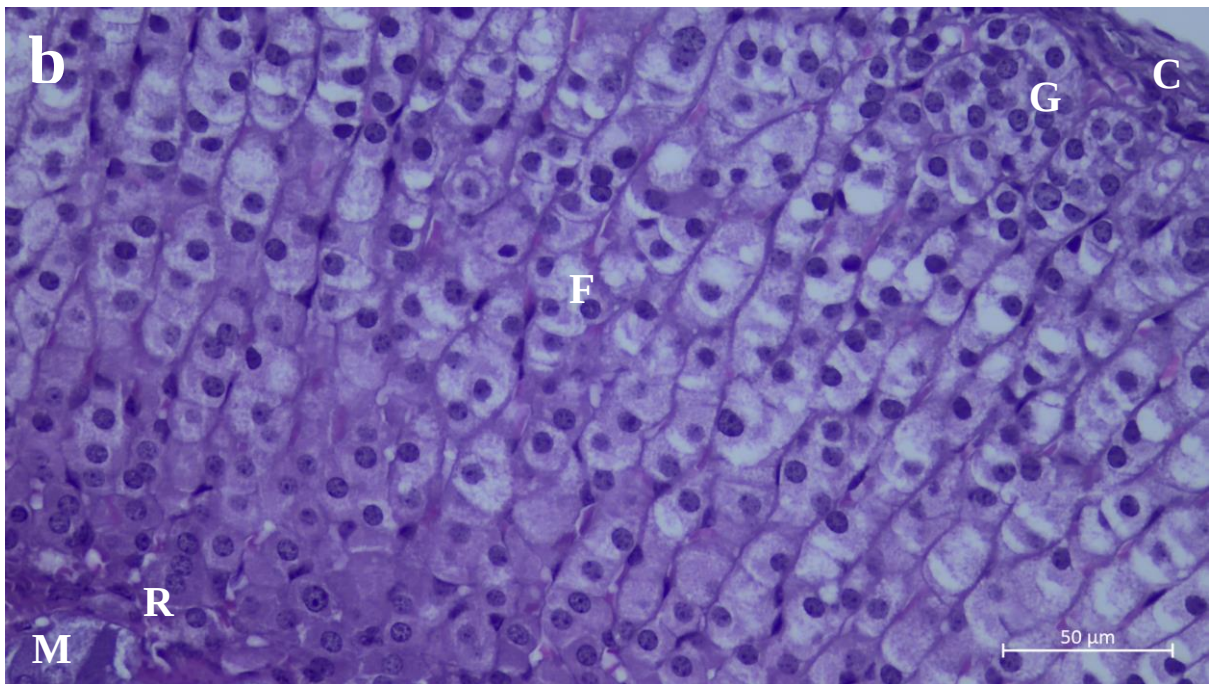
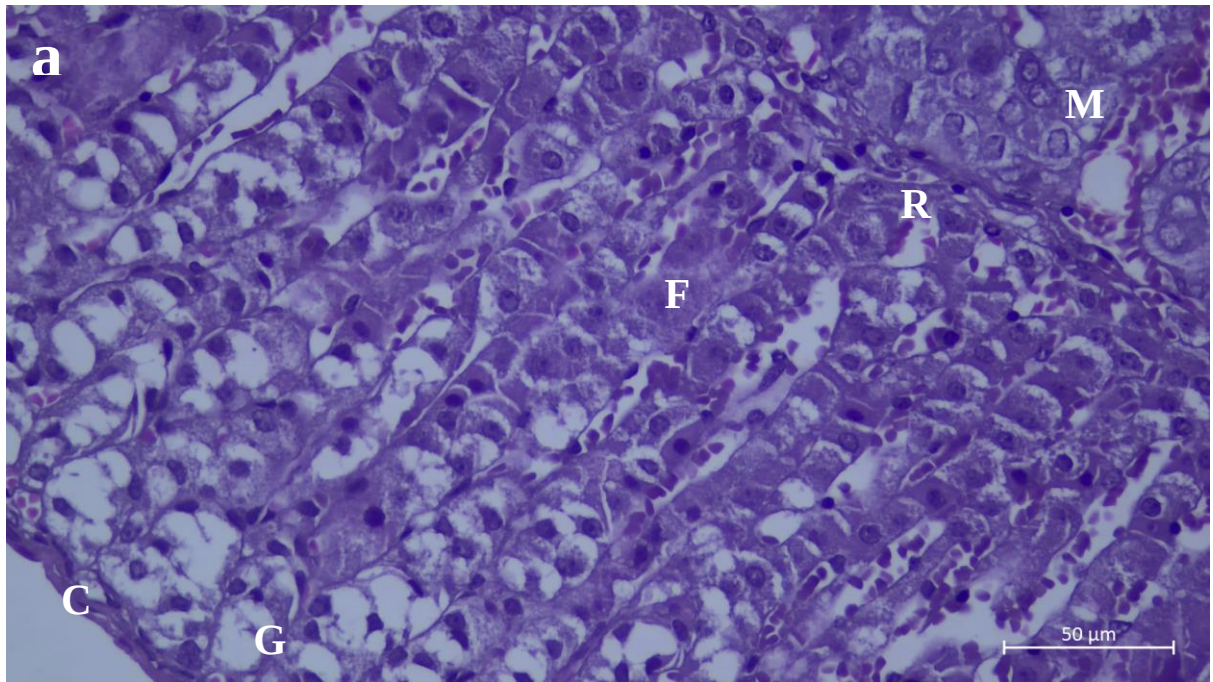


Figure 4.21: Concentration of corticosterone in the serum of experimental (for depression treatment), negative and positive and control animals. # - $p < 0.01$ compared with Group IIb

As follows from Figure 4.21, concentration of corticosterone, which is the main glucocorticoid in rodents, was significantly higher ($p < 0.01$) in the negative control (Group IIb) than in the positive control and experimental (for depression treatment) groups (Group III and IV respectively). There was no significant differences between the positive control and experimental groups, which indicated that BDNF treatment has similar effect with fluoxetine on the functional activity of the adrenal glands.

4.3.4 Histological changes in the endocrine glands and immune organs after treatment of depression

Below is Figure 4.22 that demonstrates post-treatment changes in the micromorphology of the adrenal glands and the thymus.



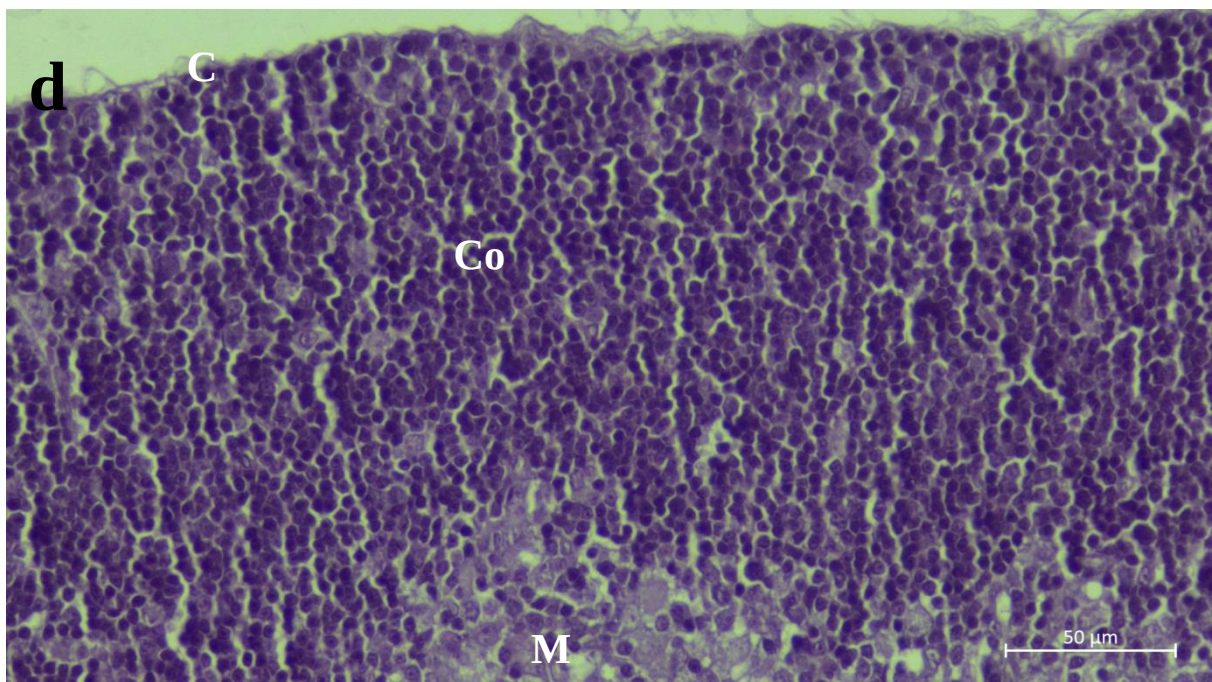
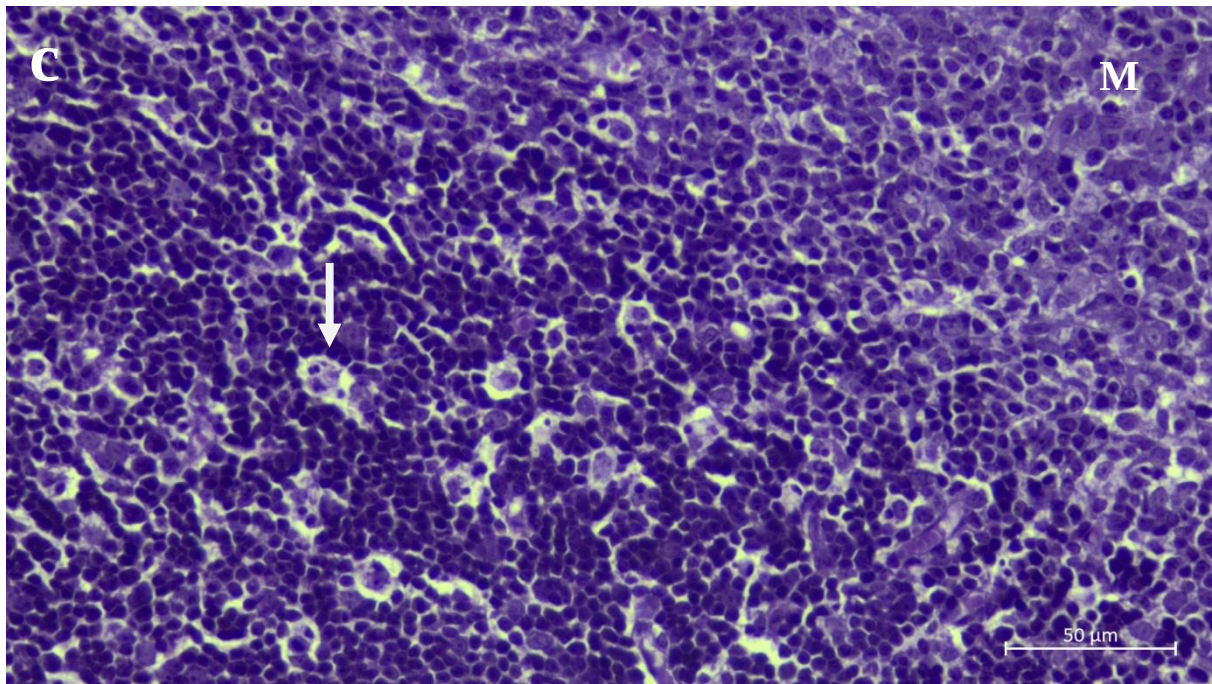


Figure 4.22: Adrenal gland (a,b) and thymus (c,d) of Group IIb (a,c) and Group IV (b,d) animals. Micrographs. Hematoxylin-eosin staining. Objective x40. Co- cortex, M – medulla, arrow – macrophage with apoptotic bodies, C – capsule, G,F,R – zonae glomerulosa, fasciculata, reticularis

Figure 4.22 allows us to qualitatively compare the changes in the adrenal cortex

and thymus of the experimental animals with different types of medications for treatment of experimental depression. Animals of Group IIb (Figure 4.22a) showed pronounced hyperplasia of the adrenal cortex, an obvious dominance of the zona fasciculata, hypertrophy of its cells and their nuclei, a foamy cytoplasm, and capillary plethora; while the animals of Group IV (Figure 4.22b) demonstrated that hyperplasia of the zona fasciculata was less pronounced, the foaminess of the cytoplasm was moderate, and there were no apparent differences in the structure of the zona glomerulosa and zona reticularis compared to Group IIb. Animals of Group III had a comparable with Group IV histological picture of the adrenal glands. Figure 4.22 also shows a comparative picture of the thymus of mice of Group IIb (Figure 4.22c) and Group IV (Figure 4.22d). Figure 4.22c displays the hypocellularity of the cortex, a washed-out border contours between the cortex and medulla, and a large number of macrophages with apoptotic bodies. At the same time, in Figure 4.22d, thymocytes of varying degrees of maturity are tightly adjacent to each other, separated by scarce reticuloepithelial cells and rare macrophages with apoptotic bodies. In animals of the fluoxetine group, changes in the thymus microstructure were similar to those in the BDNF group.

To get a more precise information on the microstructural changes in the adrenal and thymus cortices in the mice of Groups III and IV compared to Group IIa, we undertook digital morphometry of the histological slides of the respective organs and glands.

4.3.5 Image analysis of the changes in the endocrine glands and immune organs

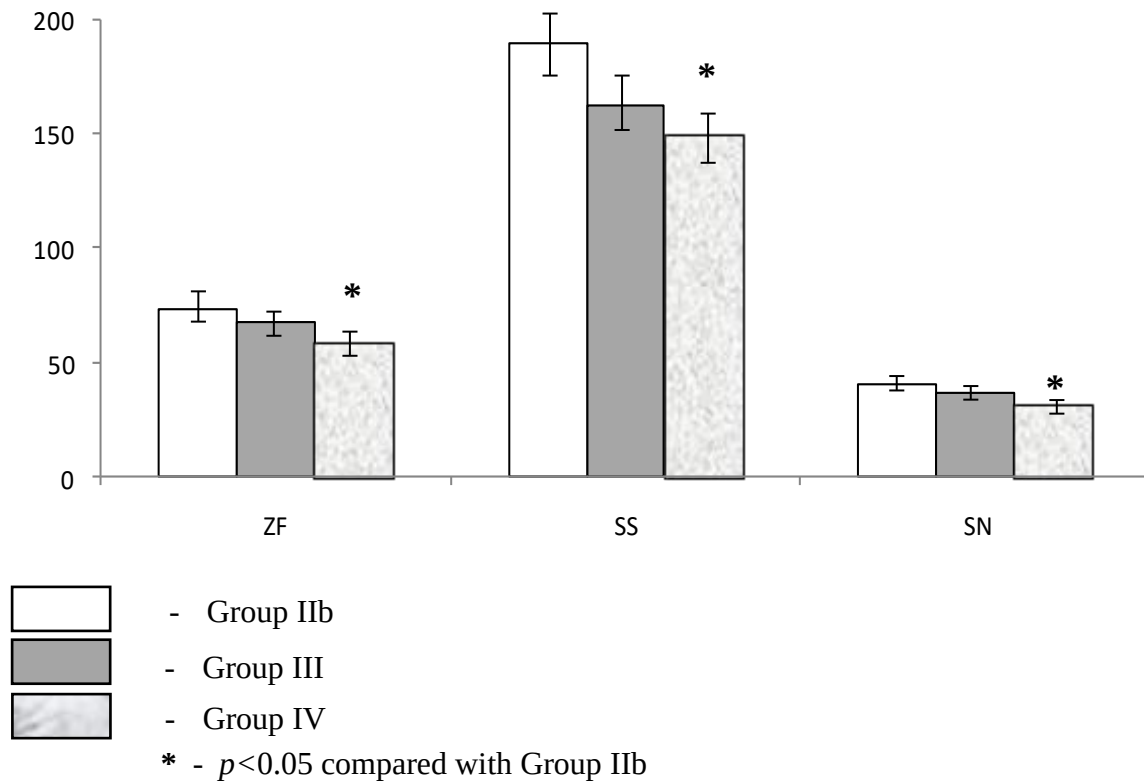


Figure 4.23: Volume density of the zona fasciculata (ZF, %), area of spongiocytes (SS, mcm²), and their nuclei (SN, mcm²) in the adrenal cortex, M+/-m

As follows from Figure 4.23, the volume density of the zona fasciculata, the area of its cells, and their nuclei were significantly lower in the Group IV compared to Group IIb ($p < 0.05$). However, in the Group III, these parameters were not significantly lower in comparison with either Group IIb; they also did not show the significance of difference when compared with Group IV.

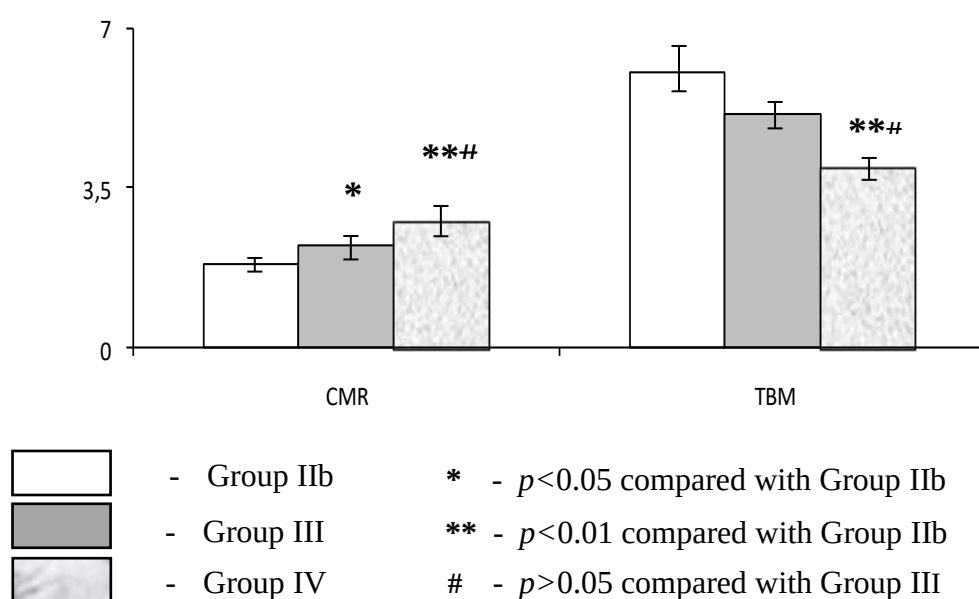


Figure 4.24: Cortico-medullary ratio (CMR) and volume density (%) of tingible body macrophages (TBM) in the thymus, M+/-m

Figure 4.24 shows comparative results of digital morphometry of the thymus. It demonstrates that the cortico-medullary ratio in the thymus of Group IV mice is significantly higher than in Group III and meaningfully higher than in the Group IIb ($p < 0.05$ and $p < 0.01$, respectively). At the same time, the Group III also significantly differed in this parameter from the Group IIb ($p < 0.05$); besides Group IV had significantly higher cortico-medullary ratio than Group III ($p < 0.05$).

The volume density of macrophages with apoptotic bodies was the highest in Group IIb. In animals of the Group III, it was insignificantly lower than in mice of Group IIb and insignificantly higher than in mice of Group IV ($p > 0.05$); while in the Group IV it was significantly lower than in Group III ($p < 0.05$) and Group IIb ($p < 0.01$). These results indicate that based on the data of the image analysis of the microstructural changes in the adrenal and thymic cortices, BDNF treatment was significantly more effective than

fluoxetine in reverting the depression-related histological changes in the endocrine glands and the immune organs.

Thus, our results demonstrate the crosstalk between the activity of HHAA and the behavioural reactions of the animals with reserpine-induced depression without exposure to any stress. The complicated correlations between the level of the activity of the HHAA and the behavioral reactions remain poorly understood (Armario et al., 2023). Our study showed that the reserpine model of depression (El-Marasy et al., 2021) caused significant behavioral changes in the experimental animals, indicating the development of depression, such as significant inhibition of horizontal and vertical activities, increased frequency of urination, defecation and grooming, and the extended passive phase in a forced swimming test. At the same time, the microscopic picture of adrenal glands showed development of the adrenocortical hyperplasia (increased volume density of the zona fasciculata, hyperplasia of the spongiocytes), while the thymus exhibited immunosuppressive changes (decreased cortico-medullary ratio, decreased cellularity of the thymic cortex, increased volume density of tingible body macrophages with ingested apoptotic bodies). Similar changes have been described in the immune and endocrine systems of experimental animals under chronic stress (Cheiran Pereira et al., 2020; Smaniotto et al., 2023), which leads to activation of the HHAA and is accompanied by the development of a depressive type of behavior. However, our target was to find out how depression not associated with stress history affects the HHAA, which is a novelty of our study.

Though numerous papers demonstrate that depression-associated activation of HHAA, none of these works provide evidence that depression in the presented context is not associated with a history of stress. At the same time, in our study, the applied reserpine

model of depression is obviously free from stress exposure, as before the start of the experiment, the animals underwent habituation. Regarding the effect of the injections themselves, the control animals also received injections of normal saline instead of reserpine, but did not have any signs of depression. A number of studies have demonstrated the effect of depression modelled by the administration of lipopolysaccharide (LPS) on the HHAA, in particular a recent study of the LPS model of depression and its correction with antalarmin, a CRHR1 antagonist (Sun et al., 2023). In this model, as in our study, the experimental animals showed increased corticosterone levels and developed behavioral reactions of depressive type which subsided after treatment with antalarmin. However, the administration of LPS itself is considered a stressor (Zaichenko et al., 2023) and causes immune disorders that are superimposed on the immunosuppressive changes caused by HHAA activation, and therefore this model does not allow proper evaluation of the neuroimmunoendocrine consequences of depression not associated with stressors or immunomodulators. Recent studies (Qin et al., 2021) have shown one of the possible links in the development of depression – an increase in the expression of PSD-93 in co-localized CRF-neurons, which is accompanied by a surge of cortisol and has been found in patients with depressive disorders. In an LPS mouse model of depression, PSD-93 knockdown reduced depressive symptoms. However, this study does not answer the main question: what factors can cause increased expression of PSD-93 in patients with depressive disorders, and whether depression in these patients is stress-related.

Our results are consistent with the data of the researchers (Ge et al., 2014), who found activation of the HHAA in depression caused by hypothyroidism, which developed as a result of hemi-thyroid electrocauterization. In the past study, experimental animals showed behavioral changes (in forced swimming and tail suspension tests) characteristic of

depression (except for sucrose preference), and pronounced hypertrophy of the adrenal glands with the detection of a large number of lipid vacuoles in the reticular zone of their cortex. However, in their model of depression, contrary to our model, the stress exposure cannot be completely excluded.

In a healthy body, the immune and neuroendocrine systems function in a coordinated manner, and their dysregulation may contribute to the pathophysiology of depression, as the latter is accompanied by neuroinflammation and increased cortisol level (Cheiran et al., 2022). However, other researchers (Nandam et al., 2020) believe that serum cortisol values are not always an adequate marker of depression level, and even if the use of antidepressants decreases cortisol values, it does not necessarily correspond to the level of clinical effect of the applied drugs. That is why we undertook a parallel study of the microscopic structure of the adrenal cortex, the results of which also showed adrenal hyperplasia, accompanied by immunosuppressive changes in the thymus, which correlated with the results of behavioral tests. Our findings regarding immunosuppressive changes in animals with modelled depression are in agreement with other investigators who demonstrated the association between development of depression and immunological response (Biltz et al., 2022), but on the contrary to their findings, immunosuppression developed in our mice without any stress exposure.

According to our results, BDNF and fluoxetine similarly affected the concentration of corticosterone in blood, as shown by the ELISA test. At the same time in our study BDNF was more effective than fluoxetine in terms of reverting hyperplasia of the adrenal cortex in mice with reserpine-induced depression. This discrepancy between the other researchers' our findings may also be explained by the data of Nandam et al. (2020)

regarding reliability of the cortisol (in humans) or corticosterone (in rodents) test for the severity of depression and effectiveness of antidepressant treatment.

With regard to the microscopic structure of the adrenal cortex in mice, the traditional understanding of its zonal pattern has recently been revised. According to earlier studies (Fuchs et al., 2017; Dumontet et al., 2018), in the cortex of mice, like in humans and other primates, there are three zones: *zonae glomerulosa*, *fasciculata*, and *reticularis*. However, in more recent works, including the papers of the same researchers (Lee et al., 2021; Dumontet et al., 2021), it was shown that the *zona reticularis* is not present in rodents, which do not develop the “adrenarche” phenomenon, and therefore it is proposed to distinguish either two zones (*zonae glomerulosa* and *fasciculata*), or three of them (*zona glomerulosa*, *zona fasciculata externa* and *interna*). This conclusion was made based on molecular biological studies showing that CYP17A1 is not expressed by adrenal cortical cells in mice and rats. In our study, we adhered to the traditional understanding of the tri-zonal structure of the adrenal cortex in mice; its results showed that hyperplasia of the adrenal cortex in modelled depression occurs at the expense of the traditional *zona fasciculata* (or external *zona fasciculata* according to the newly proposed classification), while the volume density of the *zona reticularis* (or internal *zona fasciculata*), on the contrary, is relatively decreased, i.e. the changes in the “outer” and “inner” *zona fasciculata* are multidirectional, and therefore combining them into one zone does not seem appropriate.

After we clarified all the issues of the cross-talk between the behavioural, hormonal and microscopic changes and their mechanisms in the animals with reserpine-induced depression and their control counterparts, we undertook the second series of

experiments, in which the depressed animals were treated either with a traditional antidepressant fluoxetine, or a suggested by us formulation PLGA-BDNF with a poloxamer 188 as a surfactant, or normal saline (negative control group). This is the first experimental research on treatment of depression with nano-BDNF. Previous studies showed a successful outcome of experimental nano-BDNF treatment of the brain trauma, parkinsonism and ischemic stroke (Khalin et al., 2016; Kapitonova et al., 2018, Kamarudin et al., 2020), but it was never used before for the treatment of depression. Our study's key findings reveal that nanoparticulate BDNF has a positive impact on the modelled depression in mice. This is supported by significant improvement of behavioral reactions in the open field and forced swimming tests. Commonly, depression is associated with stress, since interpersonal conflicts, losses, financial problems, unemployment, and loneliness, as well as childhood psycho-emotional trauma, are often stated in the case histories of the patients with depression. Stress activates the HHAA and causes maladaptive changes in various neurobiological systems, which provoke the development of depression and trigger a cascade of disorders in the immune system (Hinkelman et al., 2024; Menke et al., 2024). In our study, similar changes were observed. However, the experimental animals were not stressed, since we assessed the effect of depression modelled without a stress trigger aimed at the HHAA and the respective immunomodulation mediated by it. Treatment of depression with BDNF made these changes reversible.

The present experiment showed that the efficacy of nanoparticulate BDNF with a surfactant is comparable with that of the traditional antidepressant fluoxetine, a selective serotonin reuptake inhibitor. BDNF simultaneously reduced the activation of the HHAA caused by depression, as evidenced by a significant decrease in corticosterone in the blood,

which was similar in BDNF and fluoxetine groups and reduced hyperplasia of the adrenal cortex, which was significant in the BDNF-treated animals while in the fluoxetine group, this reduction was not meaningful. At the same time, BDNF reduced corticosterone-associated immunosuppression, as evidenced by the digital morphometry of the thymus, significantly more effectively than fluoxetine. Thus, the effect of BDNF and fluoxetine in modelled depression was comparable in several parameters, such as improved behavioral reactions (except grooming frequency) and decreased corticosterone levels in the blood. In contrast, other parameters (hyperplasia of the adrenal cortex, thymic involution) showed higher BDNF efficacy compared with fluoxetine. The obtained data allow us to conclude that the effect of BDNF in the treatment of modelled depression is mediated by its action on the HHAA. Our results are consistent with the data on the ability of BDNF to antagonize the action of glucocorticoids under certain conditions (Tsimpolis et al., 2024).

Earlier studies (Kapitonova et al., 2018; Kamarudin et al., 2020; Han et al., 2021) demonstrated the effect of nanoparticulate BDNF in the treatment of experimental pathology of the central nervous system, such as traumatic brain injury, parkinsonism, and ischemic stroke. The present study shows another potential application of this drug in experimental depression, which has not been done so far. Demonstration of the antidepressant effect of BDNF prompts for search of low molecular weight agonists of its receptors as a potential medication for the treatment of depression.

4.4 Chapter summary

This chapter presents the results obtained using the methods described in Chapter 3. It includes the two parts. In part I we compared the results of examination of the experimental group (with modelled depression, reserpine-induced model) and normal

control group, which received normal saline instead of reserpine. All tests showed that the model caused depressive changes in behaviour, elevation of the corticosterone level in blood and microstructural changes in the adrenal glands and the thymus, indicating presence of adrenal hyperplasia and immunosuppressive changes in the thymus, which were statistically significant.

In Part II we treated modelled depression either with normal saline (negative control group) or fluoxetine (positive control group) or PLGA-BDNF (experimental group). The results showed that treatment of depression with fluoxetine and PLGA-BDNF gave comparable results according to the behavioural tests (except grooming in the open field, which gave better dynamics with PLGA-BDNF) and corticosterone test. Microscopically, both medications reverted the changes in the adrenal glands and the thymus, which were retained in the negative control group. Digital morphometry showed better dynamics in animals treated with BDNF compared to the fluoxetine.

In the discussion part we explain why, in our opinion, corticosterone level was almost the same in BDNF and fluoxetine groups, while histological changes in the adrenal glands were statistically better in BDNF group and correlate our findings with the data of other researchers, emphasizing that in our experiments we used the model of stress not associated with stress exposure, while other researches used stress-related models which do not allow to discriminate the consequences of stress and the depression itself.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

This study unequivocally demonstrates the effectiveness of BDNF in treating modelled depression in mice. In some aspects, BDNF's efficacy is comparable with the traditional antidepressant fluoxetine, as evidenced by improved results of the behavioral tests and decreased corticosterone levels in the blood. BDNF demonstrates comparable efficacy to fluoxetine in other aspects, such as reversing hyperplasia of the zona fasciculata of the adrenal cortex, hypertrophy of its cells, and immunosuppressive changes in the thymus. These findings strongly suggest that BDNF could be a promising treatment for depression in humans.

The obtained data enable us to understand the mechanisms of action of BDNF on the neuroendocrine axes in depression modelled without stress exposure and formulate new approaches to treatment of depression and alteration of the neuroendocrine axes modulated by it. The results obtained give us opportunity to recommend nanoparticulate BDNF to clinical pharmacologists for the future trials on treatment of depression using the formulations of neurotrophins penetrating through the blood-brain barrier.

5.1 Chapter summary

This chapter contains the conclusions regarding higher efficacy of the PLGA-BDNF in treatment of reserpine-induced depression in experimental animals and recommends continuing investigation of the PLGA-BDNF with poloxamer 188 as a potential antidepressant, which is comparable with the existing antidepressant or even exceeds it in certain parameters but does not have its side effects. Toxicological studies are recommended for implementation, which are a precondition of the subsequent clinical trial.

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