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NEW PHARMACOTHERAPEUTIC APPROACHES IN TARGETING NEURODEGENERATIVE PROCESSES

DOCTORAL THESIS

Promotion to the degree of Doctor of Basic Medical Science including Pharmacy (Ph.D. in Medicine)

Subfield of Pharmacology

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„Nothing in life is to be feared, it is
only to be understood. Now is the
time to understand more, so that
we may fear less.”

Marie Sklodowska Curie

The doctoral thesis study was carried out at the Department of Pharmacology, Faculty of Medicine, University of Latvia, from 2017 to 2020.

The thesis contains the introduction, 5 chapters, reference list, and the list of scientific publications.

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The thesis is available at the Library of the University of Latvia, 19 Raina blvd.

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ANNOTATION

The increase in the frequency of progressive neurodegenerative diseases evokes a significant economic and social burden to governments, patients, and their families. The most common neurodegenerative diseases are sporadic type of Alzheimer's disease (sAD) and Parkinson's disease (PD), which remain incurable up to this day. sAD and PD both have common etiopathological causes linked to late-onset progressive proteinopathies. The research in the past three decades has led to new insights on how to halt neurodegeneration: future treatments should target the disease in its earliest stages, prior to the occurrence of irreversible brain damage or mental decline. Thus, pathological processes in the early stages of sAD and PD – neuroinflammation, oxidative stress, impaired cerebral glucose, and insulin metabolism– could serve as targets for the early therapeutic interventions of these diseases.

We have chosen an innovative approach in targeting neurodegeneration – with anti-hyperglycemic drug metformin and extracellular vesicles (EVs). These substances can cross the brain-blood barrier and have proven to exhibit neuroprotective properties *in vitro*. Hence, we suggest that these compounds could possess neuroprotective and cognition-improving effects *in vivo*. We hypothesize is that amelioration of neuroinflammation, normalization of brain glucose metabolism and the expression of proteins involved in neuronal cell homeostasis, are essential for intracellular communication that determines cognitive and motor functions and, hence, cell survival.

We have studied metformin in a sAD-type rat model generated by injecting streptozocin (STZ) intracerebroventricularly and EVs intranasally in PD-type model obtained by injecting 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle of rats. We have discovered that metformin treatment improved spatial learning/memory and social behavior, normalized STZ-induced pathological changes in the expression of brain proteins involved in glucose transport and uptake, neuroinflammation, synaptic plasticity and acetylcholine cleavage. Metformin also demonstrated acetylcholine esterase activity inhibiting properties *in vitro*.

Administration of EVs normalized, in a time-dependent manner, 6-OHDA-induced disturbances in animal gait and spatial learning/memory performance, preserved expression of tyrosine hydroxylase (TH), a key enzyme involved in dopamine synthesis, as well as Nissl body expression in the nigrostriatal structures.

The data obtained in this thesis demonstrate, for the first time, neuroprotective and neuroregulatory properties of metformin and EVs, highlighting the usefulness of these substances in halting early pathological processes of sAD and PD, respectively.

Keywords: Parkinson's disease (PD); Alzheimer's disease (AD); STZ; 6-OHDA; extracellular vesicles; metformin; gait; spatial learning/memory.

ANOTĀCIJA

Progresējošo neirodeģeneratīvo slimību izplatības pieaugošā tendence izraisa milzīgu ekonomisku un sociālu slogu valdībām, slimniekiem un to ģimenēm. Visizplatītākās neirodeģeneratīvās un joprojām neizārstējamās slimības ir sporādiskās formas Alcheimera slimība (sAS) un Pārkinsona slimība (PS).Tām abām ir raksturīgi kopīgi etiopatoģenētiski cēloņi, kas ar laiku noved līdz proteinopātijām un vēlīnu slimības sākumu, kas raksturojas ar kognitīvo spēju samazināšānos līdz pat demencei. Taču pēdējo trīsdesmit gadu pētījumi ir radījuši jaunu izpratni un nākotnes stratēģiju, kā apturēt neirodeģeneratīvo procesu attīstību, uzsākot terapiju slimības agrīnajā fāzē pirms izveidojušies neatgriezeniski smadzeņu bojājumi un mentālie traucējumi. Tādējādi terapija jāmērķē uz tiem patoloģiskajie procesiem, kas ir kopīgi un raksturīgi gan sAS, gan PS to agrīnajās stadijās: neiroiekaisumu, oksidatīvo stresu, traucētu smadzeņu glikozes un insulīna vielmaiņu.

Mūsu hipotēze balstās uz šo neironu dzīvotspējai kritisko procesu aizsardzību, lai saglabātu šūnu interkomunikācijas spējas, kas savukārt nodrošina kognitīvās un motorās funkcijas. Darbā esam izvēlējušies inovatīvu pieeju, izmantojot ekstracelulārās vezīkulas (EVs) un antihiperglikēmisko preparātu metformīnu. Šīs vielas šķērso hematoencefālisko barjeru un tām piemīt neiroprotektīvas īpašības *in vitro*, kas atļauj domāt, ka EV un metformīnam piemīt neiroprotektīvas un kognīciju uzlabojošas īpašības *in vivo*.

Iegūtie dati rāda, ka EVs intranazāla ievadīšana PS modeļžurkām normalizēja 6-OHDA-izraisītos gaitas un iemācīšanās/atmiņas traucējumus, saglabājot dopamīna sintēzes atslēgenzīma – tirozīna hidroksilāzes (TH) un Nissl ķermeņu ekspresiju nigrostriālajās struktūrās arī pēc EVs ievadīšanas pārtraukuma.

Metformīna ievadīšana sAS-tipa modeļžurkās, kas iegūtas intracerebroventrikulāri ievadot neirotoksīnu streptozocīnu (STZ), uzlaboja telpisko iemācīšanos/atmiņu un sociālo uzvedību, normalizēja STZ-izraisītās patoloģiskās izmaiņas to garozas un hipokampa proteīnu ekspresijā, kas ir iesaistīti glikozes transportā un uzņemšanā šūnās, neiroiekaisumā, sinaptiskajā plasticitātē un acetilholīna šķelšanā. Metformīns uzrādīja acetilholīnestarāzes aktivitāti inhibējošas īpašības arī *in vitro* eksperimentos.

Iegūtie dati pirmoreiz parāda EV un metformīna neiroprotektīvās un regulējošās īpašības sAS un PS modeļdzīvniekos, kas norāda uz šo vielu perspektivitāti un lietderīgumu sAS un PS agrīno stadiju patoloģisko procesu savlaicīgā apturēšanā.

Atslēgas vārdi: Alcheimera slimība (AD); Pārkinsona slimība (PD); STZ; 6-OHDA; ekstracelulārās vezīkulas; metformīns; gaita; mācīšanās/atmiņa.

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INTRODUCTION

The actuality of the study

The prevalence of neurodegenerative disorders is increasing, partly due to the extented lifespan all over the world. The neurodegeneration involves progressive death of neuronal cells, followed by a decline in cognitive functions, finally leading to dementia. That is an alarming fact because neurodegenerative diseases are still incurable. Therefore during the last three decades, one of the trends in neurosciences is focused on neurodegenerative disorders, mostly on Alzheimer's disease (AD) and Parkinson's disease (PD). AD represents approximately 60-70% of dementia cases. The manifestation of AD in the vast majority of patients (around 95-99%) have late-onset and sporadic origin, i.e., sporadic AD (sAD). In contrast, only 1-5% of all cases are familial with an identified genetic predisposition (fAD).

Although a great amount of research has been done to identify the main etiopathological mechanisms involved in the neurodegeneration, an exact understanding of the dynamics of molecular interactions and factors promoting the disease progression remains elusive. Because current therapies can only delay but not halt neurodegenerative in the brain, there is an urgent necessity to find new approaches to design and use anti-neurodegenerative drugs, capable of stopping pathological events before dementia occurs.

The aim of the study

The thesis aimed to study the neuroprotective efficacy of extracellular vesicles in PD model-rats, and metformin in sporadic AD (sAD) model-rats.

Tasks of the study

To assess *in vivo* the effects of intranasally administered extracellular vesicles (EVs) in 6-hydroxydopamine (6-OHDA)-induced PD model rats:

1.1. on motor function (gait) and learning/memory;

1.2. *ex vivo* to evaluate the influence of EVs on crucial protein expression in the nigrostriatal structures: tyrosine hydroxylase (TH), the enzyme involved in dopamine synthesis, and the Nissl bodies, representing survived neurons;

1.3. to find time-dependent efficacy of EVs.

2. To determine the effects of perorally administered anti-diabetic drug metformin in streptozocin (STZ)-induced AD model-rats:

2.1. on rat spatial learning/memory and sociability performance;

2.2. *ex vivo* to evaluate metformin effects on the cortical and hippocampal density of proteins involved in:

2.2.1. microgliosis (glial fibrillary acidic protein, GFAP) and astrogliosis (ionized calcium-binding adaptor molecule-1, Iba-1);

2.2.2. glucose transport protein-1 and -3 (GLUT-1 and GLUT-3), and glycogen synthase kinase-3 (GSK-3), an enzyme involved in glucose and insulin metabolism;

2.2.3. synaptic plasticity: synaptophysin-1 (SYP-1), growth-associated protein 43 (GAP43);

2.2.4. expression of AchE, an enzyme responsible for acetylcholine cleavage.

2.3. Determination of the influence on acetylcholine esterase *in vitro.*

3. To identify correlations between behavioral and biochemical effects of the studied compounds to understand their essential targeting properties in the pathological events.

Hypothesis

1. EVs can quickly enter and target the brain regions responsible for maintaining normal gait and memory functions.

2. Anti-hyperglycemic drug metformin provides normalization of cerebral glucose transport and metabolism and insulin signaling (GSK-3), as well as reduces neuroinflammation, thus regulating the key mechanisms to halt neurodegeneration.

Thesis for the defense

1) Intranasally administered EVs may reverse 6-OHDA-induced gait impairments and improve rat spatial learning/memory performance in PD model-rats. These effects persist even after discontinuation of EVs treatment.

2) EVs might normalize the expression of dopamine synthesizing enzyme TH and provide neuronal survival in the nigrostriatal structures of PD model-rats.

3) Metformin possesses a regulatory/restorative effect on protein expression involved in cerebral glucose transport and uptake, neuroinflammation, synaptic plasticity, and is capable of inhibiting AChE expression and activity.

Methods

1) *In vivo:* Morris water maze test for the assessment of spatial learning and memory, three-chamber sociability test for the assessment of social interaction and social novelty preference, and CatWalk gait test to determine motor function;

2) *Ex vivo:* immunohistochemical and histochemical methods for the evaluation of brain protein expression;

3) *In vitro:* determination of the influence of metformin on acetylcholinesterase activity.

Approbation of results

Publications

I. V.Pilipenko, **K. Narbute,** J.Pupure, I.K.Langrate, V.Klusa. Neuroprotective potential of antihyperglycemic drug metformin in sporadic Alzheimer's disease model-rats. *Eur.J.Pharmacol*., 2020; 881. https://doi.org/10.1016/j.ejphar.2020.173290.

II. **K.Narbute,** V.Pilipenko, J.Pupure, T.Klinovics, J.Auders, U.Jonavice, K. Kriaučiūnaitė, A.Pivoriunas. Time-dependent memory and gait improvement by intranasally-administered extracellular vesicles in Parkinson's disease model rats. Cellular and molecular neurobiology, 2020. https://doi.org/10.1007/s10571-020-00865-8.

III. **K. Narbute**, V. Pilipenko, J. Pupure, Z. Dzirkale, U.Jonavičė, V. Tunaitis, K.Kriaučiūnaitė, A.Jarmalavičiūtė, B. Jansone, V.Klusa and A.Pivoriūnas, Intranasal administration of extracellular vesicles derived from human teeth stem cells improve motor symptoms and normalize tyrosine hydroxylase expression in the substantia nigra and striatum of the 6-hydroxydopamine-treated rats. *Stem Cells Translational Medicine*, 2019; 8(5): p. 490-499. https://doi.org/10.1002/sctm.18-0162.

Conference thesis

1. **K.Narbute**, V.Pilipenko, J.Pupure, T.Klinovics, J.Auders, A.Pivoriunas, V.Klusa. Influence of extracellular vesicles on motor functions and memory in 6-OHDA-induced Parkinson's disease model-rats. Advances in Alzheimer's and Parkinson's therapies: An AAT-AD/PD focus meeting, April 2-5, 2020, Vienna, Austria.

2. V.Pilipenko**, K.Narbute,** J.Pupure, B.Kanepe, I.K.Langrate, V.Klusa. Metformin ameliorates neuroinflammation, oxidative stress and synaptic density in sporadic Alzheimer's disease model-rats. Advances in Alzheimer's and Parkinson's therapies: An AAT-AD/PD focus meeting, April 2-5, 2020, Vienna, Austria.

3. I.K.Langrate, **K.Narbute**, V.Pilipenko, V.Kluša. Antidiabetic drug metformin improves sociability and social interaction in Alzheimer's disease model-rats. RSU International student conference, March 27-28, 2020, Riga, Latvia.

4. V. Pilipenko**, K. Narbute**, J. Pupure, B.Jansone, V.Klusa. Neuroprotective action of metformin, an antidiabetic compound, in Alzheimer's disease-type male rat model. 14th International Conference on Alzheimer's & Parkinson's Diseases, March 26-31, 2019, Lisbon Portugal.

5. **K.Narbute**, V.Pilipenko, J. Pupure, A.Pivoriūnas, B.Jansone, V.Kluša. Extracellular vesicles reverse dysfunctions in 6-OHDA-induced Parkinson's disease model-rats. International Scientific Conference on Medicine, 77th International Scientific Conference of the University of Latvia, Riga, February 22, 2019. Medicina (Kaunas) Vol. 55, Suppl.1.

6. **K.Narbute**, V. Piļipenko, A. Pivoriunas, B.Jansone, V. Z.Kluša. Human dental pulp stem cell-derived exosomes administered intranasally can cross the blood-brain barrier in rats.76th International Scientific Conference on Medicine of the University of Latvia. Book of Abstracts 2018 P.34.

Selection of compounds

1) Extracellular vesicles (EVs) derived from human exfoliated deciduous teeth stem cells (SHEDs) (Fig. 1);

2) Metformin, the antihyperglycemic agent of the biguanide class (Fig. 2);

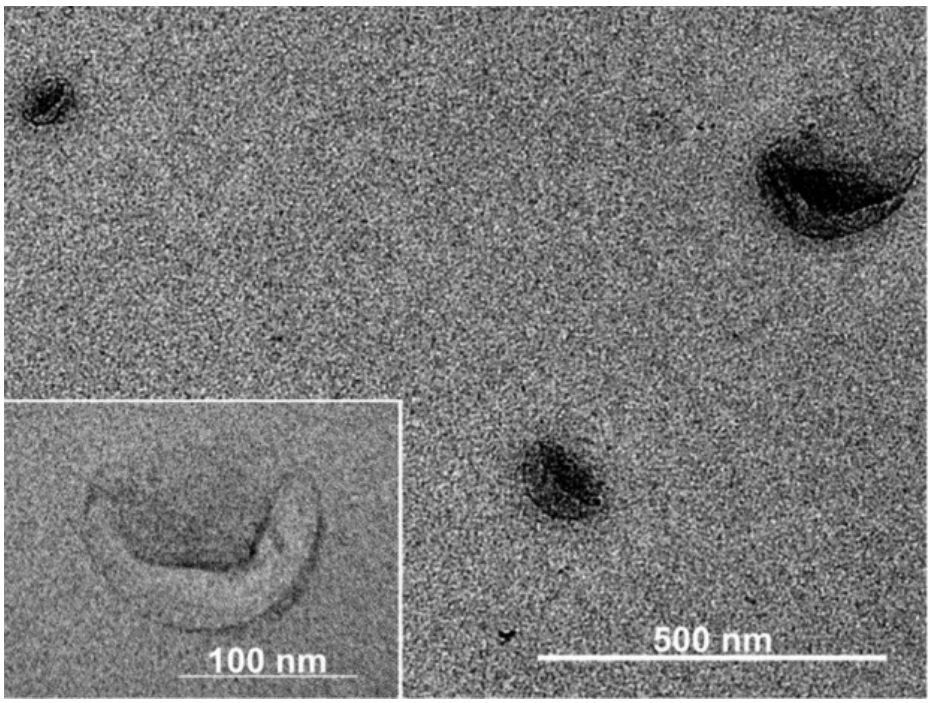


Figure 1. Transmission electron microscopy of extracellular vesicles isolated from stem cells from the dental pulp of human exfoliated deciduous teeth (×30,000 magnification). A magnified image of EV is shown on the left panel (×120,000 magnification).

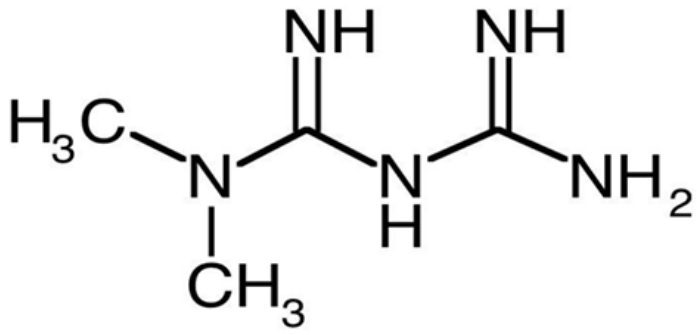


Figure 2. Chemical structure of metformin.

ABBREVIATIONS

AChE – acetylcholine esterase

aCSF – artificial cerebrospinal fluid

AD – Alzheimer's disease

ANOVA – analysis of variance

APP – amyloid precursor protein

Aß42 – amyloid beta peptide 1-42

ATP – adenosine triphosphate

Aβ – amyloid beta protein

CA – cornu ammonis

CB – citric buffer

CNS – central nervous system

DG – dentate gyrus

fAD – familial Alzheimer's disease

GABA – gamma-aminobutyric acid

GAD67 – glutamic acid decarboxylase 67

GAP43 – growth associated protein 43

GFAP – glial fibrillary acidic protein

GLUT – glucose transporter

GSK-3 – glycogen synthase kinase-3β

Iba-1 – ionized calcium-binding adapter molecule-1

icv – intracerebroventricularly

MCI – mild cognitive impairment

MFB – medial forebrain bundle

PBS – phosphate-buffered saline

PD – Parkinson's disease

BBB – blood-brain barrier

ROS – reactive oxygen species

sAD – sporadic Alzheimer's disease

sAPP – soluble APP

SHEDs - human exfoliated deciduous teeth stem cells

SNpc – substantia nigra pars compacta

STZ – streptozocin

SYP1 – synaptophysin 1

1. LITERATURE OVERVIEW

1.1. Ethiopathology of neurodegenerative diseases

Neurodegenerative diseases are one of the leading causes of incurable and debilitating conditions worldwide that cause dementia-related disabilities. These complex diseases are mainly age-associated. Their prevalence is rapidly increasing, in part because the proportion of the geriatric population has increased in past years and will continue to expand in the next decades due to the increasing life span (Mahishale, 2015). Neurodegeneration is characterized by loss of neuronal population throughout the brain and synaptic dystrophy with subsequent cognitive impairments and movement disorders. The most common neurodegenerative diseases are Alzheimer's disease and Parkinson's disease. Although currently, the exact etiology of these diseases remains elusive, early manifestations have been linked to genetic predisposition, impairments in glucose metabolism, neuroinflammation and epigenetic factors (Millan, 2014).

1.1.1. Alzheimer's disease (AD)

AD is a multifactorial progressive and until this day irreversible neurocognitive disease. It is the most common form of neurodegenerative dementia (Liu et al., 2018). AD prevalence tends to increase by age and the incidence is higher in women than in men. It is estimated that 50 million people worldwide are diagnosed with dementia, and this number will likely triple by 2050 (Mendiola-Precoma et al., 2016). Although AD was first characterized more than 100 years ago in 1908 by German physician Alois Alzheimer, the exact mechanism of AD remains unknown. At that time, Alois Alzheimer described AD as a progressive cognitive disorder with local neurological symptoms, psychological, and social disability. In the brain biopsy, he found senile plaques and neurofibrillary tangles. Unfortunately, clinical staging of the disease still cannot be definitively diagnosed, since AD pathology can only be determined in *post mortem* by neuropathological evaluation (Murphy and Levine, 2010). Therefore, despite the scientific progress towards understanding the cellular and molecular bases of AD, therapies that effectively halt the progression of AD are still lacking. Currently, available treatments are designed only to improve clinical symptoms rather than to stop the progress of the disease. In the 1970s, the cholinergic system was the main pharmacotherapeutic target, and the use of anticholinesterases is still recommended. Around the 1980s, dysfunction in the glutamatergic system became as dominant, and a lot of glutamate receptor antagonists were

designed and tested. Since the 1980s, the research is focusing on targeting misfolded proteins and their fragments, such as amyloid protein and its amyloid-beta (Aβ) 1-42 fragment and tau protein. However, data from recent years show that this theory is also incomplete and requires many corrections.

1.1.1.1. Familial Alzheimer's disease (fAD)

Familial early-onset AD (fAD) affects individuals between 30 and 50 years old and generally accounts for a small proportion of all cases – approximately 1-5% (Frozza et al., 2018). Pathogenesis of fAD is associated with the so-called "amyloid cascade" (Fig.3.) with mutations either in the *amyloid precursor protein* (*APP*) or in presenilin genes: *presenilin-1* (PS1) or *presenilin-2* (PS2), that eventually leads to synaptic loss and cognitive decline. Through this amyloidogenic pathway, different Aβ fragments are formed, of which the most toxic is larger, hydrophobic, and highly fibrillogenic Aβ1-42 peptide (Aβ42). These can cause severe neuronal toxicity, disruption in synaptic transmission, neuroinflammation, and impaired cognition. Thus, two hallmark pathologies are used for diagnosing AD: the density of extracellular Aβ1-42 plaque deposits and intracellular hyperphosphorylated neurofibrillary

tangles of tau protein.

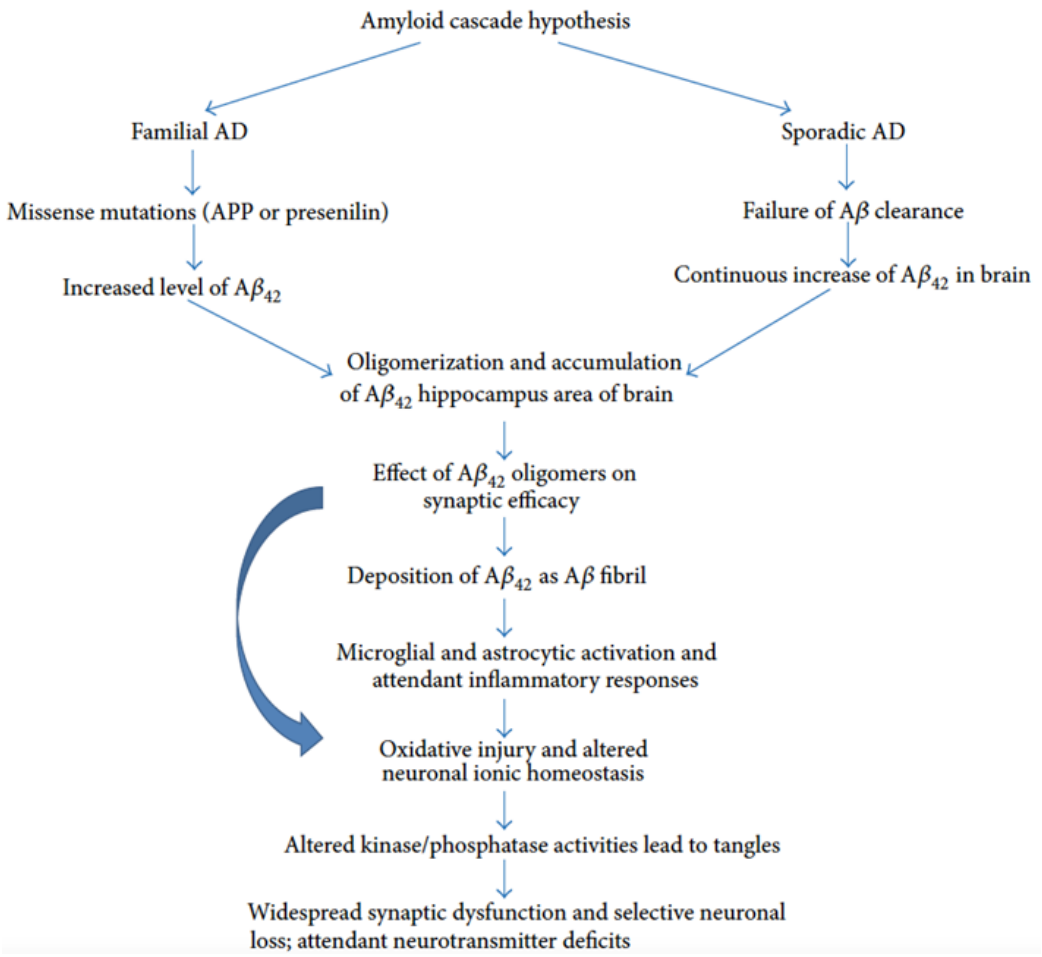


Figure 3. Amyloid cascade of Alzheimer's disease. Adapted from (Singh et al., 2016).

Although the amyloid cascade hypothesis was governing for about 20-30 years, the last decade studies have challenged this theory by showing an almost complete failure of therapies designed to target Aβ clearance. Several studies have shown that Aβ accumulation

in the brain does not correlate with neuronal death and cognitive deficits. Moreover, different pharmacological and immunological (e.g. using passive immunization with monoclonal antibodies, such as solanezumab and crenezumab) approaches and clinical trials have found that dissolving the amyloid plagues, did not correlate with the improvement of cognitive performance and/or stop in synaptic loss (Prins and Scheltens, 2013). Furthermore, with the help of novel imaging systems,–it was shown that in elderly non-demented patients, accumulated amyloid distribution can be as extensive as that of AD patients. In this context, pharmacological studies by the use of transgenic mice may represent the human pathology only partially (Kametani and Hasegawa, 2018; Ricciarelli and Fedele, 2017).

1.1.1.2. Sporadic AD (sAD)

The vast majority (95-99%) of all AD cases are a late-onset or sporadic form of AD that occurs in individuals over 65 years of age, however pathological processes of asymptomatic preclinical AD start up to 20 years before clinical manifestation. Although amyloidosis is not considered to be specifically connected to sAD, progressive Aβ accumulation can occur in later phases of the disease (Singh et al., 2016). The prodromal AD stage is characterized by short-term memory loss without affecting everyday life. Mild cognitive impairment (MCI) is a state between "normal aging" and a demented state. It is characterized by mild memory impairments and non-cognitive symptoms, such as anxiety, apathy, and depression (Chung and Cummings, 2000). MCI is a predictive indicator of developing AD, but not all patients develop AD.

Several factors have been identified which are associated with AD progression, and they to a great extent are linked with epigenetic factors characterized by deoxyribonucleic acid (DNA) modifications that modulate gene expression but don't influence the genetic code. These modifications include anomalies in DNA methylation, DNA demethylation, chromatin remodeling, and histone post-translational modifications. For instance, inhibition of DNA methylation has detrimental effects on histone modifications and synaptic plasticity in both AD and PD (Landgrave-GÃ³mez et al., 2015), that in turn, impairs cerebral glucose metabolism, neuroinflammation, impaired synaptic transmission, and neurotransmitter imbalance. Studies showed that neurodegenerative processes promote the decrease in neuroprotective gene expressions, such as *forkhead box O3* (*FOXO)*, which mediates oxidative stress resistance, anti-apoptotic *BCL2*, as well as the gene for expression of *brain-*

derived neurotropic factor (BDNF), which is critical for learning and memory (Berson et al., 2018). Moreover, these processes can be affected by environmental factors (e.g., nutrition, exercise, drugs).

Current knowledge indicates that most effective therapy could be focused on early pre-dementia stages of sAD, and new disease-modifying drugs should be found (Frozza et al., 2018).

1.1.2. Parkinson's disease (PD)

The second most common age-related neurodegenerative disease is PD. Although sAD and PD have common etiopathological causes linked to late-onset progressive proteinopathies, these diseases exhibit distinctive characteristics and symptoms. PD typically affects people over 60 years of age, and the prevalence increases over the age of 85. The prevalence is higher among men than women (proportion 1.5 to 1.0). The exact causes of PD pathology remain obscure. It is taken as proved that main cause for PD is the loss of dopamine synthesizing neurons in the nigrostriatal structures, accumulation of Lewy bodies (LB) or Lewy neurites (LN) throughout the brain, composed of misfolded α-synuclein protein which tends to form a fibrillary, β-pleated sheet conformation and bind other proteins, such as synphilin-1, parkin, and antiapoptotic chaperone. β-pleated sheet formation of aggregated α-synuclein is also found in AD brains (Rizek et al., 2016). Dopamine neuronal cell bodies are generally located in the substantia nigra pars compacta (SNpc) regions with axonal projections to caudate nucleus and putamen. Dysbalance between decreased dopaminergic activity and increased cholinergic activity results in motor impairment (muscle rigidity, tremor, and bradykinesia) and non-motor symptoms (cognitive impairment, depression and sleep disorders). Motor symptoms are evident after about 50% of the dopaminergic neurons, and 75% of striatal dopamine is lost. The manifestation of non-motor symptoms is evidently linked to disbalance in other neurotransmitter systems, such as serotoninergic, noradrenergic, and cholinergic (Perez-Lloret and Barrantes, 2016). Although only a small number of PD patients are associated with genetically inherited diseases, mutations in one or more of three genes have been identified to be associated with the genetic form of PD. They are *SNCA* gene that codes α-synuclein protein, *parkin,* and *ubiquitin carboxyl-terminal hydrolase L1* (*UCH-L1*). The severity of the disease varies depending on

disease phenotype and age of onset. Taking into account that genetic or familial form of PD is only accountable for approximately 1% of all cases, α-synuclein aggregation is also associated with the disease's sporadic form. Usually, α-synuclein degradation undergoes direct proteolysis, autophagy, and proteosome-mediated degradation. Failure in any of these steps results in the accumulation and aggregation of α-synuclein in neurons.

Moreover, mutations in the *SNCA* gene also is a major risk factor of pathological accumulation of misfolded α-synuclein oligomers. Using experimental models of PD, researchers have suggested that α-synuclein selective degeneration of dopaminergic neurons is accomplished *via* elevated levels of reactive oxygen species (ROS), preferential expression of parkin and α-synuclein in these neurons and lack of calbindin D_{28K} (know as calcium homeostasis maintaining and neuronal death preventing protein) (Steece-Collier et al., 2002). It is confirmed by several studies that environmental factors have an even stronger influence on PD etiopathology. Contaminated water with heavy metals and pesticides, such as rotenone and paraquat, is the main environmental risk factor of developing PD. These pesticides are known to impair the mitochondrial respiration chain and by causing oxidative stress (Ascherio and Schwarzschild, 2016).

Despite the massive amount of research directed to identifying the key neurodegenerative mechanisms involved in the PD's pathogenesis, this disease is still not curable. Currently, available therapies are only symptomatic, partially effective, and focused on the reduction of dopamine deficiency (e.g., by the usage of dopamine precursor, dopamine agonists, inhibition of dopamine cleaving enzymes). Similarly, as in AD, also PD treatment needs new therapeutic strategies.

1.3. Impaired brain cellular processes

1.3.1. Neuroinflammation

The early progression of neurodegenerative diseases has been linked to neuroinflammatory mechanisms. The term neuroinflammation defines the inflammatory processes of the central nervous system (CNS), involving overactivated glial cells (astrocytes and microglial cells). However, the question of whether the neuroinflammation is a cause or a consequence of neurodegeneration, remains unanswered (Guzman-Martinez et al., 2019).

Typically, glial cells have a fundamental role in maintaining brain homeostasis. Thus, microglial cells are the primary resident immune cells in the brain, wherein physiological and pathological conditions regulate astrocytes. Microglia exhibit a resting phenotype. They continuously scan their surrounding microenvironment and are major players providing synaptic plasticity (also synaptic pruning) by releasing neurotrophic and anti-inflammatory factors (Streit, 2002). Thus in acute inflammation stage due to the presence of pathogens or tissue damage, glial cells become activated and communicate by releasing a wide range of molecules, such as neurotrophic factors, cytokines, chemokines, growth factors. The mutual communication between both types of glial cells generates reciprocal modulation for various lesions in the CNS. In early AD phases microglia and astrocytes are also able to bind to and accumulate around Aβ oligomers and Aβ fibrils, release pro-inflammatory and anti-inflammatory cytokines and phagocyte them, thus acting as a neuroprotective mechanism (Bouvier and Murai, 2015). However, the sustained release of inflammatory molecules affects neurons, stimulates further production of Aβ, and induce more activation of microgliosis (Glass et al., 2010). Overactivated astrocytes release the factors that change the BBB's permeability, resulting in the migration of immune cells into the brain parenchyma. Pathogens such as bacteria, viruses, endogenous proteins, antibodies, cytokines, and chemokines, including toll-like receptors (TLRs) and receptor for advanced glycation end products (RAGE), activate microglia and compromise brain homeostasis (Shastri et al., 2013). Activated microglia then regulate the expression of different surface markers, such as the major histocompatibility complex II (MHC-II) and receptors for identifying pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), which produce proinflammatory cytokines, such as interleukins (IL-1β, IL-6, IL-12), interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α), as well as cytotoxic factors, such as reactive oxygen species (ROS), superoxide radicals (O_2^-) and nitric oxide (NO), resulting in neuronal damage and subsequent neurodegeneration. A simplified neuroinflammatory process is shown in Figure 4.

Since the 1990s, it has been proposed that usage of anti-inflammatory agents and the reduction of inflammation could improve the outcome of neurodegenerative diseases (Moore et al., 2010). Unfortunately, only high doses and long-term administration of COX inhibitors or glucocorticoids slightly improved cognition and slow the progression of neurodegeneration. In addition, the treatment was followed by typical for these drug's side-

effects. Several pre-clinical studies with anti-inflammatory/antioxidant agents, such as curcumin, resveratrol, have shown memory-improving properties, while non of these agents have shown considerable effectiveness (Moore et al., 2010; Walker and Lue, 2007).

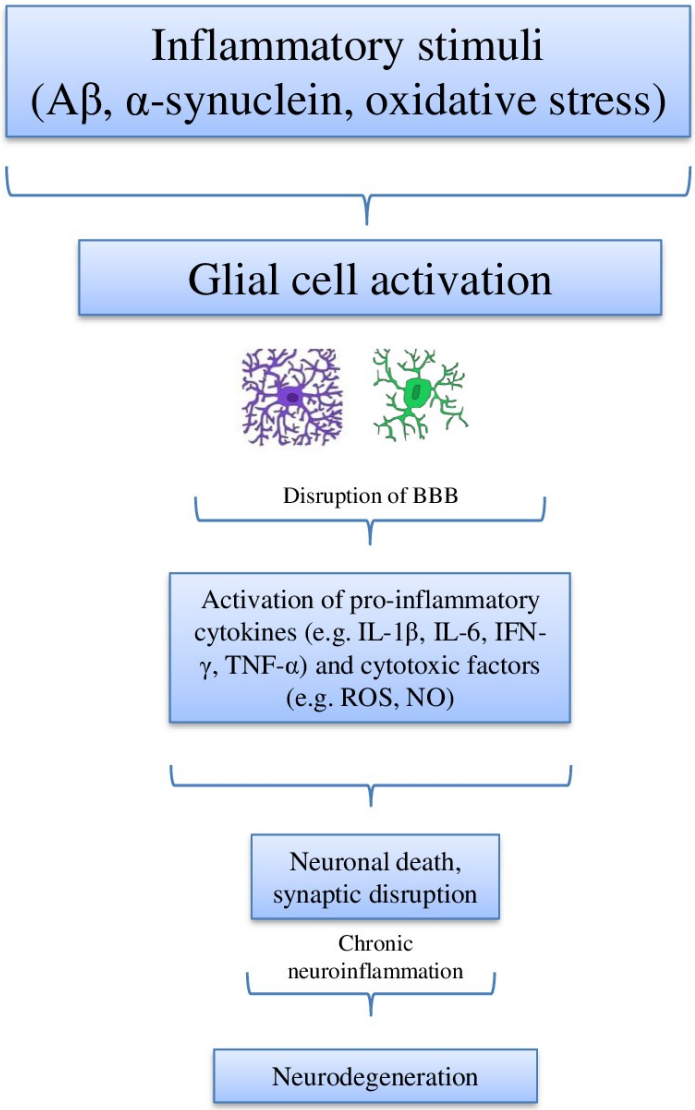


Figure 4. The simplified neuroinflammatory process that leads to neurodegeneration. Adapted from (Jung et al., 2019).

1.3.2. *Oxidative stress and mitochondrial dysfunction*

Mitochondria are very dynamic organelles that continuously undergo continuous fission and fusion, the equilibrium that regulates not only the number of mitochondria but also their functions, such as energy metabolism and production, Ca^{2+} signaling, ROS production and apoptosis (Chen et al., 2007). Mitochondria generate about 90% of needed cell energy, mostly in the form of adenosine triphosphate (ATP). Production of ATP requires aerobic, enzyme-mediated reactions through the mitochondrial electron transport chain (ETC). During this oxidative phosphorylation, electrons are transferred from electron donors to electron acceptors. In eukaryotic cells, these electrons are carried out by five different protein complexes. The released energy by ETC is used by complexes I, III and IV, to transport protons from the mitochondrial matrix to intermembrane space. The complex V then synthesizes ATP from adenosine diphosphate (Mancuso et al., 2006). It is estimated that in healthy mitochondria, about 2% of consumed oxygen is turned into ROS, and their amount is much higher in aged and damaged mitochondria. Increased production of superoxide ($O_2^{\cdot-}$), hydroxyl ($^{\cdot}OH$) and hydrogen ($^{\cdot}H$) radicals cause unrepaired damage to mitochondrial DNA (mtDNA) which leads to increased oxidative stress, cellular damage and genomic instability (Islam, 2017). Undoubtedly, oxidative stress and mitochondrial dysfunction are involved in the onset of age-related neurodegenerative diseases. It has been suggested that mutations in AD- and PD-associated genes can directly damage mitochondria by impairing the activity of respiratory complexes, causing oxidative damage and consequently causing synaptic dysfunction (X. Wang et al., 2009). Several mutations in mtDNA genes are involved in the progression of neurodegenerative diseases (Horvath et al., 2007). All the mentioned above impairments interact and promote neurodegenerative processes (Fig.5).

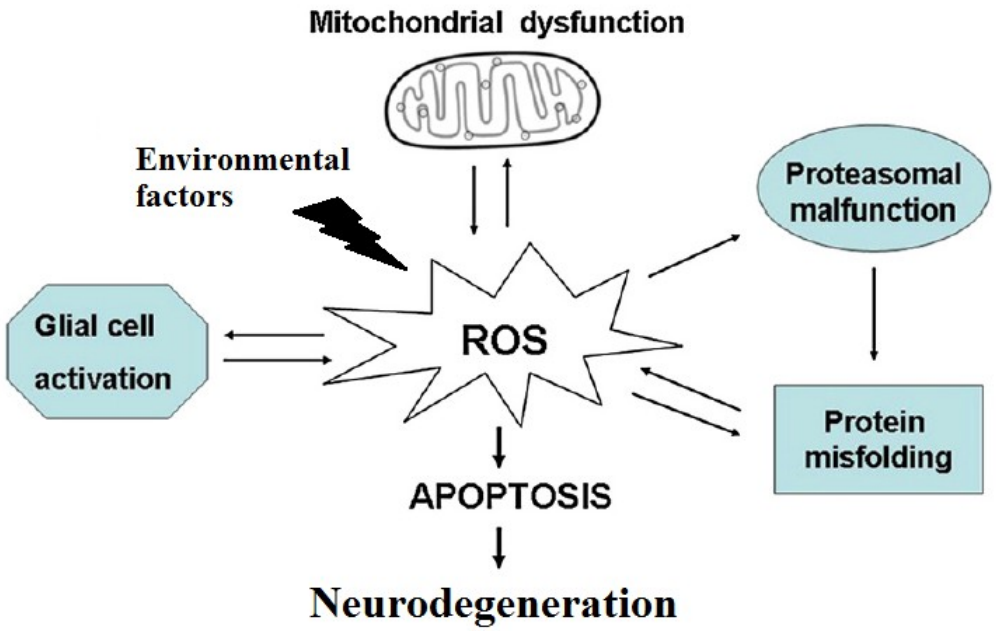


Figure 5. Reactive oxygen species (ROS) mediated neurodegeneration. Adapted from (Federico et al., 2012).

1.3.3. Impairments of cerebral glucose and insulin metabolism

In the past decade, AD has been linked to metabolic dysfunctions. The human brain is the most glucose-consuming organ in our body. Most of the energy is used to generate action and postsynaptic potentials and neurotransmitter biosynthesis (Spinelli et al., 2019). Therefore, it is not a surprise that one of the earliest hallmarks of neurodegenerative diseases is impaired cerebral glucose metabolism and insulin signaling, that leads to early cellular damage, associated with neuroinflammation, mitochondrial dysfunction, oxidative stress and impaired synaptic transmission (Athauda and Foltynie, 2016; de la Monte, 2017). Although numerous glucose transporters (GLUTs 1-14) are identified, only several of them are found in the brain. Glucose is transported through the BBB endothelial cells and in astrocytes *via* GLUT-1 in an insulin-insensitive manner and uptaken into neurons *via* GLUT-3 (Shah et al., 2012). GLUT-2 is predominantly expressed in the hypothalamus that regulates food intake, whereas GLUT-4 is found to be expressed in astrocytes, hippocampus, cerebellum and neocortex, indicating GLUTs role in glucose uptake in neurons (Sankar et al., 2002).

Interestingly, insulin stimulates GLUT-4 gene expression and protein transport from the cytosol to the plasma membrane thus modulates glucose uptake and utilization. Reduced GLUT-1 and GLUT-3 densities have been reported in the brains of AD patients. The membrane expression of GLUT-3 is regulated by AMP-kinase, and it was shown that Aβ inhibits AMP-kinase activity, suggesting one another mechanism as potential glucose inhibition in AD brain (Lin et al., 2016; Prapong et al., 2002).

The main functions of insulin and insulin-like growth factor (IGF) in the brain are to modulate neuronal and synaptic plasticity processes, including growth and survival, protein synthesis, gene expression and neurotransmitter function, thus engaging in the maintenance of cognitive functioning (de la Monte and Wands, 2005). Insulin and IGF realize their biological effects through tyrosine kinase-linked receptors. Insulin receptor substrates (IRS-1 and IRS-2) are involved in signal transmission from IGF and insulin receptors through different intracellular pathways, including phosphoinositide 3-kinase (PI3K) /protein kinase B (AKT) and mitogen-activated protein kinase (MAPK) pathways (Fig.6).

IRS signaling pathways seem to be especially important in normal brain functioning, and the altered IRS-1 and IRS-2 pathway regulation are found in AD brains and AD model-animals. IRS-1 positively influences cognitive processes by increasing synaptic plasticity, whereas IRS-2 acts opposingly. Activated mechanistic target of rapamycin (mTOR) pathway leads to aggregation of misfolded proteins and impaired autophagy-mediated cell death. AD and PD pathologies are highly associated with brain insulin resistance (IR). Oxidative stress accompanied by IR promotes dysregulation of carbohydrate and lipid metabolism (especially elevated levels of 4-hydroxy 2- nonenal, thus increasing activation of glycogen synthase kinase-3 beta (GSK-3β) and impairing anti-apoptotic signaling and mitochondrial function (de la Monte, 2017).

Molecular links between impaired glucose metabolism and insulin signaling in AD and diabetes suggests the possibility for novel therapeutic strategies for AD using antidiabetic drugs. It has been reported that intracerebroventricular administration of insulin improved rat performance in cognitive tests, and injections of insulin in the hippocampus at physiological doses improved rat spatial memory via PI3K-dependent mechanism (Spinelli et al., 2019). Furthermore, intranasal administration of insulin enhances memory and performance in healthy patients without modifying glucose or insulin levels in the blood (BENEDICT, 2004). Moreover, clinical trials have demonstrated that intranasal insulin improves memory

performance and the brain's metabolic integrity in patients suffering from AD or its prodromal stage, with mild cognitive impairment (Freiherr et al., 2013).

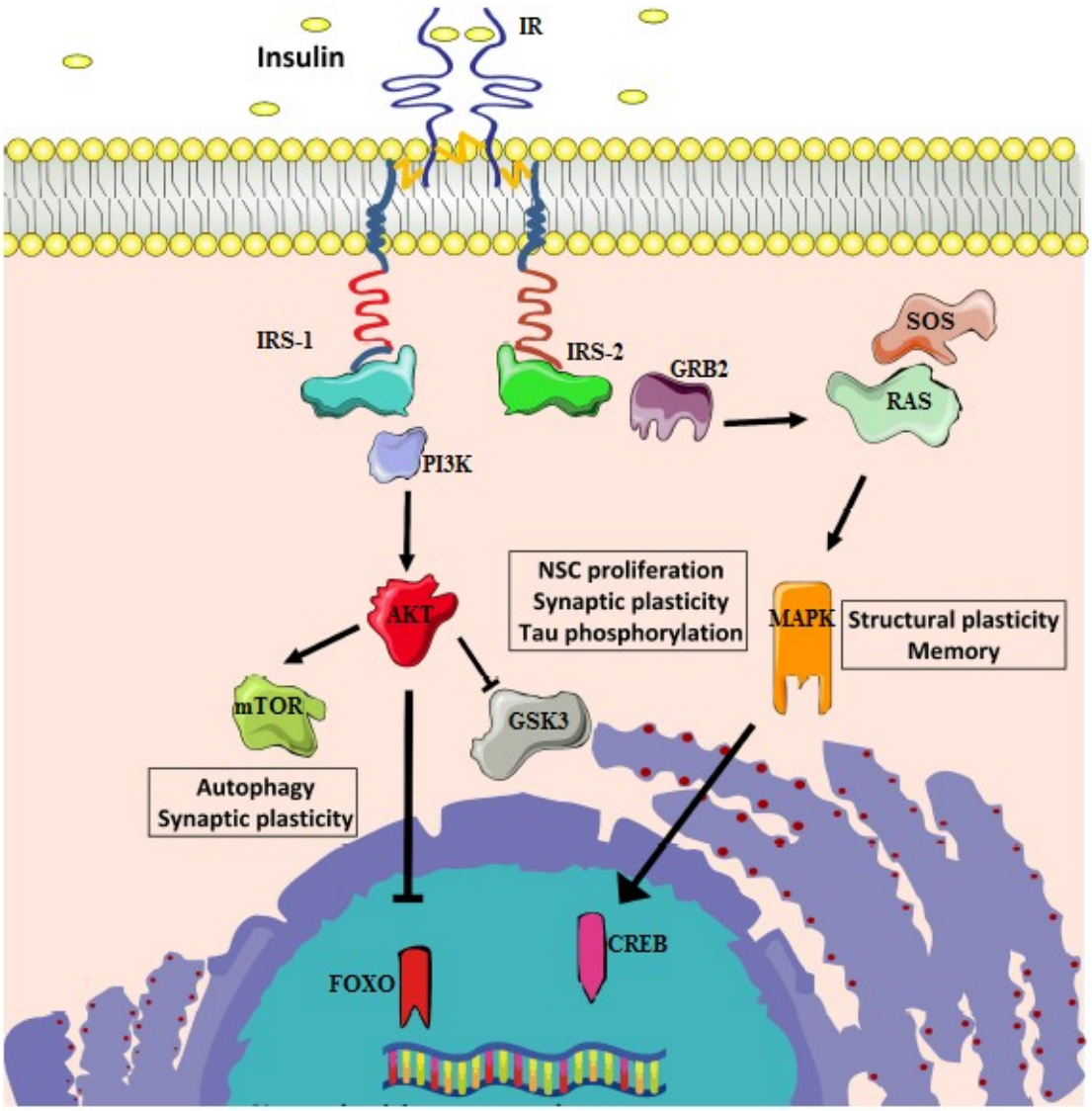


Figure 6. Insulin signaling and neuroplasticity-modulating pathway. IR , insulin receptor; IRS-1 and IRS-2, IR substrate 1 and 2; PI3K, phoshoinositide-3 kinase B; AKT, protein kinase B; mTOR, target of rapamycin; GSK3β, glycogen synthase kinase 3 beta; GRB2, growth factor receptor-bound protein 2; SOS, son of sevenless; RAS, rat sarcoma GTPase protein; MAPK, mitogen activated protein kinase; FOXO, forkhead box O transcription factor; CREB, cAMP response element-binding protein. Adapted from (Spinelli et al., 2019).

1.3.5. Neurotransmitter dysfunction

1.3.5.1. Cholinergic and dopaminergic transmission

The primary neurotransmitters which activate basal ganglia (striatum, globus pallidus, subthalamic nucleus, and substantia nigra) are dopamine (DA) and acetylcholine (Ach). The dysfunction in their transmission are associated with pathological conditions. Ach is widely distributed in cerebral areas, such as the cortex, prefrontal cortex, primary motor cortex, nucleus accumbens, and hippocampus. The cholinergic system is involved in numerous vital physiological processes, such as memory formation, learning, sensory information, and attention (Xu et al., 2012). A considerable amount of evidence exists, suggesting that cholinergic transmission plays an essential role in the pathological mechanisms of AD and PD. The loss of cholinergic neurons (especially in the basal forebrain) and consequent dysfunction of dopaminergic transmission are thought to be the main factors underlying AD- and PD-related neuropsychiatric symptoms (e.g. depression, delusions, apathy). Dysfunctioned cholinergic transmission and degeneration of the neurons from the nucleus basalis of Meynert (NBM) are associated with memory loss in AD patients (Medeiros et al., 2011; Meyer et al., 2009).

DA receptors activate different signaling cascades depending on which G-protein they activate. For instance, DA-1 and DA-2 receptors modulate synaptic plasticity and cognition via elevation of cAMP and protein kinase A (PKA), phosphorylation of dopamine- and cAMP-regulated phosphoprotein-32 (DARPP-32) and modulation of the extracellular signal-regulated kinase (ERK) and cAMP response element (CREB) (Neve et al., 2004). DAergic neurons in the substantia nigra (SN) and ventral tegmental area (VTA) and their projections to the striatal area are pivotal in maintaining motor functions and learning performance. When PD is diagnosed, approximately 70% of DAergic neurons are already degenerated, resulting in motor deficits. However, human *post mortem* studies have shown that cholinergic neurons also have critically degenerated.

A considerable amount of evidence has shown significant dopaminergic-cholinergic and dopaminergic-glutamatergic interactions, which play a vital role in learning and memory (El-Ghundi et al., 2007). It is shown that activation of presynaptic nicotinic Ach receptors can regulate the release of striatal DA via PKC (Soliakov and Wonnacott, 2001).

Cholinesterase inhibitors, such as galantamine and rivastigmine, increase Ach levels in the synaptic cleft and partially ameliorate cognitive symptoms. Nevertheless, these drugs

cannot alter disease progression and are effective only for a relatively short period of time (1-3 years) (H. Ferreira-Vieira et al., 2016). Therapies that are concentrated on DA replacement, such as DA-precursor L-DOPA or DA agonists are only effective against motor symptoms (rigidity, tremor, bradykinesia) and, unfortunately, cause well-known side effects (dyskinesias and movement fluctuations), and even impairs cognitive processes (Ferrazolli et al., 2016).

1.3.5.2. Glutamatergic transmission

One another factor involved in neurodegenerative diseases is the impaired glutamatergic system. Excitatory neurotransmission of glutamate is realized through N-methyl-D-aspartate (NMDA) receptors. Normally, glutamate regulates calcium ion (Ca^{2+}) influx, thus controlling motor and cognitive functions. Unfortunately, these functions are altered in age and AD. When NMDA receptors become extensively overactivated, resulting in Ca^{2+} excitotoxicity and cell death (Wang and Reddy, 2017). NMDA receptor antagonist memantine is currently used to to regulate glutamate transmission symptomatically. Unfortunately, it cannot improve cognitive performance and slow the progression of neurodegeneration.

1.3.5.3. GABAergic transmission

Gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the brain, and equilibrium between excitatory glutamatergic and inhibitory GABAergic systems is essential in maintaining brain homeostasis. Recently, the impaired transmission of GABA has been linked to the progression of AD. Thus emerging evidence suggests that GABA plays an important role in reducing excitotoxicity (Mazzone and Nistri, 2019). *Post mortem* biochemical assessment of AD patients' brains revealed significantly lower levels of GABA, indicating impaired GABAergic neuronal transmission and deficient synaptic function in this disease (ROSSOR et al., 1982).

Recent studies have shown that very low doses of GABA-A and GABA-B receptor agonists improve spatial learning/memory and reduce neuroinflammation in a rat model of sAD (Pilipenko et al., 2019b, 2018). These findings may open new avenues in the treatment of neurodegenerative diseases.

1.4. Novel therapeutic approaches to halt neurodegenerative diseases

The prevalence of neurodegenerative diseases is steadily increasing and, despite the great effort put on to finding new therapeutics, almost every new target has suffered a failure. Thus the new disease-modifying therapeutic approach is urgently needed. Advances in modern neurosciences allow suggesting that the simultaneous multi-targeted regulation of all cellular events, such as neuroinflammation, glucose/insulin signaling and synaptic plasticity, could be promising a beneficial in stopping the neuropathological processes at an early stage of the AD and PD.

1.4.1. Exosomes

Exosomes were first described almost 40 years ago, and initially, their function was thought to be removing unneeded intracellular proteins (James R. Edgar, 2016). Exosomes are nano-sized (30-150 nm) membranous vesicles that are secreted by almost all cell types and found in bodily fluids, such as blood, saliva, CSF, and urine (Crenshaw et al., 2018). They originate from late endosomes fusing and forming multivesicular bodies (MVB). Within MVB, intraluminal vesicles (ILVs) are formed by the invagination of late endosomal membranes while enclosing cytosolic components, such as lipids, proteins, coding and non-coding RNA and even DNA. Although exosomal content differs due to their different ancestry cells, it has been proven that ILVs formation requires the endosomal sorting complex required for transport (ESCRT)-machinery to sort-out which proteins include (Henne et al., 2011). MVB can fuse with lysosomes for degradation, but most of them fuse with the plasma membrane and are released into the extracellular space. These vesicles are referred to as exosomes (Fig.7) (Minciacchi et al., 2015). A plethora of research studies on exosome CNS functions suggests that exosomes are involved in cell-to-cell communication, participate in the modulation of neuroprotective and regenerative processes, and mediate synaptic plasticity (Holm et al., 2018). Noteworthy, exosomes can cross biological barries, including the BBB. Their cargo properties could explain these positive abilities. Exosomes can attach to other cells and release their cargo by a range of different adhesion molecules, such as tetraspanins, integrins, and CD11b/CD18 receptors and annexins (Théry et al., 2006).

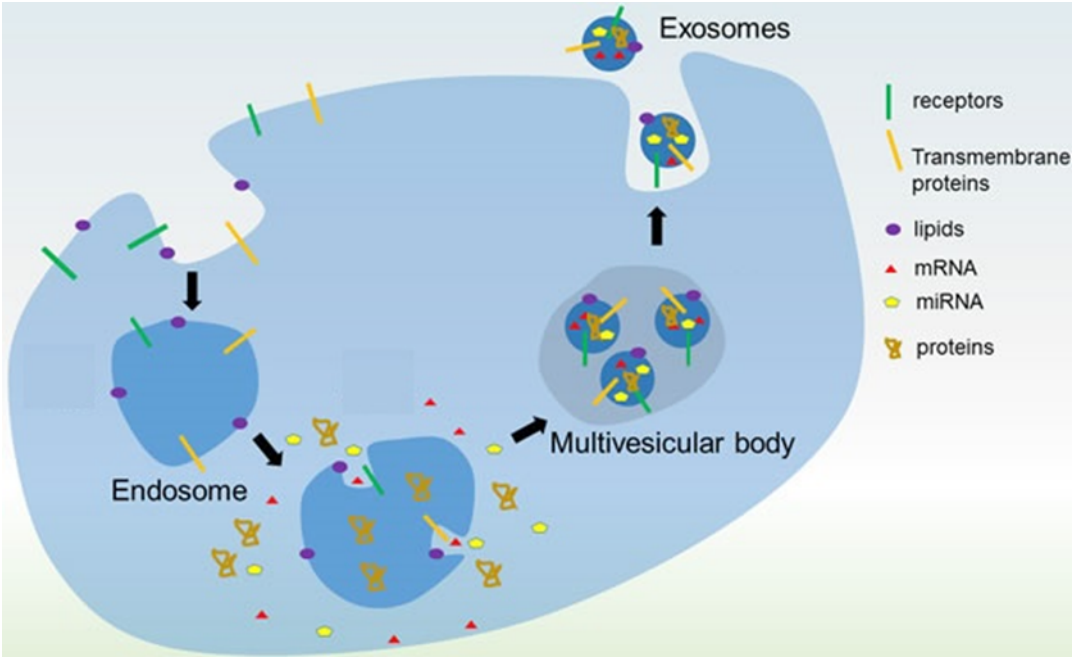


Figure 7. Schematic representation of exosome biogenesis. Adapted from (Zhou et al., 2016).

Exosomes derived from neurons are suggested to act as carriers of signaling proteins involved in synaptic plasticity. Under inflammatory response, astrocyte-produced exosomes release synapsin-1, a molecule involved in increasing neurite outgrowth, thus increasing neuronal survival (Wang et al., 2011). Moreover, exosomes carry proteins that can reduce neuronal oxidative stress, such as superoxide dismutase-1 and catalase (Holm et al., 2018).

Exosome implementation in CNS disease research is a new avenue in therapeutics – specific, effective and without serious side effects. *In vitro* study showed that exosomes can rescue human dopaminergic neurons from 6-hydroxydopamine (6-OHDA)-induced apoptosis (Jarmalavičiūtė et al., 2015). Exosome treatment also suppressed neuroinflammation and reduced cognitive impairments after traumatic brain injury (Kim et al., 2016) and after status epilepticus in mice (Long et al., 2017). Our recent studies showed that intranasally-administered exosomes derived from human deciduous stem cells can reverse gait impairments and reduce the loss of tyrosine hydroxylase expression (Narbute et al., 2019), improve learning/memory performance and preserve neuronal survival in substantia nigra pars compacta (SNpc) region even two weeks after discontinuation of exosome treatment in the 6-OHDA-induced model-rats (Narbute et al., 2020).

Due to their small size and ability to cross biological barriers, exosomes have been massively studied as drug cargo molecules. Exosomes loaded with anti-inflammatory agents, such as curcumin, can target damaged cells and reduce lipopolysaccharide-induced neuroinflammation in mice by reducing IL-6 and TNF-α expression (Sun et al., 2010; Zhuang et al., 2011). Intranasally administered exosomes loaded with catalase provided neuroprotective effects in PD model animals by specifically reducing oxidative stress and cell death in SNpc neurons (Haney et al., 2013). Macrophage-derived exosomes filled with catalase, messenger RNA, NF-κB, were capable of effectively transporting their cargo to target cells in PD-model mice, resulting in *de novo* protein synthesis and reduced neuroinflammation. Interestingly, the expression of catalase and anti-inflammatory effects in the target tissue remained even 40 days after the last administration of exosomes (Xin et al., 2013).

Exosomes have been also studied as specific biomarkers in AD and PD. It has been reported that it is possible to detect neural α-synuclein content in blood plasma by using anti-cell adhesion molecule L1 (L1CAM) antibodies and these results are associated with the severity of the disease, thus serve as a specific and sensitive biomarker (Shi et al., 2014). Undoubtedly, exosomes as diagnostic markers will open a new chapter in clinical diagnostic approaches, but, unfortunately, they are not used as specific biomarkers for diagnostics yet.

Exosome therapy opens a new perspective in neurodegenerative disease treatment (Yuan et al., 2019), however, further studies are needed to identify all pros and cons. In the future exosomes could be engineered as specific target molecules, with only desired therapeutic molecules to minimize possible safety hazards (Jarmalavičiūtė and Pivoriūnas, 2016).

1.4.2. Glucose and insulin metabolism-regulating drugs

A relationship between AD and diabetes, which characterizes impaired cerebral glucose and insulin metabolism, has been detected in the early stages of sAD (Lin et al., 2016). Moreover, previously studied antidiabetic drugs that target insulin-dependent mechanisms, such as thiazolidinediones and glucagon-like peptide-1 (GLP-1) agonists have demonstrated cognition-enhancing, anti-neuroinflammatory and glucose metabolism-improving effects in sAD mouse-model (Tumminia et al., 2018). There is growing evidence that anti-

hyperglycemic biguanide class drug metformin possesses antioxidant, anti-inflammatory, and anti-apoptotic properties and it could reverse the progression of neurodegeneration in AD and PD patients **(Rotermund et al., 2018)**. Metformin is the safest and most widely used antidiabetic drug to treat Type 2 diabetes mellitus (T2DM). Importantly, high-performance liquid chromatography (HPLC) study showed that metformin can rapidly cross the BBB and reach several brain regions (Łabuzek et al., 2010). Its effects have been explained through AMP-activated protein kinase (AMPK)-dependent and AMPK-independent mechanisms. Metformin inhibits complex I, activates AMPK signaling, and thus indirectly acts on the mTOR pathway. Through this pathway, metformin reduces ROS production. The AMPK-independent routes include the inhibition of the PI3K/AKT pathway, targeting transcription factors (Rotermund et al., 2018). Knowing that the mTOR pathway is widely studied as one of the main factors underlying neurodegenerative diseases, the ability of metformin to target the mTOR pathway indirectly, is one of the reasons to consider metformin as a promising new therapeutic agent against AD and PD (Rotermund et al., 2018). Metformin reduced overall inflammation by lowering levels of pro-inflammatory cytokines and microgliosis in a mouse model of PD (Ismaiel et al., 2016). It also reduced inflammation and protected against hippocampal cell death in a diabetic mouse model that is explained by the enhancement of the activity of transcription factors Fox-1 and NeuN (Oliveira et al., 2016). Several studies have suggested that the positive effects of metformin are due to its regulatory effect on glucose and insulin metabolism (Campbell et al., 2018; Lin et al., 2018). There is a report about metformin treatment followed by improved cognition, reduced hyperphosphorylated tau and enhanced autophagy in a mouse model of diabetes (Chen et al., 2016).

In a transgenic mouse model of diabetic dyslipidemia, metformin treatment improved cognitive outcome in one study (Chen et al., 2016), but had no effect in another study (Li et al., 2012). Several studies have shown that metformin has no effect on high-fat diet-induced cognitive deficits in rats and mice (Lennox et al., 2014; McNeilly et al., 2012; Thangthaeng et al., 2017). In a MPTP-induced PD mouse model, metformin treatment reversed TH^+ neuron loss in nigrostriatal structures (Kang et al., 2017) and reduced astrogliosis (Katila et al., 2017). Although, the effects of metformin on cognitive performance in AD patients are controversial (Moore et al., 2013), metformin effects have never been studied in sAD model-animals before. Metformin effects in sAD model-animals have first been studied by our team. We demonstrated that metformin improved rat spatial learning/memory and sociability

performance and regulated the expression of proteins involved in cerebral glucose and insulin metabolism, reduced neuroinflammation and increased the expression of proteins responsible for synaptic plasticity (Pilipenko et al., 2020).

2. MATERIALS AND METHODS

2.1. Animals

Male Wistar rats (280 ± 20 g, 9 weeks old) were used in all experiments. For the study on EVs efficacy in PD model-rats (EVs-I), animals were obtained from the State Research Institute Centre for Innovative Medicine Laboratory animals breeding house, Vilnius, Lithuania. For the study on time-dependent EVs efficacy and for the metformin study, animals were bought from the Laboratory Animal Center, University of Tartu, Estonia. All efforts were made to minimize animal suffering and to reduce the number of animals used. The experiments were conducted in accordance with the EU Directive 2010/63/EU and local laws and policies on the protection of animals used for scientific purposes. Animal protocol for this study was approved by the Animal Ethics Committee of the Food and Veterinary Service, Riga, Latvia. Rats were housed in individually ventilated two-level cages (GR1800, Tecniplast, Italy) with controlled laboratory environment (temperature 22 ± 2°C, humidity 55-65%, 12/12 h light/dark cycle (lights on at 8:00 and off at 20:00), 4 rats per polypropylypelene cage with maintenance diet (V535-000, Ssniff, Germany) and tap water provided *ad libitum*. Red polycarbonate cylinders, aspen bedding, and aspen chewing blocks were provided for each cage as environmental enrichments.

2.2. Antibodies and chemicals

The following chemicals were purchased from Sigma-Aldrich (USA): 3,3'-diaminobenzidine (DAB, D5905), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB, D8130), 6-OHDA (H116), acetylcholine esterase (C2888-500UN), acetylthiocholine iodide (01480), mouse anti-GFAP antibody (G3893), rabbit anti-GSK-3 α/β antibody (SAB4501330), mouse anti-SYP-1 antibody (S6758), mouse anti-TH antibody (T2928), apomorphine (A4393-1G), DPX mountant (06522), Triton X-100 (X100), cresyl violet acetate (C5042), ethopropazine (E5406), Mayer's hematoxylin solution (MHS16), neostigmine bromide, radioimmunoprecipitation assay (RIPA) buffer (R0278), STZ (S0130). Copper sulfate (102790), metformin (317240), potassium hexacyanoferrate III (104973), and sodium citrate (106448) were purchased from Merck Millipore (USA). Rabbit anti-Iba-1 antibody (019-19741) was supplied by Wako (Japan). S-acetylthiocholine iodide (A16802) and nickel ammonium sulfate hexahydrate (12519) were purchased from Alfa Aesar (USA). The

following reagents were obtained from Abcam (USA): rabbit anti-GLUT-1 antibody (ab652), rabbit anti-GLUT-3 antibody (ab15311), and goat anti-rabbit immunoglobulins (ab205718). Collagenase type I (17018029), goat anti-mouse Ig (31430), goat anti-rabbit Ig (31460), No-Stain™ protein labeling reagent (A44449), phosphate-buffered saline (PBS, 10010031), Pierce™ ECL Western blotting substrate (32209) and SuperBlock™ blocking solution (37515) were supplied by ThermoFisher Scientific (USA). Dulbecco's modified Eagle medium (DMEM, F0415), amphotericin B (A2612), and penicillin/streptomycin mixture (A2212) were bought from Biochrom (Germany). Applichem (Germany) provided 1% bovine serum albumin (BSA) and protease inhibitor cocktail (A7779). MSC Nutrichem® XF was supplied by Biological Industries (Israel). 0.02% ascorbic acid (Asc) solution and sodium citrate buffer (CB, pH 4.5) was prepared *ex tempore*. Apomorphine was dissolved in 0.9% saline before the injections.

2.3. Experimental designs

2.3.1. EVs efficacy in PD model-rats (EVs I study)

Experimental design is shown in Figure 8. The rats were randomly divided into four groups (n=8):

- aCSF (intra-MFB) + PBS (i.n.);
- aCSF (intra-MFB) + EVs (i.n.);
- 6-OHDA (intra-MFB) + PBS (i.n.);
- 6-OHDA (intra-MFB) + EVs (i.n.).

Each EVs dose contained 2,67 x 10^7 vesicles (in 12 µl of PBS), thus overall, for 15 days each rat received a total of 180 µl EV solution containing approximately 40 × 10^7 EVs.

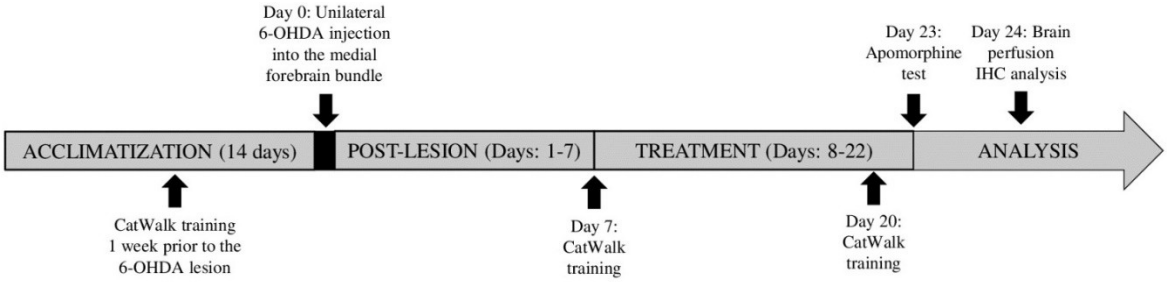


Figure 8. Experimental design of EVs efficacy in a PD model-rats study. 6-OHDA, 6-hydroxydopamine; IHC, immunohistochemistry.

2.3.2. EVs time-dependent efficacy in PD model-rats (EVs II study)

Experimental design of this study is shown in Figure 9. Rats were randomly divided into six groups. Accordingly, EVs, or phosphate buffered saline (PBS), as control were administered intranasally (i.n.) for 17 consecutive days (at 9:00 in the morning).

Experimental groups were following:

- Ascorbic acid, Asc (intra-MFB) + PBS (i.n.) (n=12);
- Asc (intra-MFB) + EVs (i.n.), n=8;
- 6-OHDA (intra-MFB) + PBS (i.n.), n=12;
- 6-OHDA (intra-MFB) + EVs (i.n.), n=12;
- 6-OHDA (intra-MFB) + PBS (i.n.) + 2-week non-treatment period (n=12);
- 6-OHDA (intra-MFB) + EVs (i.n.) + 2-week non-treatment period (n=12).

Each EVs dose contained 3 x 10^8 vesicles (in 10 µl of PBS), thus overall, for 17 days each rat received a total of 170 µl EV solution containing approximately 51 × 10^8 EVs.

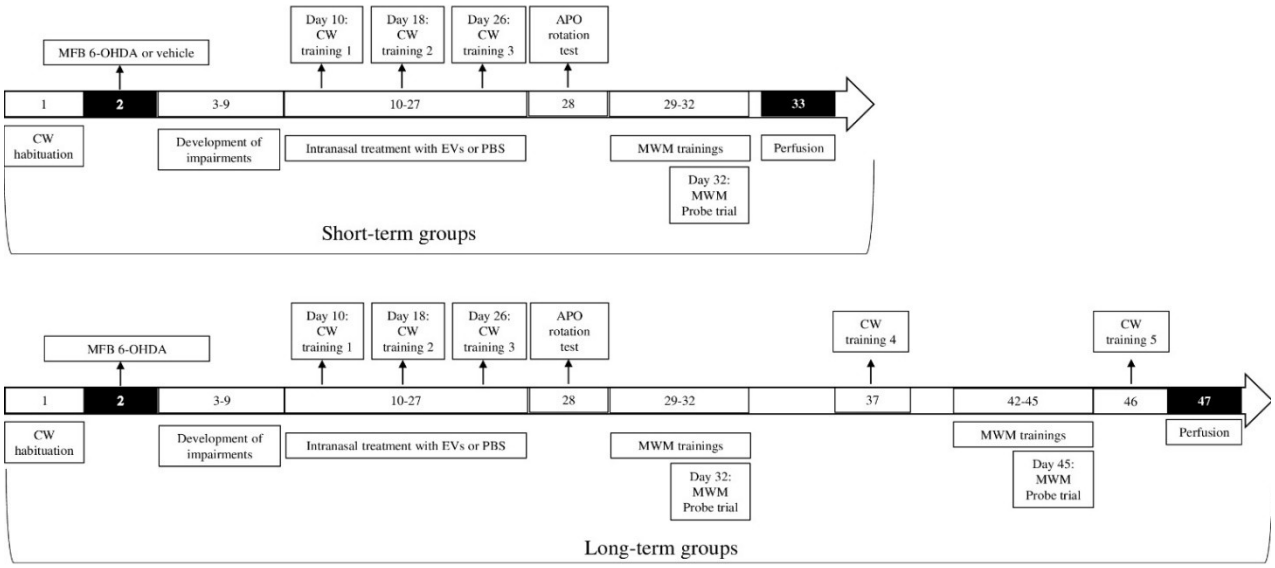


Figure 9. Experimental design of EVs time-dependent efficacy in PD model-rats. MFB, medial forebrain bundle; CW, CatWalk gait test; APO, apomorphine; MWM, Morris water maze.

2.3.3. Efficacy of metformin in sAD model-rats (METF study)

The experimental design is shown in Fig. 10. Briefly, rats were randomly assigned to one of the six experimental groups (n=9-10) and received either saline (1 ml/kg) or metformin (METF, 75 or 100 mg/kg) perorally (p.o.) using gastric gavage for 21 consecutive days starting 2 days after i.c.v. administration of STZ or citric buffer (CB, pH 4.0):

- CB i.c.v. + saline 1 ml/kg p.o. (control group, n=9)
- STZ i.c.v. + saline 1 ml/kg p.o. (lesion group, n=10)
- CB i.c.v. + metformin 75 mg/kg p.o. (n=9)
- CB i.c.v. + metformin 100 mg/kg p.o. (n=9)

- STZ i.c.v. + metformin 75 mg/kg p.o. (n=10)

- STZ i.c.v. + metformin 100 mg/kg p.o. (n=10)

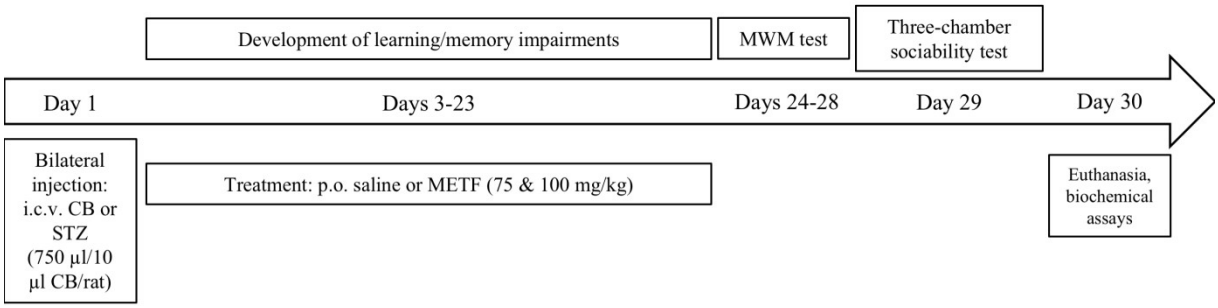


Figure 10. Experimental design of metformin (METF) study in sAD model-rats. CB, citrate buffer; STZ, streptozocin; METF, metformin; MWM, Morris water maze.

2.4. Stereotactic surgeries

2.4.1. Unilateral intra-MFB lesion

On experimental day 1 in the EVs I and EVs II studies, 30 min before the induction of general anesthesia, rats received imipramine (20 mg/kg) to protect adrenergic neurons against the development of 6-OHDA-induced lesions. The animals were anesthetized with isoflurane (3-3.5% for induction and 2% for maintenance) and placed on a stereotaxic frame (Stoelting Inc., USA). Unilateral 6-OHDA (20 µg in 3 µl of 0.2% ascorbic acid) or solvent (control, 5 µl) were injected intra-MFB during stereotaxic surgery using the following coordinates: -4.4 mm anteroposterior, +1.1 mm mediolateral, and -8.0 mm dorsoventral relative to the bregma, using a 27-gauge needle attached to a 50 µl microsyringe (Hamilton, USA). Injection flow was controlled using an electronic pump (WPI, USA) at a rate of 1 µl/min. The microsyringe needle was left in the injection site for 5 min after each injection to avoid drug reflux.

2.4.2. Bilateral intracerebroventricular lesion

On experimental day 1 of the METF study, surgery was performed for an i.c.v. injection of either STZ or CB. Briefly, animals were anesthetized with isoflurane (3 – 3.5% in 70% N_2O/30% O_2 for induction and 2% in 50% N_2O/50% O_2 for maintenance. Animals were then fixed on a stereotaxic frame (Stoelting Inc., USA). Two holes were drilled in the skull above both lateral ventricles using the following coordinates (Paxinos and Watson, 2007): –0.7 mm anterioposterior, 1.7 mm mediolateral and –4.0 mm dorsoventral relative to bregma. Using Hamilton microsyringe, STZ was injected bilaterally (750 µg/10 µl in CB for each rat) at 1 µl/min and 5 µl per ventricle. Control group received bilateral injections of CB (10 µl for each rat). Injection flow was controlled using an electronic pump (WPI, USA) at a rate of 1 µl per min. The microsyringe needle was left in the injection site for 5 min after each injection to avoid drug reflux. One day after the surgery, animals were allowed to rest for 24 h, and their condition was closely monitored.

2.5. *In vivo* assessments

2.5.1. Behavioral tests

2.5.1.1. Apomorphine- induced rotation test

Apomorphine-induced rotational behavior was used to evaluate the dopaminergic neuron lesion induced by 6-OHDA on experimental day 23 (EVs I study) and 28 (EVs II study). Apomorphine was dissolved in saline and injected subcutaneously at the dose of 0.2 mg/kg. After 5 min, the rotations were monitored for 30 min. The number of contralateral rotations (to the non-lesioned side) was recorded by examiner blinded to the groups. The animals that failed this test were excluded from the immunohistochemical assessment.

2.5.1.2. CatWalk (CW) gait test

The CW gait test is based on rodent voluntary movement through an enclosed 1.3 m long glass walkway that is illuminated with green fluorescent lighting the walkway from the side and reflecting internally after the rodent comes in contact with the glass floor. High-speed video camera was located under the walkway and was used to obtain footprint images. The rats were trained 7 days before 6-OHDA injection and on experimental day 7 in the EVs I study. In the EVs II study, rats were trained on experimental days 8 and 16. Before each training session, the animals were placed on the walkway and allowed to habituate for 2 min. Testing was performed on experimental day between 11:00 and 14:00, at least 1 hour after intranasal EVs administration. Testing was successful if the animal crossed the walkway without stopping, and at least 3-4 each paw placements were recorded. Three complete runs across the walkway were recorded for each animal. The software was used to automatically analyze the video images of the runs. Data analysis was performed with a threshold value set at 40 arbitrary units (range 0-225) per pixel. The following parameters were analyzed in EVs I and EVs II studied according to: temporal (stand duration), spatial parameters attributed to individual paws (duty cycle, %), relative spatial relationships between paws (stride length) and interlimb coordination (step cycle). Duty cycle is calculated according to the formula: stand/(stand+swing) x 100% and it represents the stand as a percentage of step cycle. In EVs TD study, swing speed and body speed were also analyzed.

2.5.1.3. Morris water maze (MWM) test

Morris water maze test trainings were used to determine rat spatial learning after the corresponding treatments: on experimental days 26-29 and 39-42 in the EVs II study, and on experimental days 24-27 in the METF study. The apparatus consisted of a blue circular tank (d=180 cm, h=75 cm) that was filled with water (23±1°C) to a depth that would cover the plexiglass platform (d=10 cm, h=25 cm) for 1 – 2 cm (Ugo Basile, Italy). Each animal underwent 4 trials (120 s for each trial) per day for 4 consecutive days. Animals were trained to find the hidden platform from different starting points in the pool. Rats were gently put in the water facing the wall of the pool. As soon as the animal found the platform, it was given 15 s to stay on it to learn the location. If the platform was not found, the animal was gently guided by the experimenter to the platform and left there for 15 s. An inter-trial period of 10 min was applied for animals between each of the 4 trainings on each day. Rat escape latency was registered as the time in seconds for each animal to find the submerged platform after being put in the pool. Swimming speed was recorded during trainings to evaluate rat motor activity. The probe trial was carried out on experimental day 29 and 42 in the EVs II study and on experimental day 28 in the metformin study. The platform was taken out of the pool and each animal could freely swim for 120 s. Time spent in the target quadrant, number of platform zone crossings, were documented. EthoVision XT 11.0 video tracking software (Noldus, The Netherlands) was used to register the parameters in the trainings and probe trial.

2.5.1.4. Three-chamber sociability test (SCT)

Three-chamber test was performed in the METF study on experimental day 29 to assess rat sociability and social novelty preference. The test was performed in an open-topped box made of clear polycarbonate (41 cm in length, 27 cm in width and 40 cm in height). The box contained 3 chambers of identical size with two clear polycarbonate walls that 10 cm high doorways. Each of the side chambers contained a cylinder made of cylindrical polycarbonate bars spaced 1 cm apart (height 20 cm; diameter 10 cm). All tested animals were habituated to the room and the box for 30 min. The test consisted of two trials: sociability and social novelty preference trial. In the sociability trial, the propensity of animals to interact with an unfamiliar rat was tested. Animals were put in the middle chamber and allowed to freely explore all chambers for 10 min. At the same time, a rat unfamiliar to the tested animal (novel rat 1) was put in the cylinder located in the left chamber. After the end of this trial, a waiting

period of 30 min, were implemented for each tested animal. During this period, novel rat 1 was returned to its cage. In the social novelty preference trial, the preference for novel social interaction was assessed. Tested animals were once again put in the center chamber and allowed to freely explore all chambers. In this case, the novel unfamiliar rat (novel rat 2) was positioned in the cylinder located in the right chamber that was previously empty. The following parameters were analyzed in both trials: 1) sniffing time near each cylinder (less than 1 cm apart) and 2) time spent in each chamber. All experiments were performed using a video recording device placed above the three-chamber box and coupled with an automated tracking program (EthoVision XT 11.5, Noldus, NL).

2.6. Ex vivo analyses

2.6.1. Immunohistochemical assessment

On experimental day 30, the rats were deeply anesthetized with intraperitoneally (i.p.) injected mixture of ketamine/xylazine (100 mg/kg/10 mg/kg, respectively) mixture, transcardially perfused with ice cold saline and fixed with cold 4% paraformaldehyde (PFA) solution. For METF study, right brain hemispheres were removed and post-fixed in 4% PFA for 24 h. After fixation, the right brain hemispheres were placed in 30% sucrose-containing solution for 48 h for cryoprotection and subsequently placed in an antifreeze solution.

For each rat brain, 30 μm thick coronal slices were obtained using a cryotome at – 26°C (CM1850, Leica Biosystems, USA). The slices were incubated in CB (pH 6.0) at 95°C for 10 min to improve antigen retrieval, subsequently cooled to room temperature and blocked with a SuperBlock® solution for 1 h to decrease backstain formation. Free-floating sections were then stained with the corresponding primary antibody (1:1000). The sections were transferred to a solution containing the primary antibody in phosphate-buffered saline (PBS) with 0.5% Triton X-100 (PBS-T). After 18 h incubation, the sections were rinsed 3 times with PBS-T and transferred to a solution containing the secondary antibody (dilution 1:5000). After 2 h incubation with the secondary antibody, the sections were rinsed 3 times with PBS-T. After rinsing with PBS-T, the sections were incubated with PBS containing DAB, 30% H_2O_2 and 1% nickel ammonium sulfate for 1-2 min. For Iba-1, the sections were also incubated in hematoxylin solution for 1 min to obtain counterstain. All stained sections were mounted on slides (3 sections on each slide) and coverslipped using DPX mountant for histology. In order to obtain similar staining, the sections from all groups were always stained simultaneously in

the same tray. Optical density of protein staining was expressed in arbitrary units (a.u.). All experiments were done in triplicate.

2.6.2. Histochemical assessment

Histochemical detection of acetylcholine esterase-containing nerve axon density was performed using a previously described method (Karnovsky and Roots, 1964), which was optimized and described elsewhere (Kadish and Van Groen, 2002). Briefly, for each right brain hemisphere, 6 coronal slices per animal (30 µm thick, AP plane: Bregma −2.92 mm to −3.48 mm in the same animal) were obtained. Obtained brain sections (n=5 brain samples per group) were rinsed with a 0.1 M maleate buffer (pH 6.0). Subsequently, the sections were incubated with 0.1 M maleate buffer containing 86.5 mM S-acetylthiocholine iodide, 30 mM ethopropazine, 30 mM copper sulfate, and 100 mM sodium citrate and 0.03 mM potassium hexacyanoferrate for 2 h. Subsequently, the staining was intensified by incubating the sections with 2.5% DAB, 0.2% hydrogen peroxide, and 5% nickel ammonium sulfate for 2 min in 0.05 M Tris buffer (pH 7.6) for 2 min. Optical density of protein staining was expressed in arbitrary units (a.u.). All experiments were done in triplicate.

Nissl body staining was performed using 0.1% cresyl violet acetate solution. 30 µm thick tissue slices were transferred onto gelatinated slides and air-dried at 37°C. Slides were then rinsed twice for 5 min with PBS and deionized in H_2O for 1 min. Sections were then stained with 0.1% cresyl acetate solution for 20 min in dark. Stained sections were rinsed with deionized water twice for 5 min to remove excess stain and further differentiated by wash in 70% ethanol until desirable staining was achieved. Then, slides were dehydrated in 90% and 96% ethanol. Finally, slides were cleared with xylene 3 times for 3 min each and coverslips were mounted onto the slides using DPX mountant.

2.6.3. Western blot analysis

After rats were euthanized and brains were fixed with ice-cold 4% paraformaldehyde, left brain hemispheres were stored at –80 °C. Tissue samples (cortex, hippocampus, and cerebellum separately) were homogenized in a SpeedMill Plus Homogenizer (Analytic Jena, USA) in an appropriate amount (based on tissue amount) of RIPA lysis buffer and protease inhibitor cocktail according to the manufacturer's instructions. Equal amounts of proteins (20

μg) were then separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequently blotted onto polyvinylidene difluoride (PVDF) membranes. After blocking for 1 h at room temperature in SuperBlock™ solution, the blots were incubated overnight at room temperature with the following primary antibodies: anti-GLUT-1 (1:1000), anti-GLUT-3 (1:1000), anti-GSK-3 (1:500) and anti-β-actin (1:1000). The blots were then washed with 0.1% Tris buffered solution with Tween-20 and incubated with the corresponding secondary antibody: horseradish-conjugated goat anti-mouse (1:1000) or goat anti-rabbit IgG (1:5000). Blots were incubated with a protein labeling reagent (No-Stain™) for 10 min, then washed 5 times with distilled water. Protein bands were visualized by enhanced chemiluminescence using Pierce ECL Western blotting substrate kit. All experiments were done in triplicate.

2.6.4. Quantification of ex vivo data

Stained and immunostained slides were digitally scanned with the whole slide Pannoramic MIDI II scanner (3DHISTECH Ltd., Hungary) using a 20 × microscope objective. Pannoramic Viewer 1.15.2 software (3DHISTECH Ltd., Hungary) was used to obtain the images of the retrosplenial cortex, *stratum radiatum* of hippocampal *cornu ammonis* 1 and 3 (CA1 and CA3), as well as dentate gyrus (DG) granule cell layer. The optical densities of studied proteins were then measured using these images. Quantification of immunohistochemical, histochemical and Western blot data was done using open source image-processing software (ImageJ, Germany) by a person blinded to the experimental groups.

2.7. *In vitro* analysis

2.7.1. Proteomic analysis

Liquid chromatography-mass spectrometry (LC–MS/MS) analysis was performed by prof. Augustas Pivoriunas's team. LC–MS/MS analysis was performed at the Proteomics Unit, Institute of Biotechnology, University of Helsinki, Finland.

This method is described elsewhere (Narbute et al., 2019).

2.7.2. Inhibition of AChE activity

The acetylcholinesterase inhibitory activity of metformin was evaluated using spectrophotometric method (Ellman et al., 1961) with slight modifications. Neostigmine

bromide was used as positive reference. A reaction mixture consisted of samples containing 100 µl of 0.1 M phosphate buffer (pH 7.4), 10 µl of acetylcholine esterase solution (2 U/ml) and 10 µl of metformin or neostigmine bromide at different concentrations (0.01, 0.1, 0.2, 1, 5 and 10 mM). This mixture was co-incubated in the dark at 250 °C for 5 min in a 96-well plate. Next, 50 µl of 0.75 mM DTNB were added, and the plate was incubated for 20 min. Then, 15 µl of 1.5 mM acetylthiocholine iodide were added and incubated for 5 min in the dark. The absorbance was measured after 10 min at a wavelength of 405 nM using ELx808™ microplate reader (BioTek Instruments Ltd., UK).

2.8. Statistical analysis

All data are presented as mean values ± standard deviation (S.D.) of at least 2 independent experiments. Morris water maze training data were analyzed using 2-way analysis of variance (ANOVA) with repeated measures to account for inter-group variations (with group and training day as factors), followed by Holm-Sidak's multiple comparisons test. Morris water maze probe trial, three-chamber sociability, and social novelty preference test data, as well as the quantitative immunohistochemical, histochemical and Western blot data were analyzed using one-way ANOVA followed by Holm-Sidak's multiple comparisons test. Statistical significance was set at $p \leq .05$.

3. RESULTS

3.1. Effects of EVs on rat behavior

3.1.1. In the Morris water maze (MWM) test

Rat spatial learning and memory performances in the Morris water maze test are shown in Fig. 11. On post-treatment days 3-5 (Fig. 11A), administration of 6-OHDA resulted in longer escape latency on post-treatment days 3 and 4 ($p \leq .0001$), as well as 5 ($p \leq .05$) compared to controls, while EVs treatment shortened 6-OHDA-treated rats' escape latency on these days to control group values ($p \leq .001$ on post-treatment day 3, $p \leq .0001$ on post-treatment day 4 and $p \leq .01$ on post-treatment day 5). In the probe trial on post-treatment day 5, 6-OHDA-injected rats spent less time in platform quadrant ($p \leq .01$, Fig. 11C) and crossed platform zone considerably less than control ($p \leq .05$, Fig. 11E), whereas EVs treatment increased both of these parameters to control group values ($p \leq .01$ for time in platform quadrant and $p \leq .05$ for platform zone crossings).

On post-treatment days 15-18 (Fig. 11B), escape latencies of 6-OHDA-injected rats were also longer compared to controls ($p \leq .0001$). However, EVs-treated rats did not show any changes in escape latencies when compared to 6-OHDA group rats on these days. In the probe trial on post-treatment day 18, 6-OHDA-injected rats showed less time spent in the platform quadrant ($p \leq .0001$, Fig. 11D) and markedly less platform zone crossings (Fig. 11F) compared to controls. Treatment with EVs failed to produce changes in both parameters.

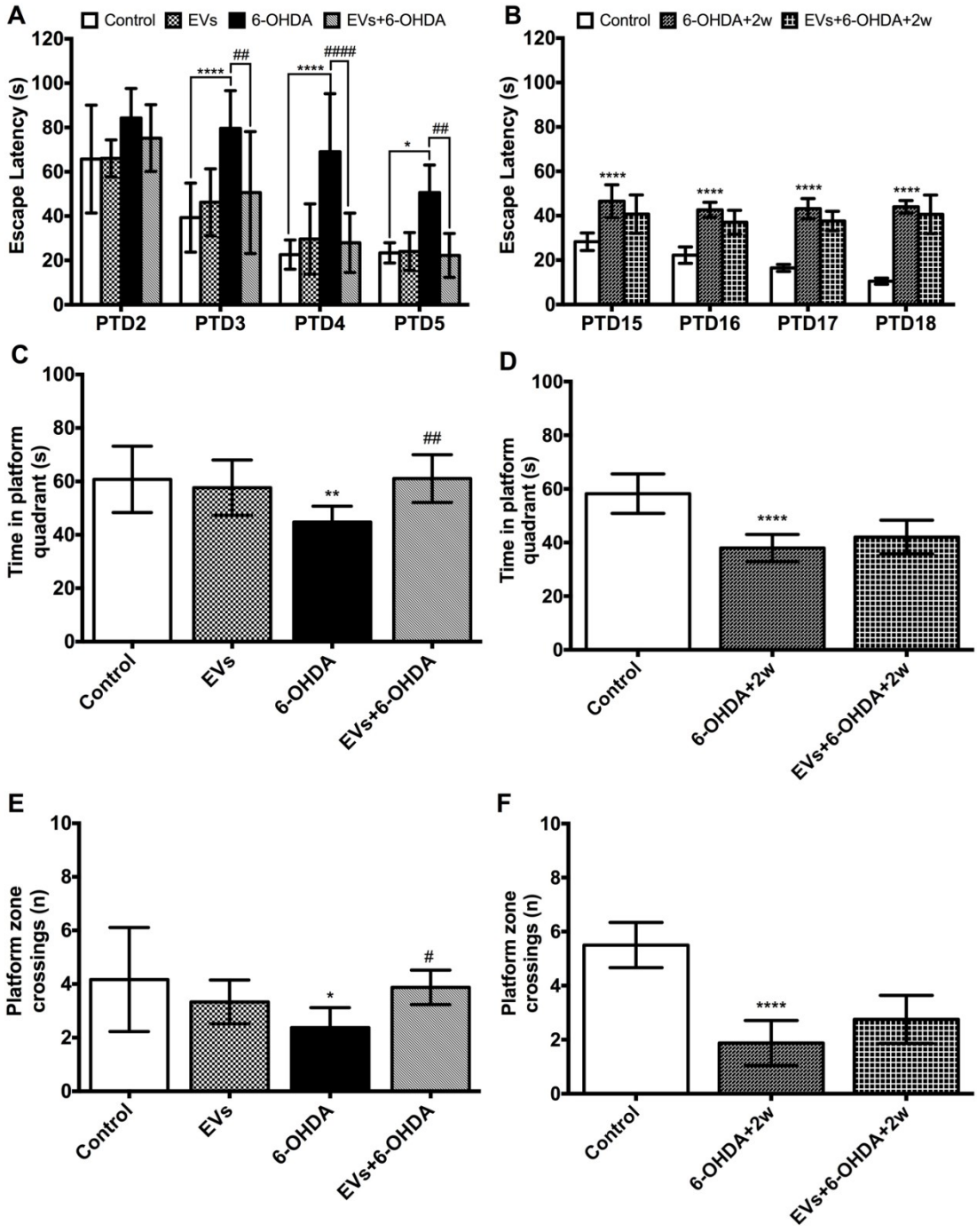


Figure 11. The effects of extracellular vesicles (EVs) on rat performance in the MWM test. Rat spatial learning was assessed on post-treatment days (PTD) 2-5 (A) and PTD15-18 (B), as well as on spatial memory on PTD5 (C-E) and PTD18 (D-F). *$p \leq .05$, **$p \leq .01$, ***$p \leq .001$ and ****$p \leq .0001$ vs. Control; #$p \leq .05$, ##$p \leq .01$, ###$p \leq .001$ and ####$p \leq .0001$ vs. 6-OHDA.

3.1.2. In the CatWalk gait test

We observed gait impairments in the CatWalk test 1 (treatment day 17, Fig.12A-B), CatWalk test 2 (post-treatment day 10, Fig.12C-D). In CatWalk test 3 (post-treatment day 10, Fig.12E-F), all gait parameters did not differ between groups. Injection of 6-OHDA prolonged the stand time of all paws except right front (RF) ($p \leq .05$, Fig. 12A) on treatment day 9, and of all paws on post-treatment day 10 ($p \leq .05$, Fig. 12C) compared with the control group. Rats from the 6-OHDA group that received EVs treatment demonstrated shorter stand time of all paws on treatment day 9 ($p \leq .05$ for right and $p \leq .01$ for left paws) and on post-treatment day 10 ($p \leq .01$). The 6-OHDA injection also decreased the stride length of all paws on treatment day 9 ($p \leq .05$, Fig. 12B) and all paws on post-treatment day 10 ($p \leq .05$, Fig. 12D). In the EVs-treated 6-OHDA rats on treatment day 9, stride length of the right hind (RH) paw ($p \leq .05$) and left front (LF) paw ($p \leq .05$) was increased compared with the 6-OHDA group, whereas on post-treatment day 10, stride length was increased in all paws ($p \leq .05$ for the left paws, $p \leq .01$ for the RF and $p \leq .001$ for the RH paws).

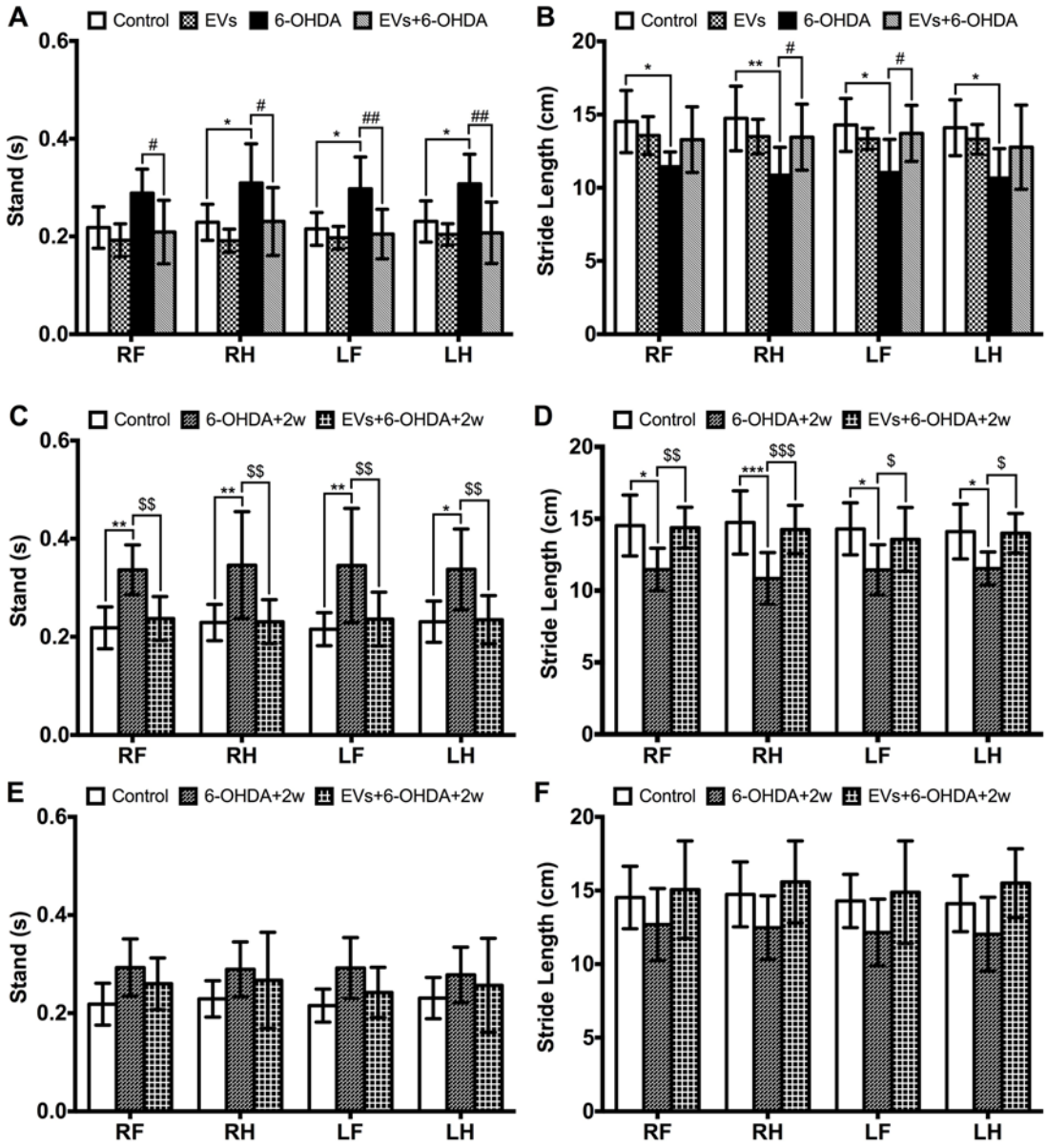


Figure 12. Effects of extracellular vesicles (EVs) on rat stand and stride length parameters in the CatWalk test. Gait parameters were assessed on treatment day 9 (A-B), post-treatment day 10 (C-D), and on post-treatment day 20 (E-F). RF – right front paw, RH – right hind paw, LF – left front paw, LH – left hind paw. $^{*}p \leq .05$, $^{**}p \leq .01$ and $^{***}p \leq .001$ vs. Control; $^{\#}p \leq .05$ and $^{\#\#}p \leq .01$ and $^{\#\#\#}p \leq .001$ vs. 6-OHDA; $^{\$}p \leq .05$, $^{\$\$}p \leq .01$ and $^{\$\$\$}p \leq .001$ vs. 6-OHDA+2w.

6-OHDA injection resulted in longer step cycle of all paws ($p \leq .0001$ for the RF, RH, $p \leq .01$ for the LF and $p \leq .001$ for the LH paw, Fig. 13A) on treatment day 9 as well as on post-treatment day 10 ($p \leq .05$ for the RF and RH, $p \leq .01$ for the LH, and $p \leq .001$ for the LF paw, Fig. 13C) compared with the control group. Intranasal EVs administration resulted in

remarkably faster step cycle on treatment day 9 ($p \leq .0001$ for the RF, RH; $p \leq .01$ for the LF and LH paws) and on post-treatment day 10 ($p \leq .05$ for the RH, LH and $p \leq .01$ for the LF paws). The 6-OHDA group rats showed an increase in the duty cycle on treatment day 9 (Fig. 13B) of the paws: RF and LF ($p \leq .01$), RH ($p \leq .05$), and LH ($p \leq .001$), on post-treatment day 10 (Fig. 13D) of paws: RF, LF and LH ($p \leq .01$). The EVs treatment reduced duty cycle parameter of the paws: RF and LH ($p \leq .0001$), LF ($p \leq .001$), and RF ($p \leq .01$) on treatment day 9 and reduced this parameter on post-treatment day 10 of LF ($p \leq .001$), RF ($p \leq .01$), RH and LH ($p \leq .05$) paws.

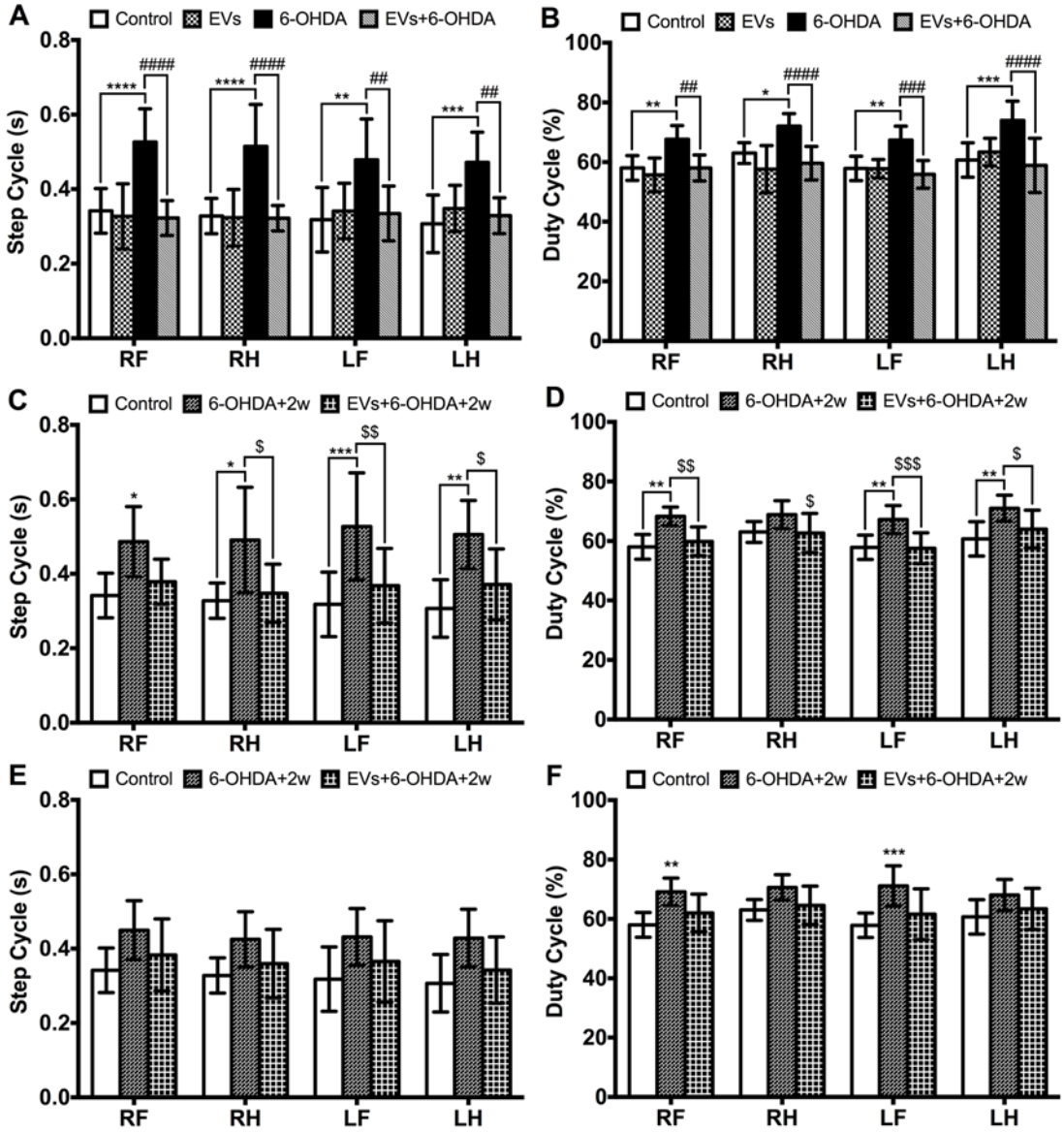


Figure 13. Effects of extracellular vesicles (EVs) in the CatWalk gait test. Gait parameters were assessed on treatment day 9 (A-B), post-treatment day 10 (C-D), and on post-treatment day 20 (E-F). RF – right front paw, RH – right hind paw, LF – left

front paw, LH – left hind paw. $^{*}p \leq .05$, $^{**}p \leq .01$, $^{***}p \leq .001$ and $^{****}p \leq .0001$ vs. Control; $^{\#\#}p \leq .01$, $^{\#\#\#}p \leq .001$ and $^{\#\#\#\#}p \leq 0.001$ vs. 6-OHDA; $^{\$}p \leq .05$, $^{\$\$}p \leq .001$ and $^{\$\$\$}p \leq .0001$ vs. 6-OHDA+2w.

The 6-OHDA group rats showed an increase in the swing speed on treatment day 9 (Fig. 14A) of the paws: RF, RH ($p \leq .05$) and LF ($p \leq .01$) and on post-treatment day 10 (Fig. 14C) of all paws ($p \leq .01$ for RF and LH paws, $p \leq .001$ for RH and LF paws). EVs therapy increased swing speed on post-treatment day 10 of paws: RF ($p \leq .01$), RH ($p \leq .001$), LF ($p \leq .05$) and LH ($p \leq .0001$). 6-OHDA also induced changes in body speed in RH and LH on treatment day 9 ($p \leq .05$, Fig. 14B) and on post-treatment day 10 of all paws ($p \leq .001$ for RF and LF paws, $p \leq .0001$ for RH and LH paws, Fig. 14D). EVs therapy increased body speed of 6-OHDA injected rats on post-treatment day 10 of all paws ($p \leq .01$).

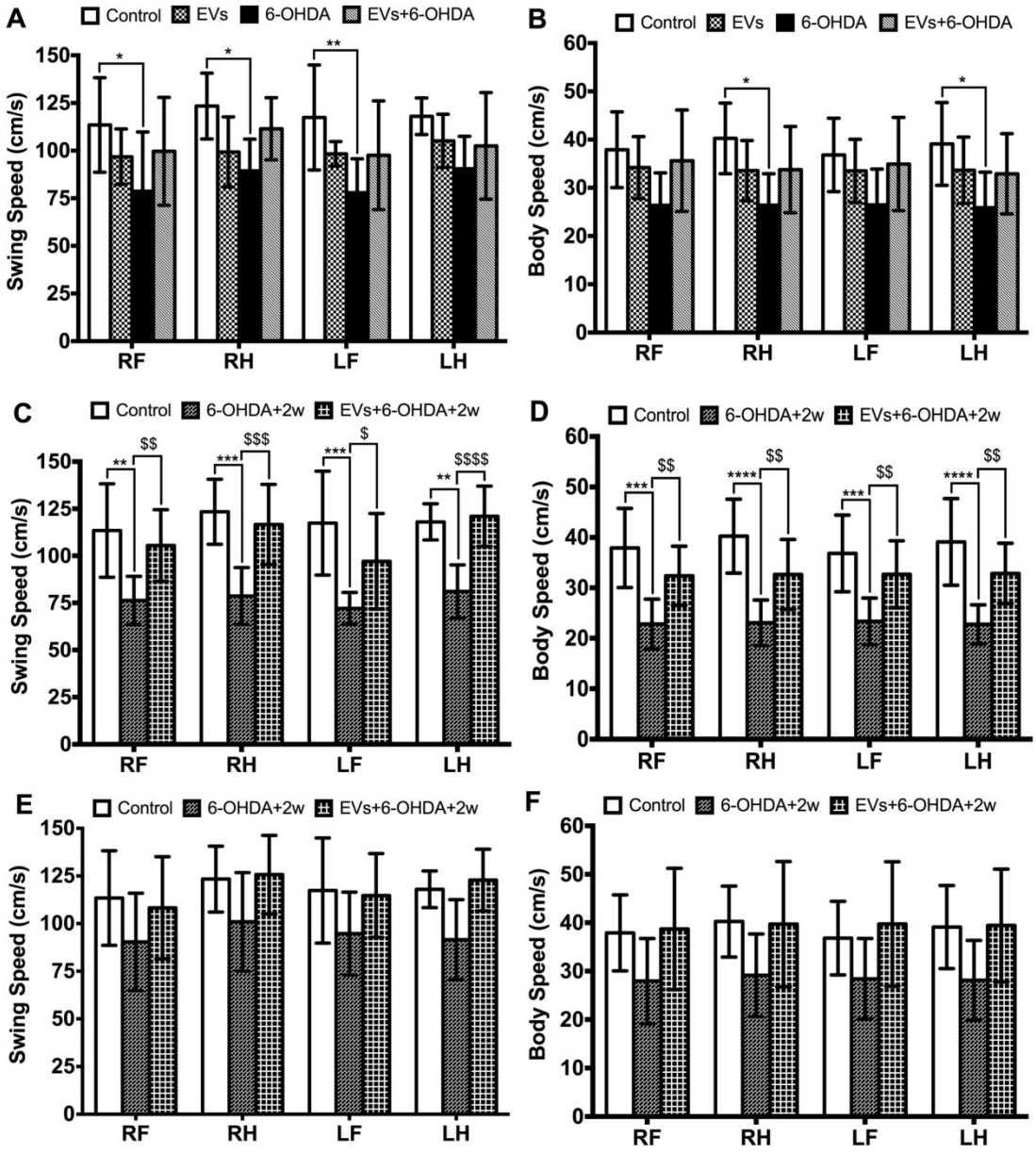


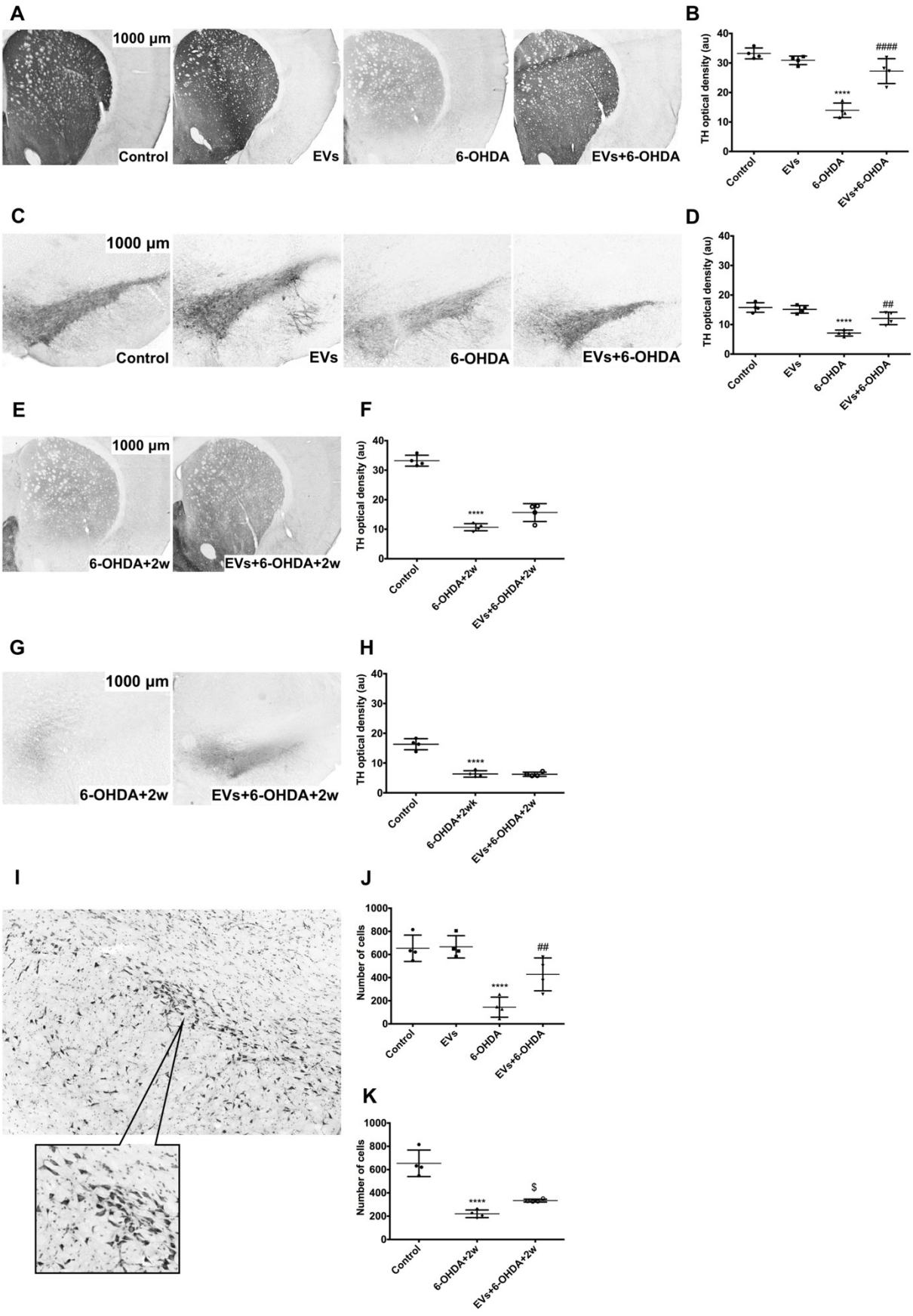
Figure 14. Effects of extracellular vesicles (EVs) in the CatWalk gait test. Gait parameters were assessed on treatment day 9 (A-B), post-treatment day 10 (C-D), and on post-treatment day 20 (E-F). RF – right front paw, RH – right hind paw, LF – left front paw, LH – left hind paw. $^{*}p \leq .05$ and $^{**}p \leq .01$ vs. Control; $^{\$}p \leq .05$, $^{\$\$}p \leq .01$, $^{\$\$\$}p \leq .001$ and $^{\$\$\$\$}p \leq .0001$ vs. 6-OHDA+2w.

3.2. Effects of EVs on rat brain protein expression

3.2.1. TH density and Nissl body count

Rats that were injected with 6-OHDA demonstrated a remarkable (by 50%) decrease in TH density in the striatum on post-treatment days 6 ($p \leq .0001$, Figs. 15A and 15B) and 20 (p

$\leq .0001$, Figs. 15E and 15F) compared to controls; similar 6-OHDA effects were observed in the SN ($p \leq .0001$, Figs. 15C, 15D and 15G, 15H, respectively). Treatment of 6-OHDA-injected rats with EVs increased TH density on post-treatment day 6 in the striatum ($p \leq .0001$) and in the SN ($p \leq .001$) to control group values, while no differences in TH density were observed on post-treatment day 20. Furthermore, 6-OHDA-induced lesion produced a decrease in Nissl body count in the SN on post-treatment days 6 and 20 ($p \leq .0001$, Figs. 15J and 15K, respectively) compared to control group values, while this decrease was fully reversed by EV on post-treatment days 6 ($p \leq .01$) and partially on post-treatment day 20 ($p \leq .05$).


A
1000 µm
Control
EVs
6-OHDA
EVs+6-OHDA
B
TH optical density (au)
Control
EVs
6-OHDA
EVs+6-OHDA
C
1000 µm
Control
EVs
6-OHDA
EVs+6-OHDA
D
TH optical density (au)
Control
EVs
6-OHDA
EVs+6-OHDA
E
1000 µm
6-OHDA+2w
EVs+6-OHDA+2w
F
TH optical density (au)
Control
6-OHDA+2w
EVs+6-OHDA+2w
G
1000 µm
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EVs+6-OHDA+2w
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J
Number of cells
Control
EVs
6-OHDA
EVs+6-OHDA
K
Number of cells
Control
6-OHDA+2w
EVs+6-OHDA+2w

Figure 15. Microphotographs and densitometry bars showing the effects of extracellular vesicles (EVs) on the nigrostriatal optical density of tyrosine hydroxylase (TH) staining and Nissl body counts. TH density on post-treatment day 6 in the striatum is demonstrated in (A) and substantia nigra in (C); on post-treatment day 20 – in the striatum (E) and substantia nigra (G). 2-week period after EVs treatment discontinuation – 2w. Nissl body staining in shown in the substantia nigra region at 250 × (I) and 600 × magnification in the inserted square below image (I). Correspondingly to the microphotographs, densitometry bars show optical density of TH in the striatum (B, F), substantia nigra (D, H) and Nissl body count on post-treatment day 6 (J) and 20 (K). $^{***}p \leq .0001$ **vs. Control;** $^{\#\#}p \leq .01$ **and** $^{\#\#\#\#}p \leq .0001$ **vs. 6-OHDA;** $^{\$}p \leq .05$ **vs. 6-OHDA+2w.**

3.3. Effects of metformin on rat behavior

3.3.1. In the Morris water maze (MWM) test

In the MWM trainings, STZ rats showed escape latency prolongation on training day 2 ($p = 0.0051$ vs. Control) and days 3-4 ($p \leq 0.0001$ vs. Control), as shown in Figure 16A. In STZ rats treated with 75 mg/kg metformin, shortening of escape latency was observed on training day 2 ($p = 0.0004$ vs. STZ), day 3 ($p = 0.0039$ vs. STZ) and day 4 ($p = 0.0005$ vs. STZ). STZ rats that were treated with 100 mg/kg metformin also displayed shorter escape latency on training day 2 ($p = 0.0056$ vs. STZ), day 3 ($p = 0.0185$ vs. STZ) and day 4 ($p = 0.0049$ vs. STZ). Metformin *per se* did not alter escape latency of control animals during all training days. Swimming speed was not altered between groups on either of the training days (Fig. 16B).

In the probe trial, STZ rats demonstrated aggravated spatial memory: they spent shorter time in the target quadrant ($p = 0.002$ vs. Control, Fig. 16C) and crossed the platform zone less frequently than the controls ($p = 0.0053$, Fig. 16D). STZ rats treated with metformin at 75 mg/kg showed an increase in the time spent in the quadrant where the platform was previously located ($p = 0.0031$ vs. STZ). Metformin treatment with 100 mg/kg also resulted in STZ rats spending longer time in the target quadrant ($p = 0.0018$ vs. STZ). Furthermore, at both doses, metformin increased the number of platform zone crossings of STZ rats ($p \leq 0.0001$ vs. STZ). Treatment of control rats with metformin did not produce significant changes in the probe trial parameters.

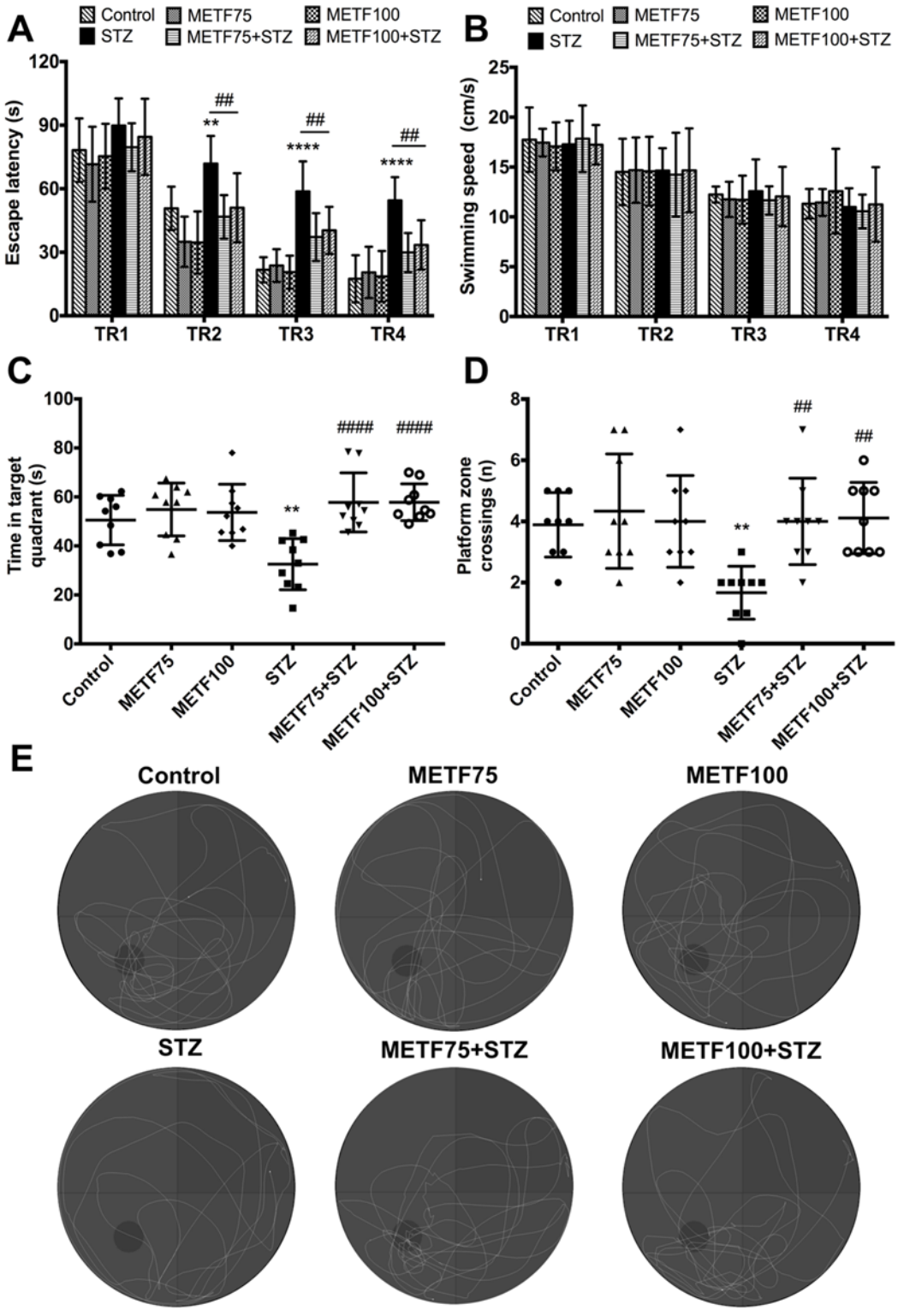

Figure 16. Effects of metformin (METF) on rat brain escape latency (A), swimming speed (B), time in target quadrant (C) and platform zone crossings (D). Tracking plots of the probe trial are shown in (E). *****p* **< 0.01 and *******p*** **< 0.0001 vs. Control; ##*p* < 0.01 and #### *p*< 0.0001 vs. STZ.**

3.3.2. In the 3-chamber sociability test

In the sociability trial, time spent with the unfamiliar rat altered between and inside groups (Fig. 17A). Within all groups, except STZ, rats spent longer time in the chamber with the unfamiliar rat than in the empty chamber: $p \leq 0.0001$ in the control, METF75, METF100, METF75+STZ and METF100+STZ groups. STZ-injected rats spent similar time in the chamber with the unfamiliar rat and in the empty chamber. The amount of time spent in the chamber with the unfamiliar rat was also shorter in STZ group rats compared to control group animals ($p \leq 0.005$). Metformin-treated STZ rats, however, spent longer time in the chamber with the unfamiliar rat than STZ group animals ($p = 0.0141$).

The number of sniffing in the sociability trial (shown in Fig. 17B) also differed within all groups. Rats sniffed the cylinder with the unfamiliar rat more frequently in the control ($p < 0.0001$), METF75 ($p = 0.0004$), METF 100 ($p = 0.0002$), METF75+STZ ($p \leq 0.0001$) and METF100+STZ ($p = 0.0005$) groups. STZ group rats had higher numbers of empty cylinder sniffing when compared to sniffing the unfamiliar rat ($p = 0.0044$) and to the control group rats ($p = 0.0036$). Metformin-treated STZ-injected rats sniffed the unfamiliar rat more frequently than STZ-injected animals ($p = 0.0035$ in METF75+STZ and $p = 0.0074$ in METF100+STZ).

Similar results were also seen in the social novelty preference trial. Longer periods of time were spent with the unfamiliar rat in the control ($p \leq 0.0001$), METF75 ($p = 0.0025$), METF100 ($p= 0.0017$), METF75+STZ ($p=0.0052$) and METF100+STZ ($p = 0.0068$) groups (Fig. 17C). In the STZ group, rats spend less time with the unfamiliar rat than with the familiar one ($p \leq 0.0001$), also when compared to controls ($p \leq 0.0001$). Metformin-treated STZ rats spent more time with the unfamiliar rat than STZ animals ($p = 0.0002$ in METF75+STZ and $p \leq 0.0001$ in METF100+STZ) compared to the STZ group rats.

As shown in Figure 17D, rats sniffed the unfamiliar rat more frequently than the familiar one in all groups except STZ: $p < 0.0001$ in the control, $p = 0.0002$ in METF75, $p \leq 0.0001$ in METF 100, $p = 0.0098$ in METF75+STZ and $p = 0.0007$ in METF100+STZ group. STZ group rats showed lower counts of unfamiliar rat sniffing ($p \leq 0.0001$) compared to controls. Higher count of unfamiliar rat sniffing was documented in METF75+STZ ($p= 0.0398$ vs. STZ) and METF100+STZ ($p = 0.0002$ vs. STZ).

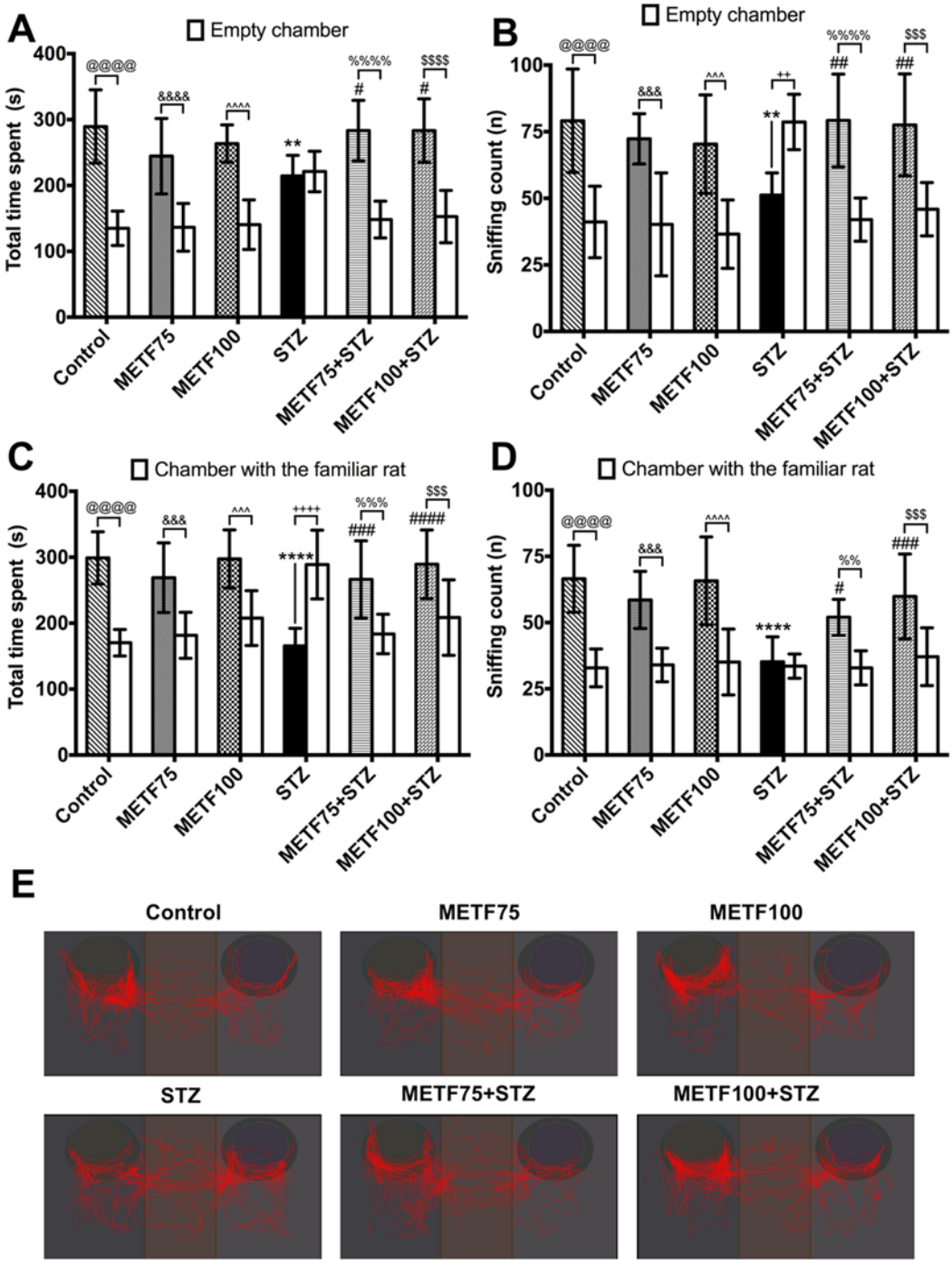

Figure 17. Effects of metformin (METF) on rat brain sociability (A, B) and social novelty preference (C, D). Blank columns show total time spent in the empty chamber (A), sniffing count of cylinder in the empty chamber (B), total time spent in the chamber with the familiar rat (C) and sniffing count of cylinder where the familiar rat was present (D). Tracking plots of the sniffing count in social novelty trial are shown in (E). *****p* **< 0.01 and *******p*** **< 0.0001 vs. Control; #*p* < 0.05, ##*p* < 0.01, ###*p* < 0.001 and ####*p* < 0.0001 vs. STZ; @@@@*p* < 0.0001 within Control group; &&&*p* < 0.001 and**

&&&&*p* < 0.0001 within METF75 group; ^^^*p* < 0.001 and ^^^^*p* < 0.0001 within METF100 group; ++*p* < 0.01 and ++++*p* < 0.0001 within STZ group; %%*p* < 0.01, %%%*p* < 0.001 and %%%%*p* < 0.0001 within METF75+STZ group; $$$*p* < 0.001 and $$$$*p* < 0.0001 within METF100+STZ group.

3.4. Effects of metformin on rat brain protein density

3.4.1. GFAP density

Figure 18 demonstrates the effects of metformin on GFAP density. No differences between groups were detected in cortical GFAP density. In STZ rats, increased GFAP density was observed in CA1 ($p = 0.0002$), CA3 ($p = 0.0068$) and DG ($p = 0.0008$) regions compared to the controls. Metformin treatment decreased the density of GFAP in the STZ rat CA1 ($p = 0.0022$ in METF75+STZ and $p = 0.0358$ in METF100+STZ), did not alter it in the CA3 and decreased it in the DG ($p= 0.0015$ in METF75+STZ).

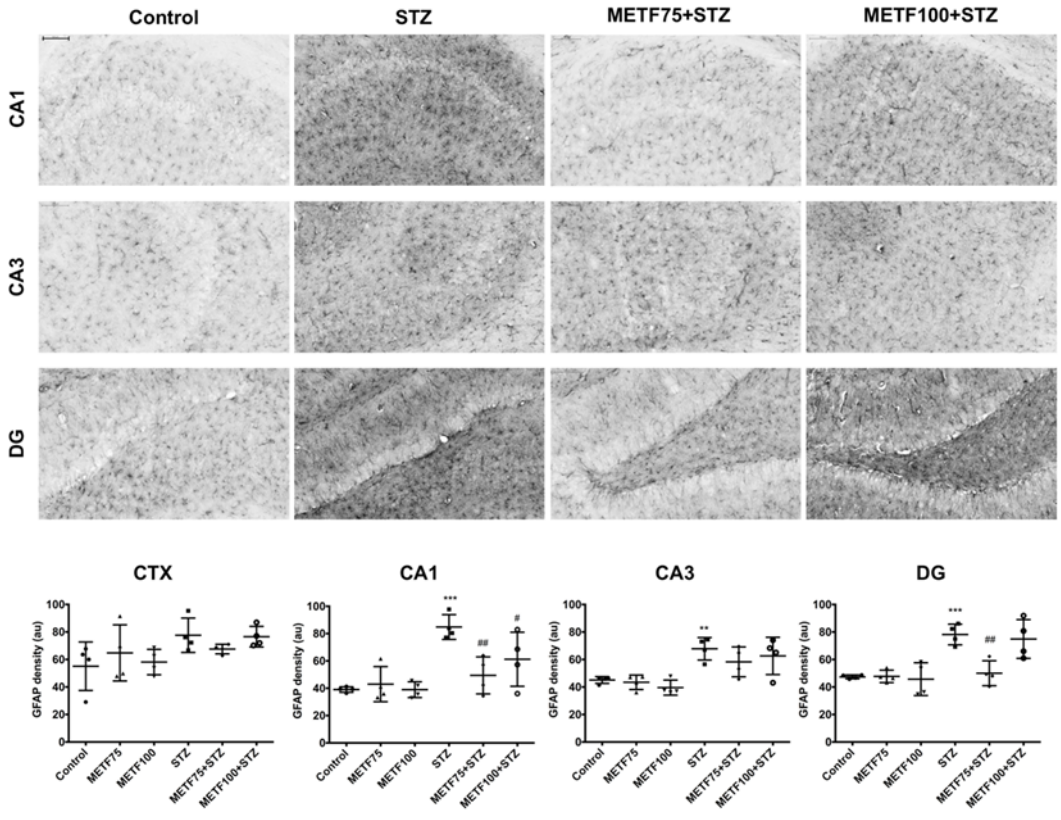

Figure 18. Effects of metformin (METF) on rat brain density of glial fibrillary acidic protein (GFAP) in the retrosplenial cortex (CTX) and hippocampal *cornu ammonis* (CA) 1, CA3 and dentate gyrus (DG) regions. Representative

microphotographs depict regions where significant differences were obtained after density measurements (200 × magnification). Scale bar is 100 µm. $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$ vs. Control; $^{\#}p \leq 0.05$ and $^{\#\#}p \leq 0.01$ vs. STZ.

3.4.2. Iba-1 positive cells

Injection of STZ produced a marked rise in cortical Iba-1-positive cells ($p \leq 0.0001$) compared to the controls (Figure 19). In STZ rats treated with metformin, the number of Iba-1-positive cells was reduced to control group values ($p \leq 0.0001$). Similarly, administration of STZ resulted in a higher amount of Iba-1-positive microglial cells in the CA1 ($p = 0.0006$), CA3 ($p = 0.0024$) and DG ($p = 0.0284$) regions of the STZ group rat hippocampi. Compared to the STZ group, the number of hippocampal Iba-1-positive cells was lower of rats treated with metformin at 75 mg/kg ($p = 0.0006$ in CA1, $p = 0.0005$ in CA3 and in $p = 0.0057$ DG). At 100 mg/kg, metformin normalized Iba-1-positive cell number in the DG of STZ group rats ($p = 0.0284$).

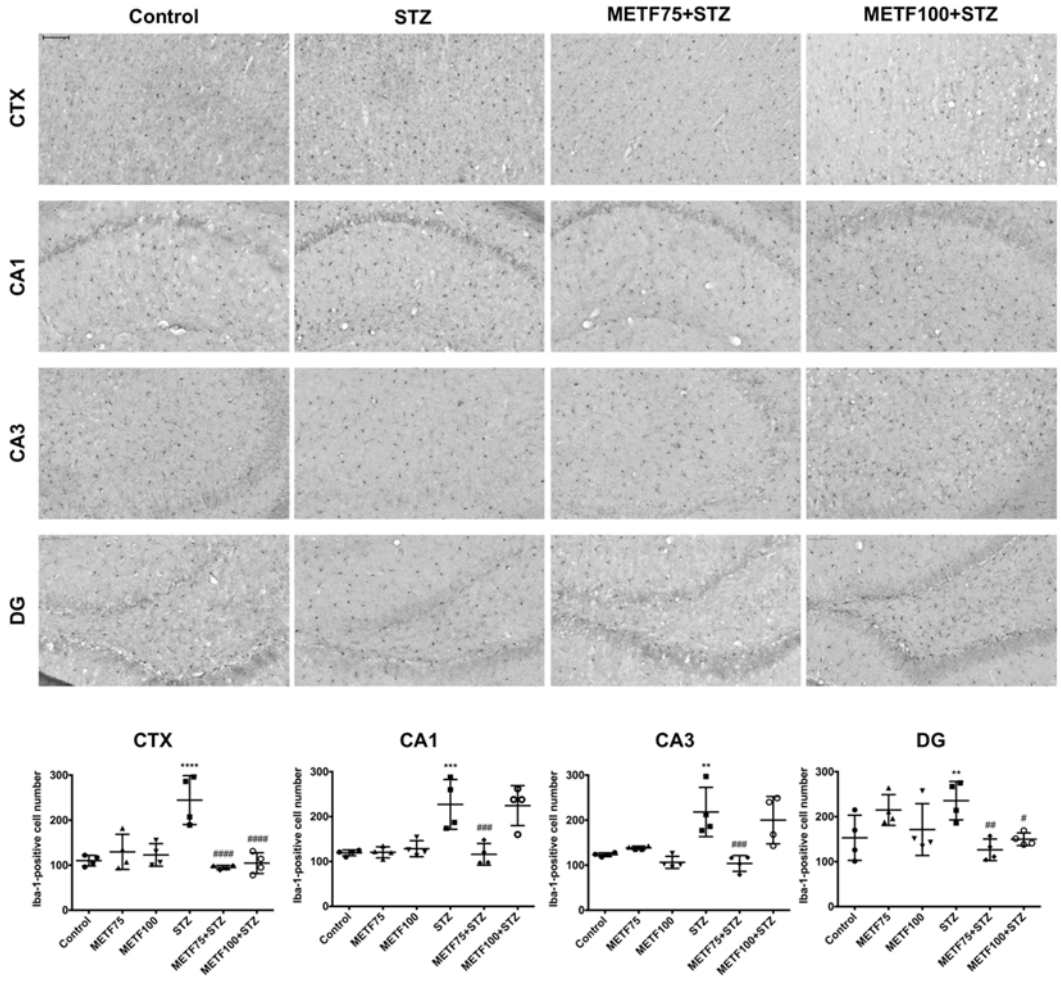

Figure 19. Effects of metformin (METF) on rat brain ionized calcium-binding protein (Iba-1)-positive cell number in the retrosplenial cortex (CTX) and hippocampal *cornu ammonis* (CA) 1, CA3 and dentate gyrus (DG) regions. Representative microphotographs depict regions where significant differences were obtained after cell counting (200 × magnification). Scale bar is 100 µm. $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$ vs. Control; $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$, $^{\#\#\#}p \leq 0.001$ and $^{\#\#\#\#}p \leq 0.0001$ vs. STZ.

3.4.3. Effects of metformin on synaptic density

Administration of STZ resulted in decreased SYP-1 density in the cortex ($p = 0.0092$) and hippocampal DG ($p = 0.027$), as shown in Figure 20. Metformin treatment did not alter the cortical SYP-1 density of STZ rats, whereas it normalized the density of SYP-1 in the hippocampal DG of STZ-injected rats ($p = 0.027$ in METF100+STZ). Moreover, metformin *per se* increased SYP-1 density in the hippocampal DG of controls ($p = 0.0017$ in METF75).

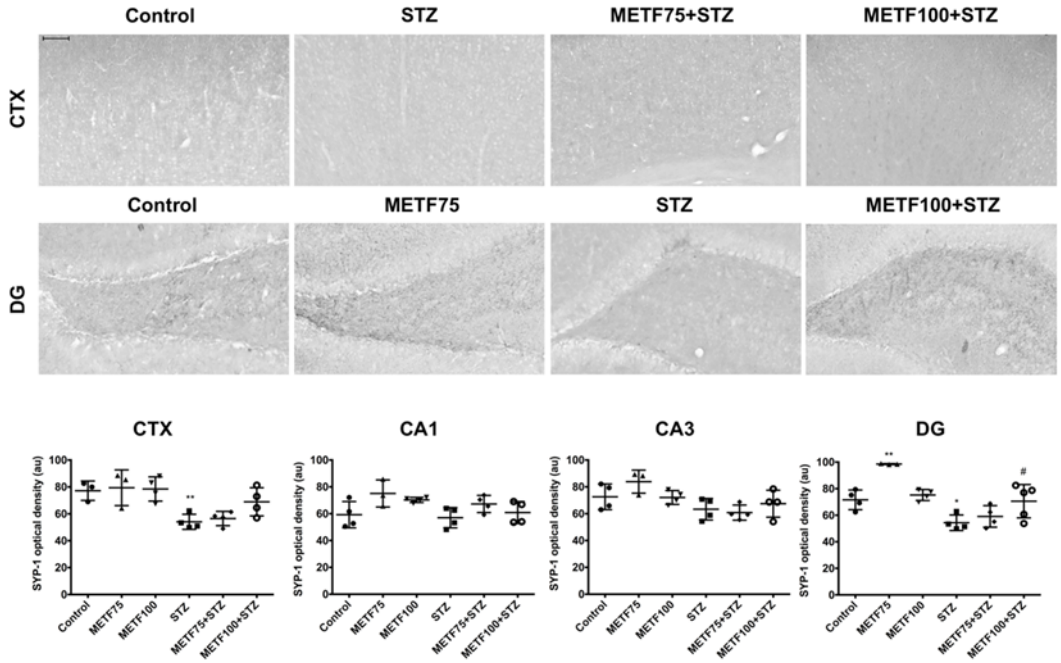

Figure 20. Effects of metformin (METF) on control and streptozocin (STZ)-injected rat density of synaptophysin-1 (SYP-1) in the retrosplenial cortex (CTX) and hippocampal *cornu ammonis* (CA) 1, CA3 and dentate gyrus (DG) regions. Representative microphotographs depict regions where significant differences were obtained after density measurements (200 × magnification). Scale bar is 100 µm. $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$ vs. Control; $^{\#}p \leq 0.05$ vs. STZ.

Changes in the GAP43 are depicted in Figure 21. Cortical density of GAP43 was decreased after the STZ injection ($p = 0.0004$ vs. Control). STZ administration also resulted in lower GAP43 density in the CA1 ($p \leq 0.0001$), CA3 ($p \leq 0.0001$) and hippocampal DG ($p < 0.0001$) in comparison to the control group values. Metformin treatment increased the hippocampal GAP43 density of STZ rats in the hippocampal CA1 ($p = 0.0141$ in METF75+STZ and $p \leq 0.0317$ in METF100+STZ), DG ($p = 0.0007$ in METF75+STZ and $p = 0.0027$ in METF100+STZ), but not in the CA3. Metformin treatment *per se* decreased GAP43 density in the hippocampal DG compared to the controls ($p = 0.0027$ in METF75 and METF100).

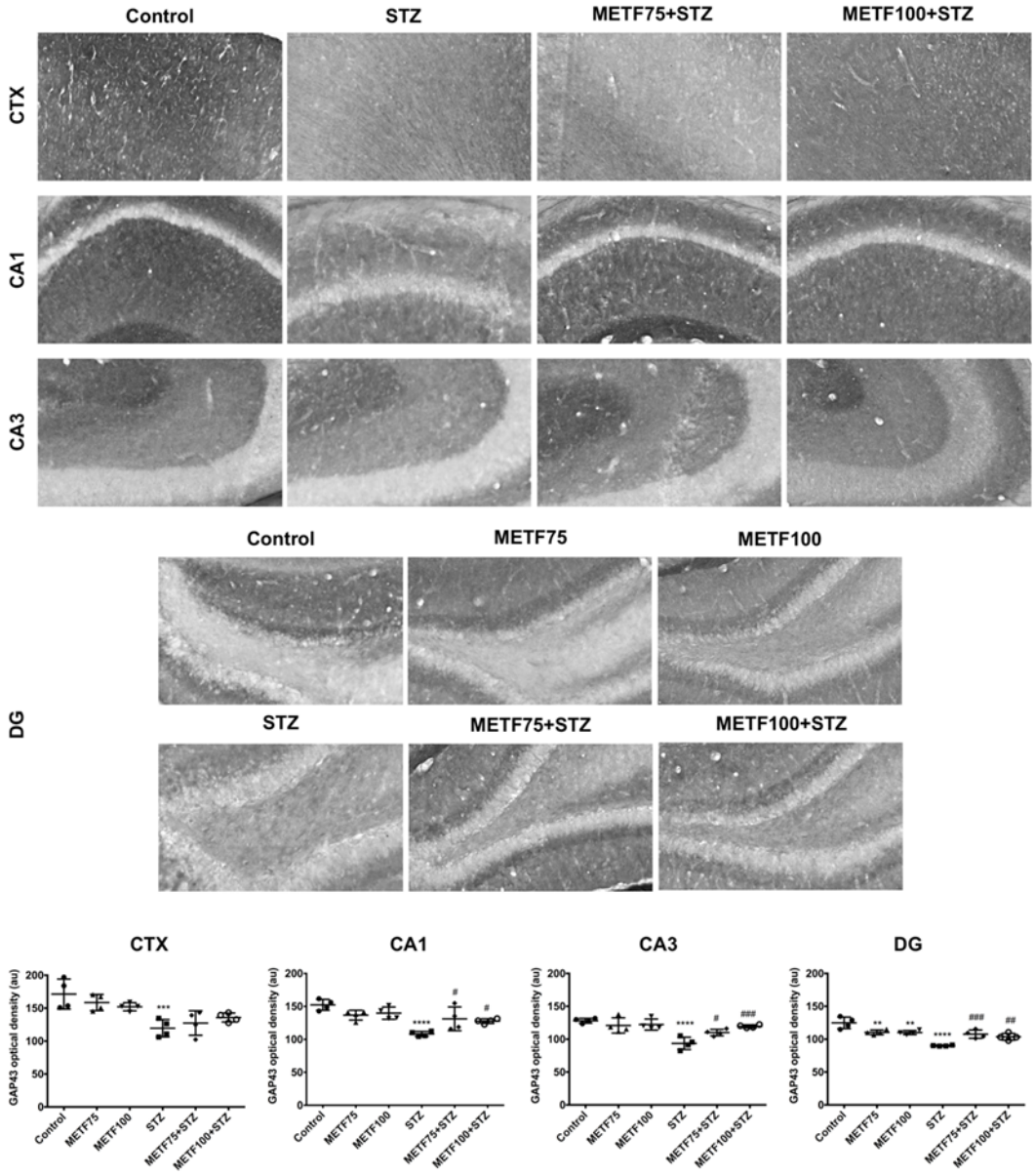

Figure 21. Effects of metformin (METF) on rat density of growth-associated protein 43 (GAP43) in the retrosplenial cortex (CTX) and hippocampal *cornu ammonis* (CA) 1, CA3 and dentate gyrus (DG) regions. Microphotographs represent regions where significant differences were obtained after density measurements (200 × magnification). $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ and $^{****}p \leq 0.0001$ vs. Control; $^{\#}p \leq 0.05$ and $^{\#\#\#}p \leq 0.001$ vs. STZ.

3.4.4. On acetycholine esterase density and activity

Changes in AChE density are depicted in figure 22. Compared to the control group, an increase in AChE density was observed in the cortex of STZ rats ($p = 0.0003$), as well as control group rats treated with 100 mg/kg dose of metformin ($p = 0.0007$). In comparison to STZ-injected rats, no differences in cortical AChE density was observed in STZ group rats that received metformin treatment. Changes in the AChE density were observed in the hippocampal CA1 ($p = 0.0010$), CA3 ($p = 0.0154$), but not in the DG of STZ rats. Metformin-treated STZ-rats showed decreased AChE density in the hippocampal CA1 ($p = 0.0002$ in METF75+STZ and $p \leq 0.0001$ in METF100+STZ), CA3 ($p = 0.0064$ in METF75+STZ and $p = 0.0076$ in METF100+STZ) and DG ($p = 0.004$ in METF75+STZ and $p \leq 0.0001$ in METF100+STZ). Metformin *per se* increased AChE density in the hippocampal CA1 ($p \leq 0.0001$ in METF75 and $p = 0.0010$ in METF100) and CA3 ($p = 0.0255$ in METF75 and $p = 0.0170$ in METF100) of control group rats.

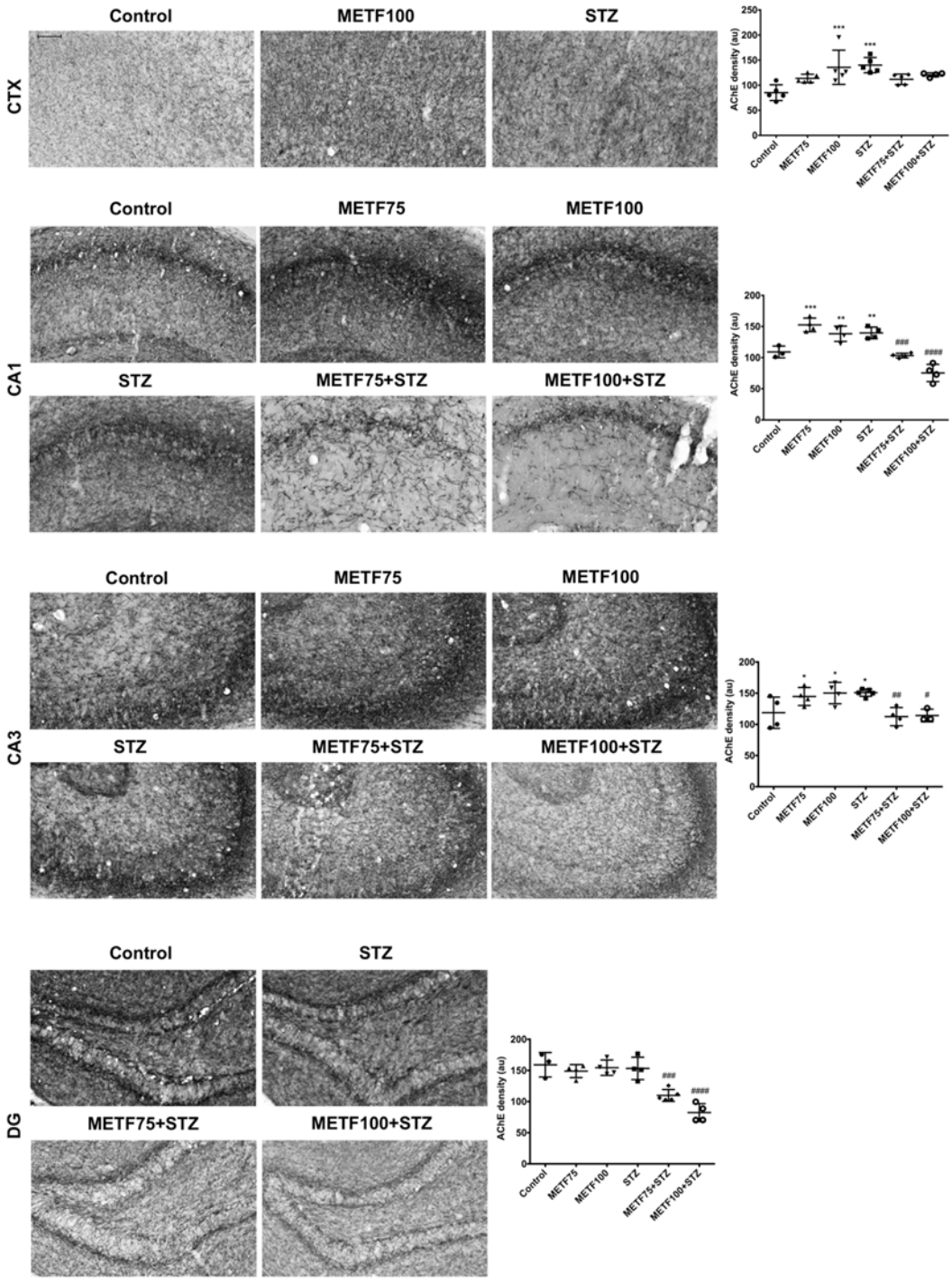

Figure 22. Effects of METF on rat brain density of acetylcholine esterase (AChE) in the retrosplenial cortex (CTX) and hippocampal *cornu ammonis* (CA) 1, CA3 and dentate gyrus (DG) regions. Representative microphotographs depict regions where significant differences were obtained after density measurements (200 × magnification). Scale bar is 100 µm. $^{***}p \leq 0.001$ and $^{****}p \leq 0.0001$ vs. Control; $^{###}p \leq 0.001$ and $^{####}p \leq 0.0001$ vs. STZ.

In vitro, metformin produced concentration-dependent inhibiton of AChE activity (Fig.21): at 0.01 mM – 1.1%, 0.1 mM – 17%, 0.2 mM – 31%, 1 mM – 54% (IC50), 5 mM – 71% and 10 mM – 77%. The calculated IC50 is 1 mM. The, reference drug neostigmine bromide also produced potent inhibition of AChE activity: 62% at 0.001 mM, 76% at 0.01 mM and 84% at 0.1 mM.

Figure 23. Effects of METF on acetylcholine esterase (AChE) activity inhibition.

3.4.5. Metformin effects on glucose transporter (GLUTs) and glycogen synthase kinase-3 (GSK-3) expression

Western blot data (demonstrated in Figure 24) showed a decrease in GLUT-1 hippocampal density in STZ rats ($p \leq 0.0001$) and lower density of GLUT-3 density in the cortex ($p \leq 0.0001$) and hippocampus ($p \leq 0.0001$). In STZ rats, GSK-3 density was higher in the cortex ($p \leq 0.0001$) and hippocampus ($p \leq 0.0001$) compared to controls. Metformin treatment elevated GLUT-1 density in the hippocampus ($p = 0.0001$ in METF75+STZ and $p = 0.03$ in METF100+STZ). GLUT-3 density was also increased by metformin treatment in the cortex ($p= 0.0003$ in METF75+STZ and $p \leq 0.0001$ in METF100+STZ) and hippocampus ($p = 0.0003$ in METF75+STZ and $p \leq 0.0001$ in METF100+STZ). Moreover, metformin normalized the density of GSK-3 in STZ rats to the control group values in the cortex ($p \leq$

0.0001 at both doses) and in the hippocampus (p= 0.002 in METF75+STZ and $p \leq 0.0001$ in METF100+STZ).

Metformin treatment *per se* did not produce alterations in the density of GLUT-1, GLUT-3 and GSK-3 in all regions.

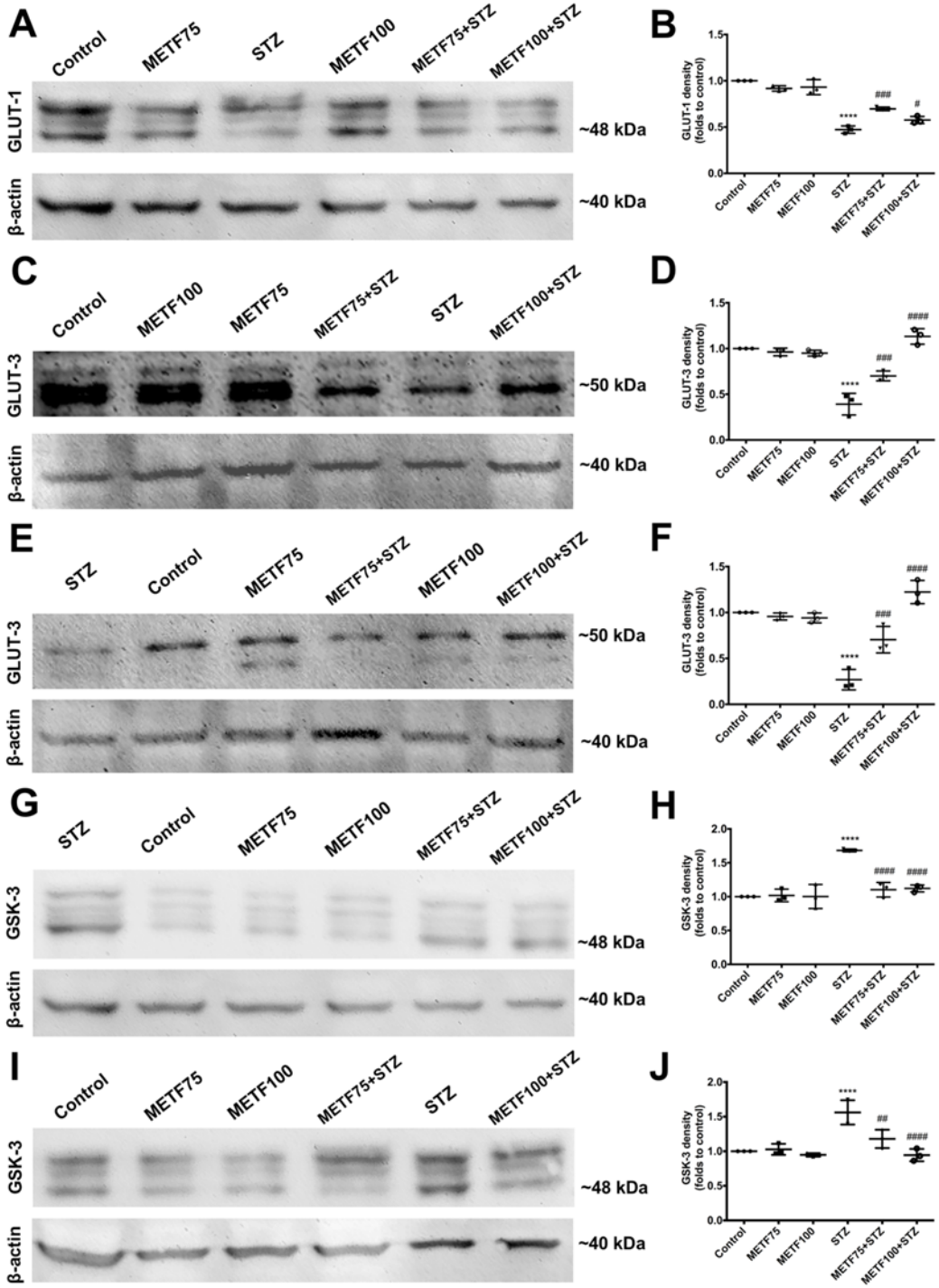

Figure 24. Effects of metformin (METF) on rat brain density of glucose transporter (GLUT)-1, GLUT-3 and glycogen synthase kinase-3 (GSK-3) in the rat

cortex and hippocampus. Representative blots depict density measurements of hippocampal GLUT-1 (A and B), GLUT-3 in the cortex (C, D) and hippocampus (E, F), as well as cortical GSK-3 (G, H) and hippocampal GSK-3 (I, J) expression. Graphs demonstrate the ratio for density normalization of the markers to the β-actin (folds to control). $^{****}p \leq 0.0001$ vs. Control; $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$, $^{\#\#\#}p \leq 0.001$ and $^{\#\#\#\#}p \leq 0.0001$ vs. STZ.

4. DISCUSSION

Sporadic AD and PD are the two most common neurodegenerative diseases that share many pathological and clinical features. Due to the occurrence of brain atrophy, AD is characterized by progressive memory loss, impairments in problem-solving and language function. PD is mainly associated with gait impairments and postural instability caused by the degeneration of dopamine neurons in the nigrostriatal brain structures. Besides, it has been shown that a substantial loss of dopaminergic neurons in PD leads not only to motor disturbances (Boix et al., 2018; Cosgrove et al., 2015), but also affects other neurotransmitter systems, thus impairing cognitive functions, particularly spatial learning and memory (De Leonibus et al., 2007). The essential mechanisms that link AD and PD, and novel approaches to stop these diseases are described below.

4.1. Antiparkinsonian effects of EVs

4.1.1. EVs improve animal gait and spatial memory performance impaired by 6-OHDA

For the first time, we have studied EVs in PD-like model-rats obtained by intra-MFB injection of 6-OHDA. This neurotoxin that is known to exert a substantial lesion of the dopaminergic system in the nigrostriatal structures and induce gait changes, hence providing the most accurate pathological scenario of PD (Boix et al., 2015). After the injection, 6-OHDA is uptaken into the dopaminergic neurons by dopamine transporters. where it undergoes rapid auto-oxidation induces the formation of considerable amounts of reactive oxygen species and causes mitochondrial respiratory chain inhibition (Mazzio et al., 2004; Schober, 2004). According to the literature, motor impairments can be detectable as early as one week after lesion but fully develop after 3–4 weeks that coincides with an almost complete DA cell loss (88.79%) after 6 weeks (Boix et al., 2018).

Our obtained data using a computerized and automated CatWalk gait analysis system demonstrated that EVs (2.85×10^8) administrated intranasally for 17 consecutive days considerably reversed 6-OHDA-induced gait impairments. They normalized stand, stride length, and step cycle, indicating improved coordination and posture. This means that the animals were able to possess higher levels of stability when walking, and were able to take a step significantly faster compared to 6-OHDA-lesioned group rats. In addition, decreased duty cycle shows how freely animals crossed the walkway. EVs treatment also reduced the

prolongation of swing speed induced by 6-OHDA, a parameter which reflects how much air-time the step takes.

Intriguingly, the gait-improving effects of EVs were presented not only during EVs administration, but also 10 days after its discontinuation. Another phenomenon we have discovered was the ability of EVs to reverse impairments in spatial learning/memory induced by 6-OHDA. However, the duration of this effect was shorter (up to the sixth post-treatment day) than that revealed for gait improvement. These findings raised the following questions: 1) what mechanisms are essential for EVs-induced gait- and memory-improving actions? 2) can the short- and long-term effects of EVs be related to the expression degree of TH, and Nissl bodies count in the nigrostriatal structures (substantia nigra and striatum) 3) which cellular events could maintain the prolonged effects of EVs after discontinuation of their administration? We tried to obtain answers to these questions, at least in part, from biochemical studies in the brain's nigrostriatal regions, as well as from proteomic analyses (see below).

4.1.2. EVs reverse changes in TH expression and Nissl body count in the nigrostriatal structures

It is well-known that gait and spatial learning/memory impairments in PD are strongly associated with the loss of dopamine-producing cells in the nigrostriatal structures. Therefore, we firstly analyzed the expression of TH, a key enzyme for the DA synthesis. We found that on post-treatment day 6, EVs produced near to full protection of TH density, which was dramatically decreased by 6-OHDA, but not on post-treatment day 20. At the same time, the amounts of other cellular proteins – Nissl bodies – were reduced 3-fold by 6-OHDA, yet preserved in EVs-treated animals 10 days after the discontinuation of EVs administration; on post-treatment day 20, however, only negligible amounts of preserved Nissl bodies were detected.

These data allow us to consider that the amelioration of gait disturbances until post-treatment day 10 is associated with preservation of nigrostriatal TH expression, and the number of living neurons in the SN, while the amount of Nissl bodies detected on post-treatment day 20 is insufficient to provide gait improvement.

4.1.3. Considerations and arguments about EVs long-term effects

When we compared the time-response data, it is interesting that the reversal of 6-OHDA-induced spatial learning/memory impairments was shorter (up to 6 days after treatment discontinuation) than that of gait performances (up to 10 days).

Since learning and memory processes, as well as gait performances do not only rely on the activity of DA alone, but also on the multiple neurotransmitters, including acetylcholine, glutamate and gamma-aminobutyric acid (GABA) (O'Gorman Tuura et al., 2018; Pilipenko et al., 2019b) one may suggest that the synthesis and release of these neurotransmitters after 6-OHDA lesion are not sufficient to maintain memory formation for a long period, while it is enough for providing motor functions (lasting up to 10 days after treatment termination). One cannot exclude the fact that Nissl bodies in the substantia nigra were detected even on post-treatment day 20. Nissl bodies are located in the granular endoplasmic reticulum, a structure that provides protein synthesis and releases via free ribosomes. Also, protein synthesis can be maintained with the aid of numerous mRNA transcripts, non-coding RNA, mitochondrial and genomic DNA, all of which are located within EVs (Murphy et al., 2019). This assumption is based on the results from the proteomic analysis of EVs, where a multitude of proteins produced by EVs were identified; some of these proteins are especially vital for the adequate expression of TH (Narbute et al., 2019). For instance, inducible form of HSP70 may reverse neurodegenerative process by increasing the number of TH-positive neurons and hence prevent the onset of motor impairments (Ekimova et al., 2018). Adaptor protein 14-3-3ζ, which acts as an endogenous activator of TH in DAergic neurons located in the nigrostriatal structures, also enhances TH expression by reducing the proteolysis of this enzyme (Obsilova et al., 2008; Wang et al., 2009).

The question about the lifespan of intranasally delivered EVs and their biodistribution and pharmacokinetics in the CNS is still a vague idea (Murphy et al., 2019). Recent data show that EVs are rapidly degraded by hydrolase enzymes (Zheng et al., 2019), while other reports demonstrate the presence of exosomes (combined with gold nanoparticles) in the lesioned brain areas 4 days after EVs administration (Perets et al., 2019). Due to the fact that in our study improvements in gait persisted for at least ten days after discontinuation of EVs administration, we assume that EVs either accumulate in the lesioned nigrostriatal structures for at least ten days, or they trigger the synthesis of essential substances capable of rescuing cell functioning.

To summarize this part devoted to EVs, we may suggest that EVs therapy could be a promising therapeutic approach against the PD's neurodegenerative processes. Nevertheless, further studies should be performed to clarify the precise mechanisms of action of EVs. Particularly, further studies to elucidate the role of other neurotransmitter systems and substances crucial for the action of EV, as well as EVs pharmacokinetics are needed.

4.2. Metformin as a possible anti-dementia drug

4.2.1. Metformin reverses STZ-induced spatial memory impairments and improved sociability performance

Metformin is the most widely used drug for the treatment of type 2 diabetes mellitus. Although, the data about the effects of metformin on cognition in diabetes patients is controversial. Several studies have reported that patients with diabetes, who received metformin treatment, had significantly lower dementia incidence and better cognitive performance than those who did not receive metformin treatment (Campbell et al., 2018; Herath et al., 2016). On the contrary, Moore et al. reported that diabetic patients who received metformin treatment had an increased risk of cognitive impairment (Moore et al., 2013). It is suggested that the cognitive impairment associated with long-term metformin use could be due to metformin-induced vitamin B12 deficiency (Porter et al., 2019). However, the knowledge of its full effects on different/multiple biochemical pathways and processes in the brain is still lacking. Metformin has only been studied in transgenic models of AD (Chen et al., 2009; Kickstein et al., 2010), but not in sAD model-animals. The most accurate model of human sAD pathology can be obtained by the i.c.v. injection of STZ, therefore creating sAD in its early stages, where disease-modifying therapies could halt the progression of the pathology. In our study, we determined the effects of peroral metformin treatment (75 and 100 mg/kg for 21 consecutive days) on behavioral and biochemical parameters in sAD model-rats.

Our current data show that the administration of STZ resulted in the prolongation of rat escape latency in the MWM training, thereby demonstrating deficits in spatial learning. Metformin p.o. administration at doses 75 and 100 mg/kg protected against STZ-induced impairments in spatial learning by shortening the escape latency of sAD model-rats by nearly 40%. Impairments in spatial memory in the probe trial were detected in STZ-injected rats as shorter time spent in the target quadrant and lesser platform zone crossings. Metformin treatment protected STZ rat spatial memory: animals spent significantly more time in the

target quadrant and crossed the platform zone twice as often. Our findings are in good agreement with those studies that were done in a rat model of scopolamine-induced learning and memory impairments, where metformin, administered at 100 mg/kg for 14 consecutive days (Mostafa et al., 2016) and at 50, 100 and 200 mg/kg for 21 days (Aksoz et al., 2019), enhanced spatial learning and memory. Metformin treatment did not affect the swimming speed of both control and STZ group animals, demonstrating no influence on motor function.

It is known that AD patients who suffer from poor facial recognition and social isolation are at higher risk of developing severe cognitive decline and generally have poorer disease outcomes (Ya-Hsin Hsiao, 2018). In addition to cognitive assessments, we studied whether metformin improves sociability and social novelty preference in the three-chamber test. In the sociability trials, we found that control animals had a strong desire to explore the chamber with the novel conspecific animal much more than the empty chamber, whereas sAD model-rats did not. Metformin treatment improved STZ-rats' sociability to a great extent: they spent almost 50% more time sniffing and socializing with the novel conspecific rat compared to STZ-rats. Furthermore, in the social novelty preference trial, control rats demonstrated a willingness to engage in a new social contact, whereas sAD model-rats did not express any preference between the novel and familiar rat. On the other hand, rats that were injected with STZ and received metformin treatment showed major improvements in social novelty preference: they spent significantly more time with the novel rat and sniffing it.

4.2.2. Metformin treatment normalizes brain glucose transport and uptake

We further investigated whether metformin's cognitive effects could be provided via the normalization of glucose transport and uptake. Human and rodent brain almost exclusively relies on glucose for energy production. Any interruption in glucose metabolism can cause immediate failure of brain function. For instance, synaptic dysfunction and consequent cognitive decline is closely associated with disrupted glucose metabolism (Chen and Zhong, 2013). A remarkable decrease in glucose uptake and transport in the hippocampal and cortical brain regions of AD patients has been demonstrated in the early stages of the disease (Landau et al., 2011; Simpson et al., 1994), correlating with decreased GLUT-1 and GLUT-3 levels (Landau et al., 2011).

In our study, GLUT-1 density was markedly reduced in STZ rats compared to controls, indicating deficits in glucose transport and uptake into glial cells. Metformin treatment at both doses partially recovered hippocampal glucose uptake and transport by increasing

GLUT-1 density. Current data correspond to the report that demonstrated normalized GLUT-1 expression in STZ-induced diabetic rats after a 6-week treatment with 100 mg/kg metformin (Sokolovska et al., 2010). In comparison to control animals, sAD model-rats also had significantly lowered cortical and hippocampal GLUT-3 density, indicating impaired neuronal glucose uptake. These deficits were prevented by metformin treatment. Moreover, the 100 mg/kg dose of metformin normalized GLUT-3 density to control group values.

Overactivation of GSK-3 is associated with the main AD hallmarks: memory impairments, increased amyloid-β production, and microglia-mediated inflammatory response (Hooper et al., 2007), as well as exacerbated insulin resistance (E. Beurel, S.F. Grieco, 2015). GSK-3 has been recognized as a promising target for the treatment of both diabetes and AD, since abnormal activation of this enzyme reduces ACh synthesis, which is in accordance with the cholinergic deficit present in AD (Hoshi M, 1996). It is noteworthy that the effects of metformin on GSK-3 have not been studied in AD model-animals previously. Results of the current study show that the administration of STZ resulted in increased GSK-3 expression in all studied brain regions. Metformin treatment lowered GSK-3 expression in all studied brain regions of sAD model-rats.

Therefore, we suggest that the normalization of GSK-3 expression might take part in the neuroprotective effects of metformin in tandem with improved glucose transport and uptake.

~~*4.2.3.*~~ *Metformin treatment showed anti-inflammatory effects*

Early pathological changes in sAD are also associated with an increase in glial activation, namely astroglial and microglial activity. At present, it is unclear whether neuroinflammation in AD occurs before, after or in parallel to impaired brain glucose metabolism. Anti-neuroinflammatory role of metformin is well-documented in diabetic model-animals (Krasnova et al., 2016; Wang et al., 2016), but has not been assessed in AD model-animals (Rotermund et al., 2018).

In our study, marked hippocampal astrogliosis (increased GFAP density) was observed in sAD model-rats. At a lower dose of 75 mg/kg, metformin normalized astrogliosis to the control group levels in the hippocampal CA1 and DG regions of sAD model-animals, whereas at a higher dose of 100 mg/kg these effects were only observed in the hippocampal CA1 region. Injection of STZ also resulted in microgliosis (overexpression of Iba-1-positive

microglia) in both cortical and hippocampal rat brain regions. Microgliosis was alleviated by metformin treatment: at 75 mg/kg it decreased Iba-1-positive microglia in all studied regions, whereas at 100 mg/kg it reduced microgliosis in the cortex and hippocampal DG of sAD-model rats. The divergent, dose-dependent effects of metformin on neuroinflammation that we have observed coincide, with those reported in a mice model of STZ-induced diabetes (Oliveira et al., 2016), where 21-day treatment with 100 mg/kg metformin ameliorated hippocampal DG microgliosis, but did not prevent astrogliosis (Oliveira et al., 2016). Based on our data, we suggest that metformin has anti-neuroinflammatory effects in sAD, thereby attenuating neurodegenerative events.

~~*4.2.4.*~~ *Metformin improves synaptic plasticity*

Normal synapse functioning requires coordinated actions of multiple pathways, involved in 1) the formation and maintenance of synaptic proteins, 2) synthesis and delivery of mRNA, proteins, neurotransmitters, and other signal molecules (Mosconi, 2013). Compromised synaptic plasticity correlates with perturbations in learning and memory processes. Neuroinflammation and abnormal insulin signaling impair adult neurogenesis in sAD model-rats, resulting in cognitive decline (Mishra et al., 2018). Therefore, we assessed the effects of metformin on the processes involved in synaptic plasticity: synaptic density (SYP-1), synaptogenesis (GAP43), and cholinergic transmission (AChE). A substantial decrease in presynaptic vesicular protein SYP-1 has been previously found in multiple AD brain regions in aged rats **(Mota et al., 2019)** and STZ-induced sAD model-rats (Pilipenko et al., 2019a).

In our study, the administration of STZ produced significant a decrease in SYP-1 density, hence contributing to deficits in synaptic density in the cortex and hippocampal DG regions of sAD model-rats. Metformin at 100 mg/kg prevented these deficits in the hippocampal DG. Interestingly, metformin at 75 mg/kg *per se* increased SYP-1 density in the hippocampal DG. It is important to stress that DG plays a critical role in memory processing. Several studies suggest that the DG acts as a pre-processor of incoming information, preparing it for subsequent processing in the hippocampal CA3 (Jonas and Lisman, 2014). One might, therefore, suggest that metformin might enhance synaptic vesiculation in healthy rats. Synaptic loss in AD also involves growth-associated protein – GAP43 – that plays a vital role in the regulation of synaptic growth (Korshunova and Mosevitsky, 2010), synaptic plasticity (Rune and Frotscher, 2005) and neurogenesis (Zaccaria et al., 2010). Our results

showed decreased GAP43 expression in sAD model-rats, highlighting impairments in both cortical and hippocampal synapse formation. Metformin treatment significantly improved hippocampal synaptogenesis in STZ group rats, but inexplicably decreased GAP43 density in the hippocampal DG of controls. The effects of metformin on GAP43 have not been described in an AD model yet. Only one report from the spinal cord injury rat model demonstrated that 14-day long metformin treatment (50 mg/kg) enhanced spinal cord expression of GAP43 (Wang et al., 2020).

4.2.5. Metformin exhibits acetylcholine esterase inhibiting properties

Acetylcholine is a neurotransmitter involved in numerous key brain processes, such as learning, memory formation, sensory information, and attention (Xu et al., 2012). Degeneration of the cholinergic system has been reported in a range of AD studies, as well as shown to directly affect neurogenesis (Kotani et al., 2008). Our current findings showed a marked increase in AChE expression in all studied brain structures of STZ-injected rats except hippocampal DG. Metformin treatment normalized AChE expression to near to control levels in all studied hippocampal regions. The anti-cholinesterase activity of metformin was reported in the brain of STZ-induced diabetic rats (Saliu et al., 2016). We also observed inhibitory activity of metformin on AChE *in vitro:* IC_{50} value was 1 mM. Thus, we have obtained important cholinergic component of the mechanism of action of metformin and its ability of accumulating acetylcholine, which can be as essential for memory-enhancing action.

To conclude, the data obtained in this study illustrate, for the first time, the multifaceted effects of metformin in sAD model-rats. We are inclined to suggest that improvements in sAD model-rat cognition and social behavior are provided by metformin targeting the regulation of glucose transport and uptake, as well as by anti-neuroinflammatory activity and maintenance of synaptic plasticity. These data indicate the promise of metformin in the treatment of sAD in its prodromal stages, where dementia is still curable.

CONCLUSIONS

1. For the first time, we have shown that intranasally administered extracellular vesicles (EVs) derived from human exfoliated deciduous teeth stem cells (SHEDs) can reverse 6-OHDA-induced gait and memory/learning impairments in PD model-rats even after discontinuation of EVs treatment (ten and five days, respectively).

2. After discontinuation of EVs treatment, maintenance of the behavioral effects can be explained at least in part of their ability to preserve expression of tyrosine hydroxylase (5 days after the treatment discontinuation) and promote neuronal survival (Nissl bodies) for up two weeks in the nigrostriatal structures.

3. Metformin treatment for 21 consecutive days at doses 75 and 100 mg/kg perorally, improved rat spatial learning/memory, sociability and social novelty performance in intracerebroventricular STZ-induced sAD model-animals.

4. Mechanisms of neuroprotective effects of metformin found in behavioral tests involve ameliorated neuroinflammation by reducing STZ-induced microgliosis (expression of Iba-1) and astrogliosis (expression of GFAP), as well as improved synaptic growth (expression of GAP-43) and plasticity (expression of SYP-1) in the cortical and hippocampal structures.

5. The ability to normalize the expression of glucose transport proteins GLUT-1 and GLUT-3, and glycogen synthase kinase-3 (GSK-3), a protein involved in cerebral

insulin signaling), in the cortical and hippocampal structures, is a vital finding in explaining metformin action.

6. Metformin exerted also AChE inhibiting properties in vivo and in vitro, indicating the role of cholinergic mechanisms in metformin memory-enhancing effects.

7. The obtained data indicate the promise of EVs for the treatment of PD prodromal stages and metformin for the treatment of sAD. Further research is needed to clarify the detailed mechanisms of neuroprotective effects of these substances.

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