UNIVERSITAT DE BARCELONA

Study on Effects of Metformin and Transplantation of NPCs Separately and in Combination on Restores Cognitive Dysfunction and Histopathological Deficits in Mouse Model of Sporadic Alzheimer's Disease

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UNIVERSITAT DE BARCELONA

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Doctoral Thesis

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Thesis presented by saghar Rabieipoor to get doctoral degree in University of Barcelona.

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“It always seems impossible until it’s Done.”

Nelson Mandela

“You never fail until you stop trying.”

Albert Einstein

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Abbreviations:

Aβ	Amyloid-Beta
ACh	Acetylcholine
AD	Alzheimer’s Disease
ADAS-Cog	Alzheimer's Disease Assessment Scale-Cognitive subscale
ADDL	Aβ-Derived Diffusible Ligands
AGEs	Advanced Glycation End products
AKT	protein kinase B
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPK	AMP-activated protein kinase
aPKC	atypical Protein Kinase C
APOE4	E4 Apolipoprotein allele
APP	Amyloid Precursor Protein
BACE1	Beta-site APP Cleaving Enzyme 1
BBB	blood–brain barrier
BIR	Brain Insulin Resistance
BLBP	Brain- Lipid-Binding Protein
CANTAB	Cambridge Neuropsychological Test Automated Battery
CAT	Catalase
CBP	CREB-Binding Protein

CDK5	Cyclin Dependent Kinase 5
CNS	Central Nervous System
COX-2	CycloOxygenase Transcription-2
CSF	Cerebrospinal Fluid
DAMPs	Danger-Associated Molecular Patterns
DG	Dentate Gyrus
DLPFC	Dorsal-Lateral-Prefrontal-Cortex
GLAST	Glutamate Transporter
GFAP	Glial Fibrillary Acidic Protein
GLUT1 and GLUT3	Glucose Transporters 1 and 3
GSH	Glutathione Reductase
GSK3	Glycogen Synthase Kinase 3
HDL	High Density Lipoproteins
hESC	human Embryonic Stem Cells
HFD	High-Fat Diet
hNSCs	human Neural Stem Cells
ICV	Intracerebroventricular
IDE	Insulin Degrading Enzyme
IκBα	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha

IL-1β	Interleukin-1β
IL-6	Interleukin-6
iPSC	induced Pluripotent Stem Cells
IR	Insulin Receptor
IRS	IR Substrate
JNK	Jun N-terminal kinase
LOAD	Late-Onset Alzheimer’s Disease
LPS	lipopolysaccharides
LTP	Long-Term Potentiation
EOAD	Early-Onset Alzheimer's Disease
NIRKO	animals lacking insulin receptors at the neuronal level
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NFTs	NeuroFibrillary Tangles
NMDA	N-Methyl-D-Aspartate
NMDAR	N-Methyl-D-Aspartate Receptor
NPCs	Neuron Progenitor Cells
Nrf1	Nuclear respiratory factor 1
Nrf2	Nuclear Factor Related to Erythroid 2
MCI	Mild Cognitive Impairment

mEPSC	miniature Excitatory Postsynaptic Currents
mTOR	Mechanistic Target of Rapamycin
oxLDL	Low-Density Lipoprotein
PAMPs	Pathogen-Associated Molecular Patterns
PI3K	phosphatidylinositol kinase
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PLB4	BACE1 knock-in mice model
PPR	Paired-Pulse Ratios
PP2A	protein phosphatase 2A
p-tau	Phosphorylated Tau
PS1	Presenilin 1
PS2	Presenilin 2
RAGE	AGE Receptor
RG	Radial Glial
RGL	RG-Like
ROS	Reactive Oxygen Species
SIRT1	Sirtuin 1
SGZ	Subgranular Zone
SOD	Superoxide Dismutase

SRT	Selective Reminding Test
STZ	Streptozotocin
SVZ	Subventricular Zone
TFAM	Transcription Factor A, Mitochondrial
tMCAO	temporary middle cerebral artery blockage
TNF-α	Tumor Necrosis Factor-α
TREM2	Triggering Receptor Expressed on Myeloid cells 2
T2DM	Type 2 Diabetes Mellitus
VHA	Veterans' Health Administration
VZ	Ventricular Zone

Index of figures

Index of table

Contents

Abstract

Background: LOAD is a type of metabolic disorder known as type 3 diabetes. Metformin, a well-known anti-diabetic medication, has been introduced as a neuroprotective agent in recent years. On the other hand, identifying the factors and solutions that can improve the amount of signaling and cognitive function through preserving brain energy and restoring/replacing the lost neurons can provide a clear perspective for the treatment of this disease. Since metformin has brought good results in insulin resistance treatment (T2DM) and shown an effective role in the process of neurogenesis, the simultaneous treatment of NPCs transplantation along with the metformin injection which improves the process of neurogenesis has been evaluated for improves cognition and memory. In addition, treatment with metformin and transplantation of NPCs were investigated separately. The goal of the current study was to evaluate effects of metformin, NPCs transplantation and metformin+NPCs transplantation on sporadic mouse models of Alzheimer's disease (SAD) using behavioral, histological and immunofluorescence studies.

Methods: Five groups of mice including Control (no treatment); sporadic AD (receiving streptozotocin (0.5 mg/kg) on days 1 and 3; STZ+MET (received STZ and metformin (MET) 200 mg/kg per day) for two weeks; STZ+NPCs (received STZ and NPCs transplantation in hippocampus (100,000 in each side) and STZ+MET+NPCs (received STZ, metformin and NPCs simultaneously) were assigned. Novel objective recognition (NOR) and Barnes Maze test were used to test learning and memory. Nissl staining was used as a histological method for counting the dark neurons (dark purple stained neurons known as dead cells) in different regions of the hippocampus in five experimental groups. Immunofluorescence staining against glial fibrillary acidic protein (GFAP), ionized calcium binding adaptor molecule 1 (Iba1) and NeuN were used to visualize reactive astrocytes, microglia (gliosis) and neurons, respectively.

Results: In NOR test, the percentage of discrimination index in the STZ group was significantly lower than the control and treatment groups. In addition, the discrimination index percentage for the novel object in STZ was significantly lower than other groups, while in the treated group it was still less than the Control. The Goal sector/non-goal sector (GS/NGS) ratio index in Barns maze was significantly higher in the metformin treatment group compared to the other two

treatment groups while escape latency was not significantly different between the groups. Traveled distance was significantly higher in the metformin group. The results of Nissl staining demonstrated the number of dead neurons was significantly increased after STZ induction and metformin and transplantation of NPCs treatment has been able significantly reduce the number of dead neurons vs STZ group. In STZ group, GFAP level was significantly higher in CA1+DG, CA3 and cortex as compared to Control and reversed in treated groups with significantly reduction in STZ+MET, STZ+NPCs and STZ+NPCs+MET as compared to STZ group. IBA1 level was significantly higher in the STZ group in CA3, and cortex regions compared to Control. Moreover, metformin significantly decreased the intensity of IBA1 in CA3 and cortex vs STZ group. NPCs transplantation also significantly reduced the IBA1 intensity in CA1+DG and cortex. Counting NeuN+ cells significantly demonstrated reduction of the number of neurons in DG+CA1, CA3 and cortex after STZ induction. NPCs transplantation in both STZ+NPCs and STZ+NPCs+MET significantly increased the number of neurons vs STZ group in CA1+DG and CA3 regions. Metformin also increased the number of neurons in limited range and non-significantly.

Conclusion: Metformin decreased inflammatory cells and reactive astrocytes as well as the dark neurons in the hippocampus region and the cortex in STZ model of sporadic AD and improved cognitive performance. Transplantation of NPCs separately and together with metformin injection also reduced inflammation in the hippocampus. And on the other hand, following the NPCs transplantation, neurogenesis in CA1+DG and CA3 areas of the hippocampus was strengthened, and the lost neurons were replaced. In this regard, improvement in cognitive memory and learning was evident in these two linked groups. In general, therefore, in the field of cognition and memory according to the new object recognition behavioral test, stem cell transplant alone showed better results than stem cell transplant combined with metformin injection and also compared to metformin treatment alone. On the other hand, metformin has been helpful in learning.

Keywords: Alzheimer’s disease; Metformin; Neural Progenitor Cells; Hippocampus.

I.INTRODUCTION

1. Alzheimer’s disease

Dementia affects 44 million people worldwide, and it is the second leading cause of mortality in those aged 70 and up (van der Flier PhD, 2021, Gaugler et al., 2021, van Bokhoven et al., 2021, Knopman et al., 2021). Alzheimer's disease (AD) is the most common form of dementia (van der Flier PhD, 2021), as well as one of the leading causes of illness and death among the aged worldwide (Cummings et al., 2021, Poor et al., 2021). Due to an ageing population, AD prevalence is expected to reach 115 million by 2050 unless new treatments to delay or cure the disease become available (Gaugler et al., 2021, van Bokhoven et al., 2021, Knopman et al., 2021). The predominant current concept attempting to explain neuronal and synapse loss, linked with cognitive and memory impairment, is based on neuropathological alterations of AD, such as tau hyperphosphorylation and Aβ toxicity (Gaugler et al., 2021, van Bokhoven et al., 2021, Knopman et al., 2021, Ballard et al., 2020, Avila and Perry, 2021).

Because the neurodegenerative process of Alzheimer's disease is strongly linked to the aging process, it's also known as late-onset AD (LOAD). LOAD has no known cause and is mostly prevalent in those over the age of 65, accounting for 95 percent of AD cases (fig.1). Patients with autosomal dominant mutations in amyloid metabolism-related genes, such as beta amyloid precursor protein (APP-chromosome 21), presenilin 1 (PS1-chromosome 14), and presenilin 2 genes (PS2-chromosome 1) included just about 5 percent of AD patients (Avila and Perry, 2021).

Even though Alzheimer's disease (AD) is frequently associated with the elderly, it may also affect younger people. Early-onset Alzheimer's disease (EOAD) affects those who are diagnosed before the age of 65 (fig.1). (Between 30 and 65). Early-onset dementia patients constitute 1-3% of AD cases in most studies, according to McMurtray and colleagues (McMurtray et al., 2006, Poor et al., 2021). APP, PS1, and PS2 are three genes that have been identified as being important in EOAD. PS1 and PS2 are two proteins that make up γ-secretase's catalytic core. These gene alterations, which include APP, PS1, and PS2, have been demonstrated to promote the production of amyloid-β (Aβ), resulting in an increase in the Aβ1–42/Aβ1–40 ratio, which favours the formation of senile plaques (van der Flier PhD, 2021). In LOAD, which has no established etiology and is considered complex (McMurtray et al., 2006), a combination of genetic,

environmental, and behavioral variables play a substantial effect (McMurtray et al., 2006, Poor et al., 2021).

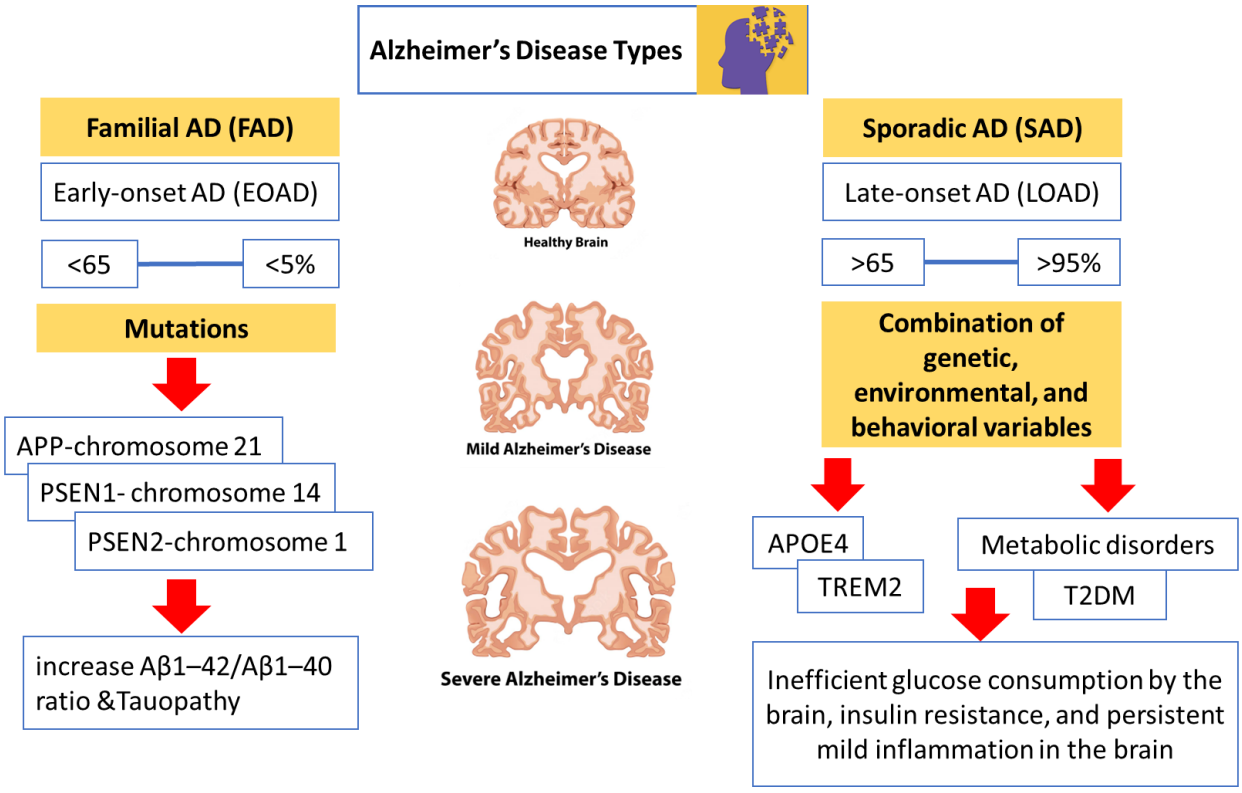


Figure 1. Alzheimer's disease types. Familial AD (EOAD) and sporadic AD (LOAD).

While the cause of LOAD is unknown, the E4 apolipoprotein allele (APOE4) and the triggering receptor expressed on myeloid cells 2 (TREM2) are two of the most important genes implicated in the disease (Irie et al., 2008, Serrano-Pozo et al., 2021). The APOE gene's ε4 allele codes the CNS's major apolipoprotein, which is involved in lipid transport and neuron homeostasis (Irie et al., 2008, Yamazaki et al., 2019). One copy of the ε4 allele in APOE has been shown to raise LOAD risk by 3~4 times (Serrano-Pozo et al., 2021, Emrani et al., 2020).

Its mutation causes APOE4 to have a larger lipid binding capacity, which is linked to less effective Aβ clearance and an increase in pathological alterations linked to cognitive decline (Serrano-Pozo et al., 2021, Poor et al., 2021). Carriers of the APOE2 allele, on the other hand, had a two-fold lower risk of LOAD than non-carriers, and it is thought to be a protective genetic factor against

the illness (Yamazaki et al., 2019). Early driving amyloid disease in the brains of APOE4 carriers has been shown in clinical and fundamental studies (Irie et al., 2008, Serrano-Pozo et al., 2021). Furthermore, APOE4 is either pathogenic or a performance-decreasing factor in multiple brain homeostatic processes, including lipid transfer, synaptic integrity and plasticity, glucose metabolism, and cerebrovascular function (Irie et al., 2008, Emrani et al., 2020).

TREM2, is a plentiful receptor on the surface of microglia and plays a key role in their activation and control (Qin et al., 2021, Xue and Du, 2021). Certain mutations affect TREM2's affinity for its ligands, reducing microglia phagocytosis of Aβ peptide and boosting a systemic inflammatory response. As a result, due to the incomplete and low function of microglial, TREM2 plays an important role in the development of LOAD. TREM2 also modulates microglia activity in LOAD and other neurodegenerative disorders, as well as inflammatory responses and metabolism, either alone or in close connection with other molecules like APOE (Li et al., 2020a, Poor et al., 2021).

LOAD and type two diabetes mellitus (T2DM) have become global pandemics, with new forecasts showing that they will continue to worsen in future decades. Obesity, type 2 diabetes, and related comorbidities have all been linked to the development of LOAD (Xue and Du, 2021). As a result, LOAD has lately been labeled a "metabolic disorder" linked with inefficient glucose consumption by the brain, insulin resistance, and persistent mild inflammation in the brain (Li et al., 2020a, Suzanne, 2014). In addition, LOAD has been referred to as "type 3 diabetes" because of insulin resistance that develops in the brain (de la Monte et al., 2018, Poor et al., 2021). In LOAD, similar to T2DM, which is characterized by a decreased ability of peripheral tissues to metabolize glucose, ability reduction of the brain to glucose metabolization (hypometabolism of brain glucose) may contribute to the neurodegenerative disorder process, along with the classic neuropathological LOAD hallmarks, such as Aβ deposits and hyper phosphorylated tau (p-tau) in neurofibrillary tangles (NFTs) (de la Monte et al., 2018, Suzanne, 2014).

It is reported thatT2DM has been linked to the onset as well as the progression of LOAD in preclinical research investigations and clinical and epidemiological trials. The prevalence of LOAD in individuals with T2DM was 56 percent higher than in persons without diabetes, according to a

retrospective analysis of 20 prospective therapeutic studies (Gudala et al., 2013). This association has been linked to brain insulin resistance, reduced insulin signaling, inflammation, hyperglycemia, vascular changes, hypoglycemic episodes, and defective amyloid metabolism (Chen et al., 2021b). As a result, both T2DM and LOAD have been demonstrated to have multifactorial risk indices and a wide range of molecular genetic linkages. The appearance of cognitive aberrations in LOAD patients with underlying T2DM might be due to the confluence of the molecular pathways of these two disorders (Burillo et al., 2021, Vinuesa et al., 2021).

2. Type 2 Diabetes Mellitus Related with Alzheimer's Disease

T2DM is a common chronic metabolic disorder marked by elevated blood glucose levels and insulin resistance (Rosenfeld, 2014, Sun et al., 2020). According to epidemiological statistics, diabetic individuals have a higher risk of getting dementia than the general normal population. As a result, Ott and colleagues were the first to describe a possible link between the two pathologies in the 'Rotterdam Study,' revealing that diabetes considerably enhanced the chance of dementia (Ott et al., 1996, Ott et al., 1999).

As a result, it was proposed that LOAD be classified as a metabolic disease because LOAD patients' brains shared some characteristics with impaired insulin signaling pathways (Gudala et al., 2013). Clinical and epidemiological research have also corroborated this link, showing that changes in metabolic indices including hyperglycemia and hyperinsulinemia are strongly linked with the development of LOAD neuropathology (Salameh et al., 2016).

In this regard, the 'Hisayama Study' found a link between diabetes and APOEε4 and Aβ plaques, but not with the production of neurofibrillary tangles (Matsuzaki et al., 2010). Willette and colleagues introduced a connection between brain insulin resistance (BIR) and Aβ brain accumulation in LOAD patients in a research study conducted in cognitively healthy middle-aged adults enrolled in the 'Wisconsin Registry for Alzheimer's Prevention study', lending support to the hypothesis that in the early stage of LOAD, BIR is known as an important risk factor (Willette et al., 2015, Willette et al., 2013).

Consequently, this study suggested that BIR is a modifiable risk factor throughout the preclinical stage of the disease, opening up a new therapeutic window for the development of novel LOAD preventive therapies (Willette et al., 2015).

Similarly, other studies imply that, in some circumstances, such as metabolic disorders, the body's diabetic state increases the risk of LOAD by interrupting glucose flow to the brain and slowing its metabolism (Gudala et al., 2013). In general, metabolic abnormalities such as glucose/lipid metabolism, protein alterations, mitochondrial dysfunction, and oxidative stress are linked to a defective insulin signaling system. BIR may also aggravate Aβ accumulation, promote tau hyperphosphorylation, disruption of glucose and energy metabolism pathway, and damage framework of hippocampal pathways (Monte, 2019, de la Monte et al., 2018, Matsuzaki et al., 2010).

Furthermore, insulin has been shown to have a variety of beneficial effects on the brain, including synaptic trophic affects and the development of dendritic spine formation (Berlanga-Acosta et al., 2020, Rebelos et al., 2021, Biessels et al., 2006). Though the evidence for a relationship between diabetes and neurodegenerative disease in LOAD is conflicting, some human postmortem results imply a link between insulin tolerance of the brain and enhanced LOAD pathology, particularly.

increased Aβ deposition (Biessels et al., 2006, Barnes and Yaffe, 2011). Furthermore, as previously indicated, multiple clinical research has found that individuals with diabetes have high levels of Aβ and p-tau in their CSF fluid, as well as worse cognitive scores (Carvalho et al., 2019, Lacor, 2007). On the other hand, Insulin and therapeutic drugs that increase insulin signaling, , have been shown in preclinical and limited clinical trials to decrease neuropathology and improve cognition in diabetes and LOAD (De Felice, 2013, De Felice and Benedict, 2015).

The most widely held belief is that Aβ is the primary cause of LOAD (Kulstad et al., 2006, Ferreira et al., 2015a). This begins with breakdown of the amyloid beta precursor protein (APP) through amyloidogenic cascade enzymes activation including β and γ secretases, to release Aβ (Viola and Klein, 2015, Cline et al., 2018, Li and Selkoe, 2020). This process raises harmful Aβ 1-42 levels

(Viola and Klein, 2015, Li and Selkoe, 2020), which allows fibrils to form extracellular deposits to neuron destruction and senile plaques organization (Viola and Klein, 2015, Poor et al., 2021).

Since amyloid plaques are seen in postmortem brains of LOAD patients and in preclinical models of AD that display cognitive abnormalities, the first amyloidogenic hypothesis indicated that amyloid plaques or insoluble amyloid fibrils were accountable for the synapse loss (Viola and Klein, 2015, Ferreira et al., 2015a).

Although AD mouse models generate amyloid plaques mostly, they also exhibit synapse disruption and cognitive abnormalities earlier than plaque development. Consequently, this concept has been challenged, and it is widely understood that soluble Aβ oligomers, known as Aβ-derived diffusible ligands (ADDL), are the potent CNS neurotoxins which accumulate in LOAD brain (Viola and Klein, 2015, Cline et al., 2018).

According to this evidence, it is estimated that the mediation of Aβ toxicity is occurred by ADDL as well as insoluble amyloid fibrils and in AD pathogenesis, synaptic failure is one of the earliest occurrences (Viola and Klein, 2015). Furthermore, the better correlation of ADDL with cognitive decline can be seen compared with insoluble Aβ peptides accumulation in pathophysiology of AD (Viola and Klein, 2015, Cline et al., 2018).

Similarly, earlier studies have shown that ADDL can affect the function of the insulin receptor in the brain (detected at the dendritic level in synapses), causing it to localize intracellular and away from the neuronal surface of dendrites. Thus, this mechanism is linked to reduction in glutamatergic neurotransmission (Viola and Klein, 2015, Ferreira et al., 2015a).

Additionally, Aβ is also associated with neuronal oxidative stress as well as impaired mitochondrial function (Cline et al., 2018). Response of unfolded protein and endoplasmic reticulum stress are involved in Aβ neuronal cell injury development and are linked to tau protein pathology. These metabolic factors may promote the incidence of LOAD in diabetic individuals (Fishel et al., 2005).

Aside from the impact of Aβ oligomers on the brain, the involvement of plasma and oligomers on peripheral IR is a fascinating topic. According to this theory, these oligomers have an inhibitory

influence on the peripheral insulin signaling system via many pathways mediated by oxidative stress and inflammatory reactions, suggesting that oligomers affect peripheral glucose metabolism in multiple ways (Takeda et al., 2009, Zhang et al., 2013).

As a result, oligomers have significant impacts on glucose metabolism in the systemic circulation, where insulin is required for glucose homeostasis via effects on the liver, skeletal muscle, and adipose tissue (De Felice, 2013, de la Monte et al., 2019). Consequently, these findings support the theory that LOAD is a metabolic disorder in which oligomers produced in the brain play a crucial role in changes in peripheral metabolism.

Cerebral hypometabolism caused by a decrease in glucose absorption is one of the symptoms of LOAD. The reduction of glucose levels in the brain is mostly due to decreased glucose uptake and decrease in glucose transporter expression (GLUT1 and GLUT3) in neurons (El Massry et al., 2021). As a result, boosting glucose transport to neurons, for example using antidiabetic medicines like metformin, has been proposed as a therapeutic strategy in AD (El Massry et al., 2021). Similarly, in LOAD patients, changes in many brain circuits linked to T2DM have been identified. As we previously stated in LOAD, a change in IR levels has been shown to impact the cognitive process owing to synaptic impairment, as well as increased oxidative stress, promoting mitochondrial malfunction and ultimately neuronal death (fig. 2) (Barber et al., 2021, Lacor, 2007).

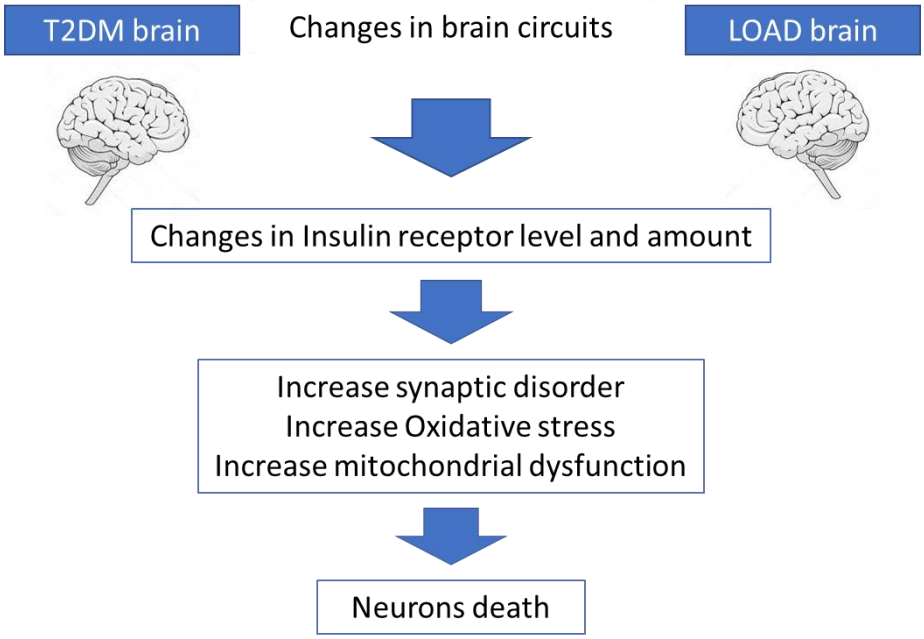


Figure 2. IR level changes in SAD and T2DM. changes in levels of insulin receptors in the brain leads to increased synaptic disorder, oxidative stress and mitochondrial dysfunction that are important factors for death of neurons.

Because chronic hyperglycemia can cause glycolipotoxicity, it can lead to the development of diabetic complications. Hyperglycemia produces advanced glycation end products (AGEs), which are another important connection between diabetes and LOAD (Li and Selkoe, 2020, Zhang et al., 2012). It's been suggested that an increase in AGE levels in the brain is linked to cognitive impairment in people with LOAD. As a result, AGEs can contribute to LOAD by boosting the production of fibrillary tangles and amyloid plaques, which are the primary neuropathological features of the disease, as well as enhancing cytotoxicity (Rojas-Gutierrez et al., 2017, Suzanne, 2014). RAGE, an AGE receptor that is also a potential receptor for Aβ, is induced by AGEs (Rojas-Gutierrez et al., 2017, de la Monte et al., 2019). RAGE levels have been found to be higher in many kinds of LOAD brain cells in previous investigations. Glial cells in the brain, for example, have higher RAGE levels, and RAGE co-localization with intracellular Aβ and tau has been seen in LOAD patients.

Furthermore, in both T2DM and LOAD, changes in the enzymes glycogen synthase kinase 3 (GSK3) and insulin degrading enzyme (IDE), suggest a relationship between the two disorders.

The insulin receptor regulates GSK3, and its activation in LOAD promotes tau protein phosphorylation and the development of neurofibrillary tangles (Carvalho et al., 2019, De Felice, 2013). IDE plays an important role in the metabolism and elimination of Aβ as well as insulin. IDE reduces both insulin and Aβ peptide, though, insulin binds IDE with greater affinity. Accordingly, in both diseases, the hyperinsulinemia separator exhibits higher affinity for insulin compared to Aβ, because of that, facilitate Aβ accumulation and increase risk LOAD (de la Monte et al., 2019).

Pluciska and colleagues revealed that brain BACE1 overexpression increased the risk of peripheral T2DM in preclinical research using a neuron-specific human BACE1 knock-in mice model (PLB4), supporting this metabolic theory of LOAD (El Massry et al., 2021). LOAD progression can induce T2DM comorbidities in mice irrespective of the typical obesogenic pathway, indicating that T2DM and LOAD may be linked.

Tau, as previously stated, is the other major biomarker associated with the process of neurodegenerative in LOAD (van der Flier PhD, 2021). Hydrophobic Tau protein plays a key role in microtubule stability and axonal transport in neurons. The "tau hypothesis" states that tau protein malfunctioning causes the development of NFTs by disrupting microtubules assembly (Knopman et al., 2021, Avila and Perry, 2021, El Massry et al., 2021, Plucińska et al., 2016).

Tau hyperphosphorylation disrupts tau's interaction with microtubules, causing the entire microtubule assembly to be disrupted (van der Flier PhD, 2021, Knopman et al., 2021, Avila and Perry, 2021, Gratuze et al., 2018, Yarchoan et al., 2014). In this way, brain insulin has been demonstrated to play an important role in tau phosphorylation control by activating its receptor in the brain. BIR is linked to the activation of tau kinases, including GSK3, via the phosphatidylinositol kinase (PI3K)/protein kinase B (AKT) signaling pathway, as previously stated (Rodriguez-Rodriguez et al., 2017). As a result of BIR, GSK3 is over activated, which promotes tau hyperphosphorylation. Due to its placement on dendrites in the postsynaptic terminals, tau hyperphosphorelation, oligomerization, misfolding, and aggregation are implicated in the disruption of synaptic plasticity and contribute to the neurodegenerative process (Yarchoan et al., 2014). The reduction in density and form of dendritic spines, as well as neuronal death, have been linked to p-tau deposits in the CA1 and CA3 areas of the hippocampus (Rodriguez-Rodriguez

et al., 2017, Yarchoan et al., 2014). BIR expression was linked to IRS1-pS616 and IRS1-pS312 expression in LOAD and brain tauopathies such as Pick's disease, corticobasal degeneration, and progressive supranuclear palsy, according to Yarchoan and colleagues (Yarchoan et al., 2014). As

As a result, the authors propose a link between BIR and tau, in which increased IRS-1 pS616 phosphorylation favors aberrant tau phosphorylation (Yarchoan et al., 2014).

Marciniak and colleagues (Marciniak et al., 2017) revealed tau's significance as a critical BIR modulator and the insulin receptor signaling pathway, as well as the ways through which tau may affect insulin receptor activity (Marciniak et al., 2017). As a result, tau deletion affects peripheral and brain insulin metabolism, hippocampus BIR manipulation can improve cognitive performance, and hypothalamic BIR regulates metabolic changes in LOAD patients and tauopathies (Marciniak et al., 2017). Chronic BIR plays a role in tau disease development by disrupting the equilibrium of kinases and phosphatases, and vice versa. Furthermore, tau hyperphosphorylation has been shown to cause an increase in insulin uptake and intra neuronal deposition of insulin as insoluble oligomeric aggregates in LOAD patients and a variety of tauopathies (Gratuze et al., 2018). This process happens in the absence of T2DM, suggesting that BIR is linked to changes in the insulin signaling pathway regardless of the existence of clinical T2DM.

Tau has also been discovered to be a major regulator of peripheral insulin signaling, with data tying tau to IR in the brain and peripheral tissues, as well as beta cell dysfunction (Rodriguez-Rodriguez et al., 2017, Gonçalves et al., 2019, Wang and Mandelkow, 2016). Tau is abundantly expressed in the pancreatic islets' insulin-secreting beta cells. Mice with worldwide tau deletion have a higher body weight, abnormalities in glucose-stimulated insulin secretion, and poor glucose tolerance at a young age (Rodriguez-Rodriguez et al., 2017, Yarchoan et al., 2014). Although it is widely known that insulin may alter tau protein phosphorylation, its involvement in insulin receptor modulation has only recently been investigated. According to this theory, animals lacking insulin receptors at the neuronal level (NIRKO mice) and IRS-2 -/- mice demonstrated enhanced tau phosphorylation via suppression of the phosphoinositide 3 kinase

(PI3K)/AKT signaling pathway (de la Monte et al., 2019, de la Monte et al., 2018, Gonçalves et al., 2019, Wang and Mandelkow, 2016).

The connection between insulin and tau signaling is primarily based on the modulation of down-stream signaling pathways involving kinases such as GSK-3, c-Jun N-terminal kinase (JNK), and AMP-activated protein kinase (AMPK), as well as phosphatases such as protein phosphatase 1 and 2 (PP1 and PP2A, respectively) (de la Monte et al., 2019, de la Monte et al., 2018).

Oxidative stress signs appear in earliest brain changes of AD which is preceded by deposition of detectible amyloid and neurofibrillary tangles accumulation (Nunomura et al., 2000, El Sayed and Ghoneum, 2020). Chronic inflammation and some neuro-disorders such as AD and Parkinson's disease are associated with oxidative stress (Polidori, 2004, El Sayed and Ghoneum, 2020).

The brain's neurons are so sensitive to excessive reactive oxygen species (ROS) and oxidative impairment which originates from the fact that neurons have a very high consumption of oxygen and production of energy (Bélanger et al., 2011, El Sayed and Ghoneum, 2020).

Neurodegenerative disorders such as AD are also associated with neuroinflammation. The neuron's inflammatory response is related to the transcription factor NF-κB. Under normal conditions, NF-κB processes an inactive cytoplasmic complex with its inhibitor, IκBα. However, when NF-κB is stimulated, the inflammatory target genes are induced such as interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and cyclooxygenase transcription-2 (COX-2). In addition, neuroinflammation is associated with autophagy in neurodegenerative diseases. Neuroinflammation can cause autophagy deficiency that exacerbates neurodegeneration, and conversely, autophagy disruption during morbidity can induce or exacerbate neuroinflammation (El Sayed and Ghoneum, 2020, Zheng et al., 2013).

Decreased autophagy has been observed in human AD and mouse models of AD and has been shown to contribute to the pathological assembly of tau aggregates (El Sayed and Ghoneum, 2020, Zare-Shahabadi et al., 2015). Autophagy is known to be regulated by mTOR, a mammalian target for rapamycin, and inhibition of mTOR has been shown to prevent neuroinflammatory inflammation in a mouse model of cerebral palsy. In addition, studies of rat cortex exposed to

ischemic brain injury have shown that GSK-3β inhibition suppresses neuroinflammation through autophagy activation (Zhou et al., 2011).

3. LOAD Risk Factors

It is vital to identify the elements that affect this disease in order to reduce the possibility of a future in which there will be a large percentage of AD sufferers. Numerous epidemiological studies pertaining to the identification of AD risk factors have been published in recent years. Susceptibility genes and environmental factors are two categories of LOAD risk factors (Mendiola-Precoma et al., 2016, Robledo et al., 2017).

Apolipoprotein E (ApoE), the most extensively researched genetic risk factor for AD, is a significant genetic contributor to LOAD. The liver, macrophages, and CNS all manufacture ApoE (Huang and Mahley, 2014, Mendiola-Precoma et al., 2016).

Although astrocytes and microglia manufacture it in the CNS, stress or neuronal injury can trigger the production of ApoE in neurons. It is generated by astrocytes and microglia in the CNS, but under specific pathological circumstances (stressors and injurious agents), ApoE can be expressed on neurons in response to stress or neuronal injury (Van Giau et al., 2015, Mendiola-Precoma et al., 2016).

Hypercholesterolemia (Dias et al., 2014, Xue-Shan et al., 2016, Mendiola-Precoma et al., 2016), obesity (Verdile et al., 2015, Walker and Harrison, 2015, Mendiola-Precoma et al., 2016), hyperhomocysteinemia (Chakrabarti et al., 2015), hypertension (de Bruijn and Ikram, 2014, Mendiola-Precoma et al., 2016) and T2DM (Butterfield et al., 2014, Sandhir and Gupta, 2015) are the key metabolic and non-genetic risk factors.

Hypercholesterolemia. High levels of serum and plasma cholesterol have been proposed as AD risk factors (Prasanthi et al., 2012, Kosari et al., 2012). Primary cholesterol production occurs in astrocytes and to a lesser extent in neurons in the adult brain. Local high density lipoproteins (HDL) transfer cholesterol into the brain (Mendiola-Precoma et al., 2016). Low-density

lipoprotein (ox LDL) levels are raised in hypercholesterolemia and cardiovascular illnesses due to enhanced oxidation and nitration-related systemic changes (Lim et al., 2014).

In an experimental cell-based investigation, it was discovered that the distribution of cholesterol in the membrane had an impact on the metabolism of APP, the movement of APP, the functions of γ- β and α -secretases, and Aβ production (Mendiola-Precoma et al., 2016). Although the exact mechanism by which cholesterol disrupts Aβ metabolism is still unknown, a number of studies indicate that changes in cholesterol levels affect the cell membrane (Mendiola-Precoma et al., 2016). As a result of impaired lipid rafts, membrane microdomains important for protein trafficking (Cline et al., 2018), signal transduction (Li and Selkoe, 2020), and neurotransmission (Cline et al., 2018, Ferreira et al., 2015b). These cholesterol-rich lipid rafts are where APP is finally produced by secretase cleavage (Mendiola-Precoma et al., 2016).

Hyperhomocysteinemia, Homocysteine levels that are elevated are influenced by a number of variables, including age, heredity, lifestyle, and sex (Plucińska et al., 2016). This risk factor's occurrence in the population has a variety of origins, including both genetic and nongenetic factors. Hyperhomocysteinemia in the general population may be brought on by vitamin B12, folate, and pyridoxine deficiency (Gratuze et al., 2018). According to pharmacological evidence, homocysteine enhances lipid synthesis (Plucińska et al., 2016), inflammatory reactions, and the activation of the N-methyl-D-aspartate receptor (NMDA) (Marciniak et al., 2017). In AD models, it has been demonstrated that NMDA receptors mediate the peptide's downstream effects, and pharmacological suppression of this receptor's activity eliminates the harmful impact of Aβ (Rodriguez-Rodriguez et al., 2017, Yarchoan et al., 2014, Mendiola-Precoma et al., 2016).

The other risk factor for LOAD is hypertension (Gonçalves et al., 2019). Several studies have connected hypertension to brain shrinkage and the production of NFTs. This relationship, nevertheless, varies with age and is complicated. It has been demonstrated that having high blood pressure in middle age increases the likelihood of developing AD (Wang and Mandelkow, 2016), although other studies have revealed no link between dementia and high blood pressure in the elderly (Rena et al., 2017, Sun et al., 2020, Mendiola-Precoma et al., 2016).

Obesity is a risk factor for several diseases, including T2DM, metabolic syndrome, hypercholesterolemia, and cardiovascular disease (de la Monte et al., 2006). This is the result of lifestyle modifications, such as insufficient exercise, an imbalanced diet, and overeating, which cause stress oxidative and inflammatory processes and affect the metabolic pathways required for homeostasis (Rosenfeld, 2014, Mendiola-Precoma et al., 2016).

Numerous studies have linked obesity to increased risk of AD and cognitive impairment (Folch et al., 2015, Lu et al., 2016, Pedditizi et al., 2016), as well as to inflammation of the central nervous system (Thaler et al., 2012, Bonda et al., 2014) due to an increase in pro inflammatory cytokines (Syal et al., 2020). Particular dietary components may play a crucial role in regulating AD risk, according to studies in both human and animal models (Moser and Pike, 2016)For instance, a diet heavy in fats is linked to obesity and, consequently, to a greater risk of AD (Barnard et al., 2014, Mostafa et al., 2016, Morris and Tangney, 2014).

According to a recent study, a high-fat diet results in damage that is comparable to that seen in Alzheimer's disease, including potentiation of APP processing by β–secretase (Maesako et al., 2015), cognitive decline (Knight et al., 2014) and mitochondrial damage linked to insulin resistance (Petrov et al., 2015). Millions of people throughout the world suffer from T2DM and obesity (Ott et al., 1999, Rosales-Corral et al., 2015).

Hyperglycemia, which causes an increase in hepatic glucose production, insulin resistance, and a reduction in pancreatic beta-cell formation are the hallmarks of T2DM (Ott et al., 1996). The primary energy source that neurons need is glucose, and any change in the metabolism of glucose impairs neuronal processes (Talbot et al., 2012). Numerous hypotheses have been put forth to explain the relationship between diabetes and dementia, including vascular lesions, inflammation, oxidative stress, elevated glycolysis end products, insulin resistance, abnormal insulin receptor signaling, and insulin degradation and its connection to Aβ protein deposits (Kapogiannis and Mattson, 2011). It's interesting to note that both illnesses have amyloidogenesis, which results in Aβ plaques (Ott et al., 1999). Diabetes is associated with neurodegeneration due to oxidative stress pathways and neuroinflammatory signals in the brain being affected by high glucose and insulin resistance (Rosales-Corral et al., 2015). Furthermore,

a lot of studies support the idea that AD is a response to the pathogenic energy imbalance in neurons that is brought on by glucose function problems (Mendiola-Precoma et al., 2016).

The control of metabolism and energy expenditure involves the molecule insulin. Insulin is regarded as a paracrine/autocrine effector in the brain because it binds to insulin receptors (IRs) and activates the IR substrate (IRS) via two established pathways, PI3K/Akt and Ras/mitogen-activated kinase cascades (Calvo-Ochoa and Arias, 2015)The anatomical and functional characteristics of synapses are thought to be regulated by central insulin, and mice with a neuron-specific insulin receptor deletion (NIRKO) exhibit insulin resistance, which increases GSK-3 activation and tau hyperphosphorylation (Kleinridders et al., 2015, Nuzzo et al., 2015). Insulin resistance reduces GLUT4, AMPA, and NMDAR export to the membrane through impairing IR/PI3K/Akt/mTOR insulin signaling. When all of these things happen simultaneously, tau hyperphosphorylation, LTP failure, and glutamate neurotransmission are all affected (Calvo-Ochoa and Arias, 2015). The fact that Aβ peptide may bind to both the IR and ApoE is particularly significant. ApoE attaches to the IR, and the various interactions between ApoE isoforms imply that the ApoE 4 genotype may result in an earlier disruption of brain insulin signaling (Chan et al., 2015).

4. Alzheimer Disease current treatment strategies

4.1. Pharmacological therapy

Pharmaceutical therapy known as pharmacotherapy that refers to the treatment of disease using drugs. It can be used to treat or prevent the development of disease, as well as to relieve pain and symptoms of a particular condition. As an age-related neurodegenerative ailment that is incurable, Alzheimer's disease demands accurate diagnosis, preferably early on, and sufficient etiological therapy. Its unique pathophysiology should also be considered. A better approach for this public health issue is prevention, as therapeutic solutions have mostly focused on symptom relief and slowing the rate at which damage is progressing. Despite this, the illness has not been

dramatically reversed (Knopman et al., 2021, Kumar et al., 2016). The progress of the illness is facilitated by toxic forms of tau or Aβ and inhibiting the production of these peptides may contribute to effective therapies. While evidence for the intricacy and multi causality of this dementia is acknowledged in fundamental and clinical investigations, the present therapies for this illness are centered on cholinesterase inhibitors and a glutamate antagonist, offering only symptomatic alleviation (Scheltens et al.). Clinical studies are presently being conducted to test etiology-based therapies, which will supplement preventative measures including exercise, a healthy diet, mental stimulation, and comorbidity management (Nelson and Tabet, 2015).

4.2. Symptomatic therapy

All currently approved AD treatments are "symptomatic" agents that aim to improve cognitive and behavioral symptoms without altering the underlying course of the disease. Most current drug development programs target disease management with agents that prevent or delay the onset or slow progression of AD (Cummings, 2021) doi.org/10.1186/s13024-021-00446-3.

4.2.1. Acetylcholinesterase Inhibitors

It is widely recognized that acetylcholine (ACh) is a key mediator of memory and learning (Mitsushima et al., 2013). Additionally, direct interaction between Aβ and cholinergic systems has been hypothesized, with negative feedback to the production of the peptide. It has been proposed that this direct interaction, with a focus on alpha-7 nicotinic acetylcholine receptors, decreased the effectiveness of cholinergic transmission (Puzzo et al., 2015, Garcia-Osta and Alberini, 2009). Based on this, cholinesterase inhibitors are an excellent therapy for AD and fit well with Davies and Maloney's (1976) early cholinergic deficiency theory explaining AD etiology. For the treatment of AD, analogs of tacrine, donepezil, rivastigmine, galantamine, xanthostigmine, para-aminobenzoic acid, coumarin, flavonoid, and pyrroloisoxazole have been created and explored. The authorized medications (FDA approved) rivastigmine, donepezil, and

galantamine increase ACh levels and enhance cholinergic function in the brain by blocking the acetylcholinesterase enzyme, which breaks down the neurotransmitter (Anand and Singh, 2013, Godyń et al., 2016). Acetylcholinesterase inhibitors, with the exception of tacrine, are often well tolerated, and side effects are dose dependent (Knopman et al., 2021).

4.2.2. Antagonist of the NMDA Receptor

It is well known that glutamate-mediated excitotoxicity causes calcium excess, mitochondrial malfunction, and increased nitric oxide production. These effects can be harmful to cells because they cause high amounts of oxidants and trigger neuronal death. Memantine, an NMDA receptor antagonist that the Food and Drug Administration (FDA) licensed in 2003 for the treatment of moderate-to-severe AD with a marginally positive effect on cognition in mild-to-moderate AD, can inhibit this overstimulation (Prentice et al., 2015, Shi et al., 2016). According to the type 3 diabetes hypothesis for AD, coordinate therapy is required for AD since it is a chronic degenerative illness associated with old age. In connection to other illnesses like metabolic syndrome, which encompasses atherogenic dyslipidemia and central obesity, hyperglycemia and insulin resistance, hypertension, a pro thrombotic state and a proinflammatory state, dyslipidemia and obesity are viewed as causal variables (Grundy, 2016, Srikanthan et al., 2016). Additionally, medications used to treat T2DM may help preserve neurons in AD. Moreover, being researched as therapies for AD are amylin and glucagon-like peptide-1 receptor agonists (Godyń et al., 2016).

4.2.3. Oligomannate sodium

Recently, increasing evidence suggests a link between gut microbiota disturbances and AD progression, but the role of gut microbiota in AD pathogenesis remains unclear. During the progression of AD, changes in the composition of gut microbiota cause the accumulation of phenylalanine and isoleucine, which stimulates the differentiation and proliferation of T helper-

1 (Th1) cells. Peripheral Th1 immune cells infiltrating the brain are associated with M1 microglia activation and contribute to AD-related neuroinflammation. GV-971, a sodium oligomannate that has shown robust and sustained improvement in cognition in phase 3 clinical trials in China, inhibits gut microbiota-associated phenylalanine/isoleucine accumulation and inflammation to reverse cognitive impairment (Wang et al., 2019). Sodium Oligomannate GV-971 is an orally administered mixture of acidic linear oligosaccharides (from dimers to reducing agents with molecular weight 670-880 Da) derived from marine brown algae. It was developed by Shanghai Green Valley Pharmaceuticals for the treatment of AD. Although the full mechanism of action of sodium oligomannate is still unclear, it has been shown to have a regenerative effect on the gut microbiome, which may limit the contribution of extracellular immunity. Sodium oligomannate also enters the blood-brain barrier via transporters, including glucose transporter type 1 (GLUT1), which binds to several subregions of Aβ to inhibit Aβ fiber formation and induce destabilize preformed fibers into nontoxic monomers (Syed, 2020).

4.2.4. 5-HT6 antagonists

5-HT6 antagonists have been the subject of several recent clinical trials. Intepirdine, idalopirdine, and masuperdine are drugs in this class that were evaluated in phase 3 development programs [32]. Not all trials have been successful in establishing a difference between drug and placebo in cognitive outcomes (Cummings, 2021). 5-HT6 receptors are found only in the central nervous system and primarily regulate gamma-aminobutyric acid (GABA) and glutamate levels and promote the secondary release of other neurotransmitters such as norepinephrine and acetylcholine that are impaired in Alzheimer's disease (Khoury et al., 2018).

4.2.5. Rotigotine dopamine agonist

The dopaminergic system may have a role in cognitive function, particularly in executive behavior mediated by the sub frontal cortical system. The dopaminergic midbrain is altered in AD, and there is substantial evidence that this structure is associated with cognitive and behavioral changes in AD [41]. Rotigotine, a dopaminergic drug approved for the treatment of motor

symptoms in Parkinson's disease, showed benefit in a frontal executive intervention in a phase 2 trial in Alzheimer's disease (Koch et al., 2020, Cummings, 2021).

4.2.6. Rasagiline monoamine oxidase inhibitor

Rasagiline, a monoamine oxidase inhibitor approved for motor symptoms in Parkinson's disease, improved his FDG-PET metabolism in a study of Alzheimer's disease and improved his performance on memory measures without improving performance in several administrations (in phase II). showed benefits in measures and quality of life measures (Matthews et al., 2020, Cummings, 2021).

4.2.7. Ladostigil multi-targeted drug

Ladostigil (TV3326), a multi-targeted drug with acetylcholinesterase inhibitor that also inhibits monoamine oxidases A and B, exhibits antidepressant benefits and is now undergoing phase II clinical studies (Folch et al., 2016). This drug has no effect on improving cognition (Schneider et al., 2019, Cummings, 2021).

5. Metformin or Glucophage (N, N-Dimethylimidodicarbonimidic diamide)

Metformin is a diabetes medication made from galengine, a natural product of the Galega officinalis plant (de la Monte et al., 2019). Metformin is a biguanide that comprises two pairs of guanidine molecules (Rena et al., 2017, Chaudhari et al., 2020) and has a chemical structure that is very hydrophilic (1, 1-dimethylbiguanide hydrochloride) (Markowicz-Piasecka et al., 2017). Metformin is the first-line therapy for T2DM, and most health guidelines recommend it since it has few adverse effects, is usually well absorbed, and is not linked to weight gain (Bendlin, 2019). Metformin inhibits hepatic gluconeogenesis and insulin resistance, resulting in decreased plasma

glucose levels (Markowicz-Piasecka et al., 2017). Metformin, on the other hand, may pass the blood–brain barrier (BBB) and has been linked to improved cognitive function (Lv et al., 2012). Metformin may also change the makeup of the gut microbiota, which may play a role in AD development (Syal et al., 2020).

5.1. Metformin as an Antidiabetic Drug Strategy for Alzheimer's Disease Treatment

Since the pathogenesis mechanism of Alzheimer's disease is not fully understood and only few drugs are available for Alzheimer's disease, there is an urgent need to identify new and potential therapeutic strategies for Alzheimer's disease. Considering the medicinal properties of metformin, which acts through various mechanisms related to homeostasis of glucose metabolism, insulin signaling, inflammation, oxidative stress, it can affect the severity of cognitive function and reduce dementia. Alzheimer's disease and type 2 diabetes share a common pathophysiology that involves impaired insulin signaling (insulin resistance) that greatly increases dementia (Ning et al., 2022).

The mechanisms of metformin effect in Alzheimer's disease can be very different and complex. This drug can act through the homeostasis of glucose metabolism, reducing the deposition of beta-amyloid plaques, normalizing tau phosphorylation and increasing autophagy. In addition to these features, reducing inflammation and oxidative stress is among the benefits of metformin in the process of increasing cognition and reversing Alzheimer's disease (Ning et al., 2022).

5.2. Preclinical Animal Studies with Metformin

A number of animal models have been established to recreate the human illness environment with the goal of developing medicines or disease modifying agents for AD treatment (Chen et al., 2021a, Nakai et al., 2021). Most AD animal models' primary goal is to generate the neuropathological characteristics that occur before cognitive loss (Correia et al., 2008, Trujillo-Estrada et al., 2021). Transgenic mice are valuable models for studying familial Alzheimer's

disease pathologies. Because they only imitate the symptomatic characteristics of AD common mutated genes, these models do not reflect all the abnormalities present in human AD and do not duplicate the sporadic types of AD. Transgenic innovation, on the other hand, provides a unique opportunity to reproduce the origin of familial AD by transfecting a mutant human APP (Nakai et al., 2021, Correia et al., 2008). Mice models helped us understand the molecular processes involved in Aβ synthesis, deposition, and clearance, as well as the influence of Aβ on the neural network and synapses (Laurijssens et al., 2013, Nakai et al., 2021). The APP mouse model was successful in producing a wide range of parenchymal and vascular amyloid deposits that were similar to those seen in human AD (Correia et al., 2008). The bulk of these animal models are transgenic mice created by over-expression of mutant human PS1, APP, and tau. Three mutant genes (human

PS1M146V, APPSwe, and tauP30L) are found in the triple-transgenic 3xTg-AD mice, which generate growing age-dependent amyloid plaques and NFTs, as well as memory problems (Chen et al., 2021c, Bomfim et al., 2012).

Many of the hallmark symptoms of AD, on the other hand, may be replicated by injecting pharmacological or chemical agents into the brain or activating lesions in particular brain areas (Chen et al., 2021c, Bomfim et al., 2012). Aβ peptide injection into the brain of a rat or a rhesus monkey, for example, has been employed in various investigations. Although these models generate some of the clinical symptoms, they do not accurately imitate the pathophysiology of AD. The chemical or physical degeneration of certain parts of the brain, such as the hippocampus, cortical, and striatal regions, which are typically either cholinergic or involved in cognitive functions, is included in lesion models (van der Flier PhD, 2021). In general, as a disease model, interventional models will be efficient in discovering symptomatic or therapeutic treatments. The Streptozotocin (STZ)-induced AD model and the scopolamine-mediated amnesia model that resulted in learning and memory loss as well as cognitive impairment (Chen et al., 2021c, Grieb, 2016) are examples of useful models. For example, endotoxins trigger inflammation in scopolamine-induced amnesia models, and brain metabolism interacts with other chemical action models (Laurijssens et al., 2013).

Because LOAD accounts for more than 95% of AD cases, animal models linked with it are useful research tools for researching pathophysiology and developing experimental treatments for sporadic AD (Chen et al., 2021c, Pilipenko et al., 2020). STZ is a diabetogenic drug that causes IR in pancreatic beta cells and is commonly used to induce diabetes in animals. Decreased glucose/energy metabolism in the brain correlates with the severity of dementia symptoms in AD and is a well-known sporadic AD brain anomaly (Trujillo-Estrada et al., 2021, Lu et al., 2020). BIR decreased brain glucose metabolism, tau and buildup, gliosis, cholinergic impairments, oxidative stress, and learning and memory problems in ICV-STZ animal models (Trujillo-Estrada et al., 2021, Salkovic-Petrisic et al., 2013). The metabolism of cerebral glucose is controlled by brain insulin signaling, and defective brain insulin signaling has been linked to Alzheimer's disease (Ou et al., 2018). In rats treated with STZ, hyperphosphorylation of tau, an increase in Aβ 42/40, and both GSK-3 and BACE1 activities were found, as well as a lack of dendritic and synaptic plasticity (Salkovic-Petrisic et al., 2013, Grünblatt et al., 2007). Dr. Hoyer proposed in January 2013 that ICV STZ is the non-transgenic metabolic type of sporadic AD (Salkovic-Petrisic et al., 2013). Hoyer's thinking began with the finding that, whereas LOAD lowers both oxygen and glucose intake in the brain, the drop in cerebral oxygen consumption is much less (Salkovic-Petrisic et al., 2013, Saffari et al., 2020). The primary biochemical change in incipient LOAD, according to these findings, is linked to the control of cerebral glucose metabolism, which leads to an alteration of a signal transduction insufficiency of the cerebral insulin receptor (Saffari et al., 2020). According to Saffari and colleagues' findings, an ICV injection of STZ reduced spatial learning and memory significantly, but metformin delivered in treatment phosphatidyl-serine nanoliposomes formulation increased learning and memory. In the STZ rat model of LOAD, metformin increased spatial learning and decreased neuroinflammation [89]. Furthermore, in a LOAD rat model, Pilipenko et al. found that metformin corrected STZ-induced deficits in spatial learning/memory capacity and sociability, as well as normalization of brain glucose transport, uptake, and metabolism, as well as improved microgliosis and astrogliosis (Pilipenko et al., 2020).

Metformin is an effective treatment for improving insulin sensitivity, according to Ditacchio and colleagues' investigations, with a larger decline in blood glucose levels in the APP AD model (DiTacchio et al., 2015). Farr and colleagues also looked at how metformin affected the

expression of APPc99, APP, Aβ, G3DPH, and p-tau in SAMP8 mice (Farr et al., 2019). They discovered that after using metformin, the expression of APPc99 and p-tau reduced. Metformin therapy in SAMP8 resulted in a considerable reduction in hyperphosphorylated tau and APPc99 proteins, which improved learning and memory functions. Furthermore, Ditacchio and colleagues discovered that App transgenic female mice given metformin had improved cognitive capacities (DiTacchio et al., 2015). These findings were confirmed in a different genetic model of AMPK activation, in which an unknown structural mechanism impaired AD-related cognitive function in these animals downstream of hepatic AMPK activation (Farr et al., 2019). Furthermore, the scientists conducted research in both males and females, and the findings revealed that metformin's positive benefits were larger in females than in males (Farr et al., 2019). This lends credence to the notion that the efficacy of this medicine may be influenced by gender.

Finally, according to Morris water maze and Y-maze data, Lu and colleagues revealed that metformin enhanced learning and memory performance in APP/PS1 transgenic mice (Lu et al., 2020). In another work, metformin increased microglial autophagy in the APP/PS1 mouse model, allowing pathogenic Aβ and tau proteins to be phagocytized, lowering a deposits and limiting tau pathology distribution (Oliveira et al., 2016). Similarly, Ou and colleagues also reported that metformin treatment in APP/PS1 exerts multiple beneficial effects in the brain neuropathology (Ou et al., 2018). Thus, metformin treatment improved the cognitive process and neurogenesis, exerting neuroprotective effects on the hippocampus. Moreover, metformin, probably through the modulation of the AMPK/mTOR/S6K/BACE1 signaling pathway, also improved the amyloidogenic pathway and prevented the neuroinflammatory process (Ou et al., 2018).

5.3. Metformin in Clinical Studies

Metformin has been proven to prevent or delay the onset of dementia in diabetic people (Chin-Hsiao, 2019). Shi and colleagues investigated the impact of metformin on older adult US veterans with T2DM and neurodegeneration in 2019 (Shi et al., 2019). According to the findings of this investigation, metformin medication for 2–4 years reduces the incidence of neurodegeneration in people with T2DM compared to those who do not get metformin treatment (Shi et al., 2019).

From 2008 to 2012, Columbia University in New York City conducted a pilot study of 80 patients with amnestic mild cognitive impairment. Despite the fact that all of the volunteers were overweight, none of them had diabetes. For one year, they were given either 2000 mg of metformin divided into two doses or a placebo. The major outcomes were the selective reminding test (SRT) for recall and the ADAS-Cog (Luchsinger et al., 2016). FDG-PET glucose absorption in the posterior cingulate/precuneus, as well as plasma levels of A42, the most lethal version of the Aβ peptide, were used as secondary endpoints. The metformin group outperformed the placebo group on the SRT by a little margin. Between courses, there were no variations in the ADAS-Cog, glucose uptake, or plasma Aβ 42. Only 10% of patients were able to take the maximum dose of metformin, with the majority of patients receiving 1000 or 1500 mg per day (Luchsinger et al., 2016, Lu et al., 2020). The study's major finding was that metformin improves recall effectiveness in the SRT.

Small research conducted at the University of Pennsylvania from 2013 to 2015 looked at the impact of metformin on biomarkers of AD in 20 non-diabetic people with mild cognitive impairment or dementia. The diagnosis of AD was confirmed using MRI, FDG-PET, and amyloid biomarkers (Luchsinger et al., 2017, Koenig et al., 2017). In a crossover trial, each participant was given metformin at a daily dose of 2000 mg for eight weeks, then placebo for eight weeks, or vice versa. The ADAS-Cog and CANTAB batteries were used to evaluate cognitive performance in a variety of learning and memory domains, as well as executive processing, attention, expressiveness, and motor speed. The amounts of Aβ, total tau, and tau in the cerebrospinal fluid (CSF) were also measured, and blood flow in the brain was assessed by arterial spin marking (Chen et al., 2021b).

In February 2020, Swedish researchers initiated a year-long study in 80 persons with T2DM and moderate cognitive impairment to see how a year of metformin medication combined with exercise and nutrition affected memory. The key consequences are recruitment, adherence, and retention rates, with metabolic improvement and memory ability as secondary metrics. (https://www.alzforum.org/therapeutics/metformin) The study will run through December 2021. (Accessed on 23 August 2021).

In turn, Samaras and colleagues compared the effectiveness of metformin in diabetes individuals with cognitive decline and dementia risk. After 6 years of research, researchers discovered that metformin treatment in older persons with T2DM was linked to a lower incidence of dementia (Madhu et al., 2022). In addition, Scherrer and colleagues found that giving metformin to elderly African American and white patients reduces the risk of dementia by 29% and 40%, respectively, in African American and white patients aged 65 to 74 years and 50 to 64 years, respectively, using data from the Veterans Health Administration (VHA) medical record. These findings are intriguing because they back up the hypothesis that metformin might reduce the incidence of dementia in elderly adults (Scherrer et al., 2019).

Sluggett and colleagues found that Finnish patients with T2DM and long-term metformin therapy had a decreased chance of acquiring AD. The findings of this investigation support the concept that glucose-lowering medicines may be useful pharmacological choices for altering the course of illness and delaying the onset of dementia (Sluggett et al., 2020).

Other investigations, such as those conducted by Koo and colleagues (Koo et al., 2019), found that metformin therapy was ineffective and even impaired the cognitive condition of elderly Korean patients. As a result, additional research is needed to determine the effects of metformin in diabetes individuals with cognitive impairment (Samaras et al., 2020, Campbell et al., 2018).

6. Molecular Mechanism Involved in Neuroprotective Effects of Metformin in Alzheimer's Disease

6.1. Metformin Effects on Amyloid and Tau

Previous research has found that AMPK is strongly expressed in the hippocampus, a brain area involved in synaptic plasticity, memory, and cognition, as well as abnormal AMPK activity in the brains of transgenic mice models of AD and AD patients (Ramamurthy and Ronnett, 2012, Gravandi et al., 2021). According to the amyloidogenic theory of AD, Aβ oligomers inhibit AMPK, thereby increasing the likelihood of metabolic dysfunction in hippocampal neurons, which might

play a crucial role in early metabolic abnormalities in the LOAD brain (da Silva et al., 2017). As a result, metformin, which can enhance AMPK activation, could be an appealing target for compensating for this energy loss in the nervous system. Furthermore, AMPK activation can lower Aβ levels by lowering BACE1 expression and thereby lowering brain Aβ levels (Culmsee et al., 2001, Gupta et al., 2011). Furthermore, AMPK may promote autophagy, which may be beneficial to LOAD (Chen et al., 2021a, Kodali et al., 2021). Previous research has found that activating autophagy reduces pathology and improves cognitive function in preclinical mice (M Wilson et al., 2014). As a result, metformin may increase brain autophagic activity, assisting in the removal of waste proteins and aiding in the treatment of Alzheimer's disease (M Wilson et al., 2014).It is widely known that various kinases control tau phosphorylation, one of which is AMPK, a tau kinase that functions by phosphorylating numerous tau sites (Cantó et al., 2009, Cantó and Auwerx, 2010). The process of tau regulation by AMPK, on the other hand, is complicated since it may be influenced by both direct and indirect pathways. Salicylate, an AMPK agonist, and wortmannin, a GSK-3 inhibitor, both lower tau phosphorylation, according to Wang and colleagues (Yarchoan et al., 2014). AMPK may also phosphorylate the Ser9 site of GSK-3, causing it to become inhibited, which could explain its role in the control of this regulatory mechanism in tau phosphorylation (Wang et al., 2020a, Vingtdeux et al., 2011). Apart from directly regulating tau phosphorylation, AMPK also stimulates SIRT1, a deacetylase enzyme that can suppress tau hyperphosphorylation by improving or augmenting the deacetylation process (Cantó and Auwerx, 2010). Similarly, the protein phosphatase 2A is involved in another process that modulates both tau acetylation and phosphorylation (PP2A). Metformin causes tau dephosphorylation by directly activating PP2A, according to a study (Vingtdeux et al., 2011). As a result, metformin might be an effective therapy option for metabolic risk factors linked to LOAD.

6.2. Metformin Effects on Mitochondria

In the therapy of LOAD, a technique based on "brain energy rescue" has been proposed (Cunnane et al., 2020). The goal of this method is to keep the brain's energy levels stable or to restore them. Metformin therapy, via enhancing mitochondrial activity and peripheral and cerebral glucose

metabolism, might be a possible brain energy rescue approach. The pathophysiology of LOAD is well recognized to entail mitochondrial metabolic abnormalities (Cantó et al., 2009, Cantó and Auwerx, 2010). As a result, it's been suggested that AMPK can control mitochondrial synthesis and autophagy's major tasks. Mitochondrial damage, which emerges before NFTs and is associated by tau protein phosphorylation, has been demonstrated in previous investigations to be an early indicator of LOAD (Cantó and Auwerx, 2010). Thus, metformin-induced AMPK activation can promote mitochondrial biogenesis via modulating the activity of peroxisome proliferator activated receptor ɣ coactivator-1α (PGC-1α, a nuclear transcriptional coactivator) (da Silva et al., 2017). Furthermore, as previously stated, metformin may increase mitochondrial autophagy via AMPK activation, thus promoting the removal of damaged/defective mitochondria, boosting ATP generation, and lowering reactive oxygen species formation (Wang et al., 2020a). As a result, it's possible that metformin's activation of AMPK causes an increase in cellular autophagy and ATP production, which helps to alleviate LOAD symptoms.

6.3. Metformin Effects on Neurogenesis: The AMPK/aPKC/CBP Signaling Pathway

Metformin is implicated in two independent molecular processes that help adult neural progenitor cells (NPCs) proliferate, regenerate, and differentiate (Kickstein et al., 2010). Metformin stimulates AMPK, which initiates the aPKC-CBP cascade to facilitate neuronal development in the first route. When AMPK is activated, aPKC is activated, which then phosphorylates CREB-binding protein (CBP) at Ser133, facilitating neurogenesis and increasing spatial memory formation in adult mice (Kickstein et al., 2010, Domise et al., 2016). Metformin enhances the synthesis of key proteins involved in adult NPC self-renewal via the second route, which up-regulates the expression of TAp73 mRNA. P73 is a transcription factor that plays an important function in neural stem cells and rises in expression as they differentiate (Domise et al., 2016). The capacity of metformin to induce neurogenesis in individuals with cognitive impairment associated with T1DM and T2DM is potentially encouraging (Vingtdeux et al., 2011, Cunnane et al., 2020, Domise et al., 2016). Previous research has shown that long-term oral metformin therapy improves hippocampal neurogenesis and spatial memory, as well as inducing

chronic microglial activation and improving the glucose-lowering effect of the phosphorus-relation of AMPK/aPKC f/k/IRS1 serine residues in the hippocampus of middle-aged diabetic mice (Domise et al., 2016). These findings are in line with recent research showing that prolonged metformin treatment protects hippocampus neurogenesis and protects against neurological problems caused by a high-fat diet (Vingtdeux et al., 2011). In light of the critical functions of AMPK in intracellular metabolism in LOAD, metformin might be introduced as an appropriate and appealing therapeutic target(Wang et al., 2020a).

Metformin improves the makeup of the gut microbiota of obese mice, according to Ma and colleagues' research. The neuroinflammatory process in the hippocampus might be inhibited by this peripheral action. In obese mice, this medicine might also prevent the degeneration of newborn neurons in the hippocampus, hence improving learning and memory. These findings support the concept that improving the cognitive process in LOAD by intervening at the microbiome level (Ma et al., 2021).

6.4. Metformin Effects on Learning and Memory

Cognition is one of the brain's most complicated functions, including perception, registration, consolidation, storage, and memory over the duration of a person's life (Cunnane et al., 2020). Any memory loss, such as amnesia, has a significant impact on a person's quality of life and is considered a major CNS disease. It is attributed to a decline in neuronal population as a result of ageing, neurodegenerative disorders, head injuries, brain defects, genetic anomalies, and other factors (Peng et al., 2020). Evidence shows that diabetic therapy can improve cognitive skills in model animals and individuals with diabetes. Similarly, pioglitazone, a thiazolidine-based diabetic treatment, reduces the incidence of dementia in diabetic patients and improves glucose metabolism and cognitive function in people with LOAD and diabetes (Peng et al., 2020, Swerdlow, 2018). Metformin treatment has been proven to significantly improve memory deficiencies. Mostafa and colleagues investigated the effects of acute metformin treatment in a scopolamine-amnesic mouse model (with impaired learning and memory abilities) for roughly

two weeks, and found that metformin had beneficial effects on memory (Mostafa et al., 2016). Metformin's neuroprotective impact was due to a variety of molecular mechanisms, as it displayed substantial antioxidant and anti-inflammatory activity. However, the authors believe that its protective effect against scopolamine-induced cognitive impairment is due to the Akt/GSK3 beta signaling pathway and the inhibition of tau protein phosphorylation. Similarly, scopolamine medication has been demonstrated to lower p-AMPK and CREB levels in the hippocampus, while metformin treatment has successfully restored p- AMPK and the transcription factor CREB levels (Mostafa et al., 2016, Ma et al., 2021). Metformin was also shown to boost the amounts of antioxidant enzymes in the hippocampus nucleus, such as superoxide dismutase (Ma et al., 2021). The hypothesis that metformin might be a viable preventative medicine for cognitive and memory impairment (Katila et al., 2020, Aksoz et al., 2019, Ma et al., 2021, Chung et al., 2015, Grillo et al., 2015) is supported by these findings. Metformin has also been demonstrated to protect cognitive damage in the chronic L-methionine model of memory impairment, most likely via reducing oxidative damage (Chung et al., 2015).

C57BL6/J mice with late middle age enhanced recognition memory in old age following 10 weeks of metformin therapy, according to Kodali and colleagues (Kodali et al., 2021). Metformin therapy decreased astrocyte hypertrophy and shifted microglial cells to an anti-inflammatory M2 phenotype in the hippocampus. Furthermore, by activating AMPK and inhibiting mTOR signaling, it lowered the concentration of proinflammatory cytokines and increased autophagy activities.

Similarly, because the hippocampus is a key region of the brain for memory and cognition and is commonly impaired in Alzheimer's disease, increasing neuronal activity and synaptic transmission in the hippocampus is critical. Metformin enhanced synapsis, memory, and cognitive impairments in patients with disrupted hippocampal synaptic transmission, according to Chen and colleagues (Chen et al., 2020). Enhanced presynaptic glutamate release might explain the increased miniature excitatory postsynaptic currents (mEPSC) into CA1 pyramidal neurons in the hippocampus (Chen et al., 2020). Similarly, Asadbegi and colleagues found that metformin therapy improved long-term potentiation in rats following an Aβ-injection considerably. Furthermore, rats were fed a high-fat diet (HFD), and metformin therapy protected hippocampus synaptic plasticity from the negative effects of Aβ and HFD (Asadbegi et al., 2016).

In turn, Li and colleagues looked examined the effects of a 200 mg kg1 d1 intraperitoneal injection of metformin for 18 weeks in db/db mice, which show many AD-related brain abnormalities such altered cognitive skills, elevated phosphotau and Aβ, and reduced synaptic proteins. Metformin reduced total tau, phosphotau, and activated c-Jun-N-terminal kinase levels in the hippocampus membrane (Li et al., 2012). Metformin therapy also enhanced synaptophysin, a synaptic protein, in the hippocampus of db/db mice (Li et al., 2012).

6.5. Metformin Effects on Synaptic Density and Dendritic Spines

Clinical and experimental research have supported the idea that stimulating insulin receptors improves cognition. As a result, insulin is widely acknowledged as improving emotional function in active and old persons, as well as Alzheimer's sufferers (Boccardi et al., 2019). Insulin signaling can impact synaptic plasticity by regulating glutamate receptor production and trafficking, and insulin receptors are abundant in hippocampal synapses, where they are thought to regulate synaptic plasticity through interactions with the glutamatergic system (Aksoz et al., 2019, Boccardi et al., 2019, Chen et al., 2020). Furthermore, synaptic markers and/or dendritic spine abnormalities arise before the formation of plaques and NFTs, showing that these processes are strongly connected to cognitive loss in AD (Poor et al., 2021). Selective reduction of thin spines is linked to impaired learning capacity in aged rhesus monkeys (Boros et al., 2017, Walker and Herskowitz, 2021). Furthermore, according to Morrison and Baxter, lowering spine shape might have a negative influence on prefrontal synaptic plasticity, which is necessary for appropriate functioning in the elderly (Morrison and Baxter, 2014). The preservation of thin and mushroom spine populations (another spine type) in the dorsal-lateral-prefrontal-cortex (DLPFC) distinguishes cognitively normal older persons with AD pathology from patients with AD dementia (Boros et al., 2017). This alteration could be linked to the mild cognitive impairment (MCI) that can be detected early in Alzheimer's disease patients (Boros et al., 2017), confirming that synaptic loss is a key factor in the disease's progression (Rodriguez-Rodriguez et al., 2017) and providing cellular evidence that dendritic spine remodeling could be a cognitive resilience process. All of these data back up the theory that synapse function and behavior are linked to

cognition ability (Dumitriu et al., 2010, Scheff et al., 2014). As a result, the cellular and molecular mechanisms that govern synapses might be exploited to treat cognitive impairment in people with AD (Walker and Herskowitz, 2021).

The loss of synaptic activity in the AD brain has been linked to cognitive impairments (Yamazaki et al., 2019, Dumitriu et al., 2010, Scheff et al., 2014). Through synaptic plasticity, metformin has been demonstrated to promote memory formation (Cardoso and Moreira, 2020, Soo et al., 2020). In the AD-associated neurodegenerative phase, abnormal adult hippocampal neurogenesis is also implicated, in addition to synaptic dysfunction and loss of neuronal integrity in mature neuronal circuitry. CDK5 is a serine/threonine kinase that is activated by p35/p39 neuron-specific activators and is involved in synaptic plasticity, neuronal activity, and cognitive function (Wang et al., 2020b). Synaptic depression is caused by the proteolytic cleavage of p35 to p25, which results in delayed and abnormal activation of CDK5 and closely resembles early AD pathophysiology (Liu et al., 2019). As a result, CDK5 inhibition may be a potential technique for the development of anti-medications. Alzheimer's in the hippocampus of APP/PS1 mice, metformin reduced CDK5 hyper-activation and CDK5-dependent tau hyper-phosphorylation (Wang et al., 2020b, Cai et al., 2020). CDK5 activation by hyperglycemia is implicated in neuronal death, according to Liu and colleagues (Liu et al., 2019). Furthermore, CDK5 phosphorylates the PPAR receptor on serine residue 273, preventing anti-obesity actions from being transcribed and encouraging weight gain. In this regard, Cai and colleagues suggested that hyperacetylation of H3K9 histone on the CDK5 promoter might represent a connection between AD and T2DM (Cai et al., 2020).

Spine loss is seen in transgenic APP/PS1 mice, as seen by lower spine density in CA1 pyramidal neurons. Chronic metformin therapy for 10 days in the hippocampus of APP/PS1 mice reduces synaptic abnormalities, including surface GluA1 expression, decrease spine disappearance, and reduction in basal synaptic transmission, according to Wang and colleagues (Wang et al., 2020b). Furthermore, theta burst stimulation-induced CA3-CA1 LTP was reduced in highly primed hippocampal slices from APP/PS1 mice, but the LTP deficit was restored by continuous treatment with metformin for 10 days (Wang et al., 2020b). Using paired-pulse ratios (PPR), increased presynaptic glutamate release from terminals innervating CA1 hippocampal pyramidal neurons

was detected, but the excitability of CA1 pyramidal neurons was not changed. This data suggests that metformin increases glutamatergic signaling in hippocampal CA1 rather than GABAergic signaling, offering new information regarding metformin's effects on neurons.

6.6. Metformin Effects on Neuroinflammation

Metformin has anti-inflammatory properties, according to Ha and colleagues (Ha et al., 2019). Microglial cells, which are resident phagocytes in the CNS, are required for the neuroinflammatory response. When microglial cells are stimulated by danger-associated molecular patterns (DAMPs) like S100A8, S100A9, Aβ, or pathogen-associated molecular patterns (PAMPs) like lipopolysaccharides (LPS), they begin an innate immune response (Ha et al., 2019). Metformin therapy reduced various inflammatory responses in BV-2 microglial cells, including the release of pro-inflammatory cytokines such as TNF and interleukin IL-6, suggesting that it might be a key autophagy regulator and anti-neuroinflammatory medication (Ha et al., 2019). Metformin lowered the incidence of clinical stroke in individuals with diabetes and slowed post-stroke brain atrophy volume in mice with temporary middle cerebral artery blockage, according to Liu and colleagues (tMCAO). Metformin treatment improved longevity in normal mice by inhibiting chronic inflammation and stimulating neurogenesis through regulation of the CREB-binding protein (CBP)-protein kinase C (PKC) pathway (Liu et al., 2014). In APP/PS1 mice, accumulating raises the levels of the proinflammatory mediators IL-1 and IL-6 (Chen et al., 2021c). Metformin has been shown to reduce the levels of IL-1 and IL-6 in APP/PS1 mice(Chen et al., 2021c). Moreover, multiple studies have emphasized metformin's anti-inflammatory and antioxidant properties, with various pathways playing a crucial role in AMPK activation (Ha et al., 2019, Wareski et al., 2009). Metformin inhibits inflammation and reduces or eliminates inflammatory mediators in some circumstances, mostly through dependent pathways and frequently independently of AMPK at the cellular and systemic levels (Liu et al., 2014, Hettich et al., 2014). Metformin also controls the cell's antioxidant activity, which helps to reduce the quantity of oxidative stress factors (Rena et al., 2017, Markowicz-Piasecka et al., 2017). Metformin's anti-inflammatory effects may be attributed to a reduction in nuclear factor kappa

B (NF-kB) expression (Markowicz-Piasecka et al., 2017). Multiple inflammatory pathways, cell death, and tissue degradation are all mediated by NF-kB (Ha et al., 2019). Furthermore, AGEs have been shown to be one of the most critical inflammatory contributors in the development of diabetes. Macrophages have a role in the inflammatory process by boosting the production of pro-inflammatory cytokines (IL-1, IL-6, and TNF-), as well as enhancing the expression of RAGE receptor and activating the NF-kB pathway. The inflammatory activity of AGE-stimulated macrophages/microglial cells is aided by RAGE/NF-kB signaling. Metformin inhibits the RAGE/NF-kB pathway by activating AMPK and inhibiting NF-kB, resulting in reduced AGE effects and, at the brain level, decreased microglia activation, preferring the M2 (anti-inflammatory) phenotype over the M1 (classic or inflammatory) phenotype (Hettich et al., 2014). On the other hand, metformin has been shown to reduce ROS generation by directly inhibiting the chain of complex I's electron transfer complex (NADH ubiquitin oxidoreductase) (Miziak et al., 2021, Fatt et al., 2015). Other ways for reducing ROS include the activation of antioxidant enzymes like catalase, which is the major decomposer of H2O2, stimulating the endogenous antioxidant system, which comprises glutathione reductase (GSH), superoxide dismutase (SOD), and catalase (CAT) (Miziak et al., 2021, Wareski et al., 2009). Metformin has also been shown to maintain the nuclear factor related to erythroid 2 (Nrf2), an oxidative stress sensor, and promote its gene expression via AMPK. Increased levels of antioxidant system enzymes such as CAT, GSH, and SOD have been linked to Nrf2 pathway induction (Miziak et al., 2021, Fatt et al., 2015). Metformin enhances the start of this pathway by inducing AMPK activation, which may explain its antioxidant properties. Local inflammation, as well as microglia proliferation, activation, and phagocyte infiltration, were employed in various investigations on mice with traumatic spinal cord injury (Dziedzic et al., 2020). Metformin treatment decreased demyelination and inflammation in a demyelinating setting produced by lysolecithin and maintained the functional integrity of the optic tract, as evaluated by visual evoked potential recording (Esmaeilnejad et al., 2021). Furthermore, metformin's potential utility in multiple sclerosis was recently studied (fig.3) (Dziedzic et al., 2020).

All these findings suggest that metformin has antioxidant and anti-inflammatory properties in various situations. As a result, we may infer that metformin may be a viable treatment option for

numerous neurodegenerative illnesses in which inflammatory pathways and oxidative stress play a role in aetiology (Hasanpour Dehkordi et al., 2019, Wareski et al., 2009).

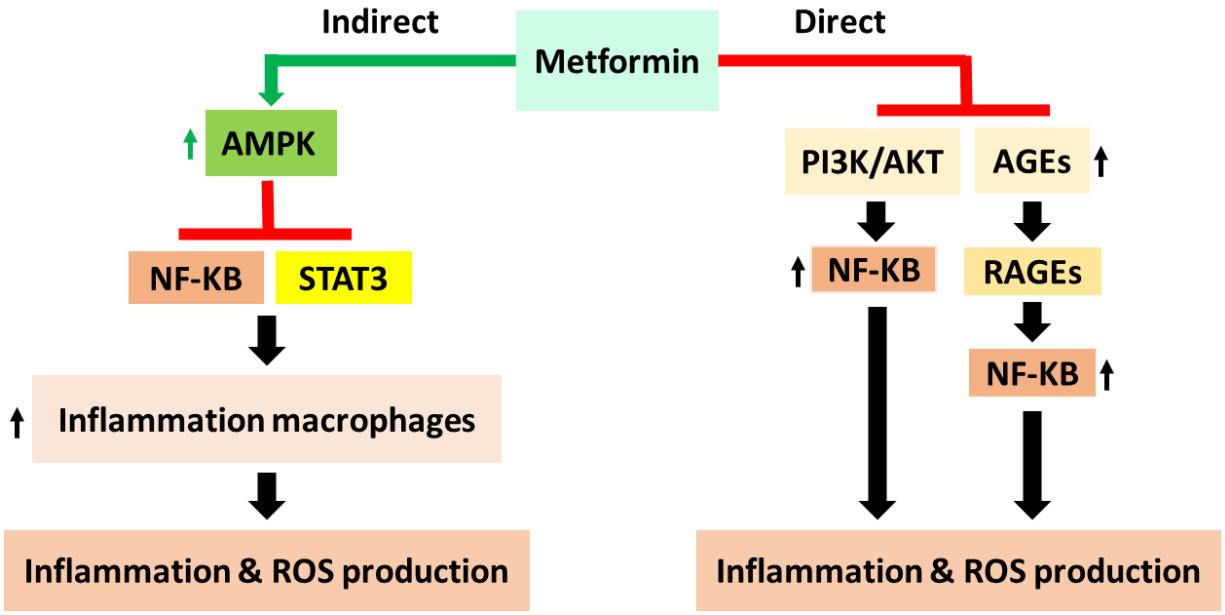


Figure 3. Metformin mechanisms to decrease inflammation and production and increase ROS.

6.7. Neuroprotective and Neurorestorative Potential of Metformin

To further understand the role of metformin in neuroprotection, Chung and colleagues looked at genes and proteins whose expressions or functions were either directly or indirectly impacted by the AMPK pathway (Chung et al., 2015). Many basic cell type functions (e.g., mitochondrial biogenesis, cellular synthetic activity, anti-inflammation, anti-oxidative stress, cell growth, and proliferation) and molecular pathways (e.g., incorporation of proper effects through AMPK-PPAR, AMPK-PGC1 alpha, AMPK-PFK, AMPK-FOXO, and AMPK-mTOR signaling cascades) can all work independently of AMPK (Chung et al., 2015). Down regulation of AMPK and downstream signaling

pathways results in the production of AGEs, as well as an increase in the mortality of human neural stem cells (hNSCs) and mitochondrial dysfunction. AGEs have also been found to impair mitochondrial capacity in several investigations, and Wareski and colleagues showed that AMPK stimulation enhances mitochondrial activity by activating PGC1 (Wareski et al., 2009). Metformin improves AMPK, PGC1, NRF-1, and Tfam expressions in age-treated hNSCs, which may contribute to the observed increase in mitochondrial functions. Furthermore, metformin-enhanced neuroprotective gene expression can protect hNSCs from AGE-induced toxicity (Hasanpour Dehkordi et al., 2019). Metformin has been shown to have a possible neurorestorative effect by Fatt and colleagues (Fatt et al., 2015).

Metformin therapy enhances NPC proliferation, self-renewal, and neuronal differentiation, according to the researchers (Fatt et al., 2015). Metformin treatment controlled this process primarily by activating TAp73 gene expression in mature NPCs and by activating AMPK which activating the aPKC-CBP cascade (Fatt et al., 2015).

Diabetes, asthma, dyslipidemia, and cardiovascular disease are only a few of the comorbid illnesses connected to dementia in the aged (van der Flier PhD, 2021, Knopman et al., 2021, Avila and Perry, 2021). As a result, all these problems may make LOAD therapy more difficult. As a result, it has been suggested that a multi-drug combination therapy may be required to reduce or stop the progression of the disease (Gaugler et al., 2021, Ballard et al., 2020). In this regard, adding a medicine like metformin to a combination therapy with three or four agents (anticholinergics, memantine, aducanumab, sodium oligomannate (GV-971), and anti-inflammatories) might be beneficial in treating hypometabolism and enhancing glucose absorption in the brain. As a result, metformin (and other anti-diabetic medicines) can provide value by enhancing glucose transport to neurons and ATP levels (Poor et al., 2021). Metformin, according to the research, can be used to slow the course of dementia and can be a unique treatment drug for LOAD-related cognitive impairment (Markowicz-Piasecka et al., 2017). Metformin is generally thought to be a safe and well-tolerated medicine. It has, however, been noted that gastrointestinal side effects, such as diarrhea, nausea, and vomiting, have appeared (Dziedzic et al., 2020). Headache, hypoglycemia, weakness, and rhinitis are among the less common symptoms. However, caution is advised because metformin comes with a serious

warning about the danger of lactic acidosis (Corcoran and Jacobs, 2021). This is an uncommon yet significant adverse effect that occurs in 1 out of every 30,000 people. As a result, metabolic acidosis causes a drop in blood pH, resulting in nonspecific signs and symptoms such as respiratory distress, high lactate levels, and acidosis (Corcoran and Jacobs, 2021). Metformin, which has beneficial effects at both the central and peripheral levels, might be a good option for preventing not just LOAD but also other neurodegenerative illnesses, according to its multi-directional routes. Metformin is a drug that passes the blood-brain barrier and operates centrally by having a neuroprotective effect. It may also aid neurogenesis and the formation of spatial memory. Metformin may perform a neuroprotective effect by addressing the markers of brain damage, in addition to the cognitive and behavioral impairments that accompany the onset of LOAD (metabolic dysfunction, synaptic dystrophy and cellular loss). Preclinical studies from metformin therapy in transgenic mice show that spatial memory, as well as neuroprotection and neurogenesis in the hippocampus, can be enhanced. Metformin can also reduce amyloidogenesis and inflammatory responses by regulating AMPK/mTOR/S6K/Bace1 signaling and blocking NF-kβ. In terms of clinical trials, the authors recommend that future research include biomarkers of Alzheimer's disease in the CSF or imaging markers such as PET linked with amyloid ligands, so that the findings support metformin's modifying function in the LOAD. In turn, trials of metformin in older persons with diabetes found that the treatment improved global cognition and lowered the risk of dementia when compared to older adults with diabetes who were not given the medicine. As a result, further clinical trials are needed to validate the relevance of metformin in a prospective combination treatment for the prevention of LOAD.

7. AD treatment based on stem cell therapy

The underlying cognitive decline in AD is the result of the loss of neurons and neural processes due to a range of factors. To date, all attempts to develop therapies targeting specific AD-related pathways have failed in late-stage human trials (Tong et al., 2015). Accordingly, an emerging consensus in the field is that treating AD patients with currently available drugs may come too

late, possibly due to significant neuronal loss in the brain. In this regard, cell replacement therapies, such as neurons derived from human embryonic stem cells (hESC) or induced pluripotent stem cells (iPSC), show potential for the treatment of AD patients. With the advent of stem cell technology and the ability to transform these cells into different types of CNS and glial cells, several successes in stem cell therapy have been reported. (Reported in AD animal models)(Tong et al., 2015).

As mentioned earlier, several factors are involved in the pathogenesis of AD; with the advancement of stem cell technology and the ability to generate different types of neurons and glial cells from stem cells, it is hoped that stem cell therapy is a new treatment for AD disease.

Thus, some success with stem cell therapy has been achieved in various animal models of Alzheimer's disease as a proof of concept(Tong et al., 2015).

Current treatments target only symptom relief using various drugs and do not cure the disease. Recently, stem cell therapy has emerged as a potential approach for various diseases, including neurodegenerative disorders, as well as IPSC and hESC, neural progenitor cells (NPCs) also can replace lost neurons.

8. Neural stem/progenitor cells (NSPCs)

Neural progenitors are cells that are capable of dividing a limited number of times and have the capacity to differentiate into a restricted repertoire of neuronal and glial cell types (fig.4).

"Stem cells" can undergo self-renewal division to produce additional stem cells with the same properties and potential, and to divide to produce daughter cells that differentiate into multiple cell type’s cell. Stem cells can be "pluripotent progenitor cells" that give rise to all cell types in an organism, or in other words pluripotent progenitor cells have the ability to differentiate into a subset of cell types. Embryonic stem cells present in the inner cell mass of the blastocyst are an example of pluripotent stem cells. Many types of pluripotent stem cells exist and may also be

referred to as "progenitor cells". Embryonic layers and specific tissues, such as CNS tissue, which develops from cell division of progenitor cells (Martínez-Cerdeño and Noctor, 2018).

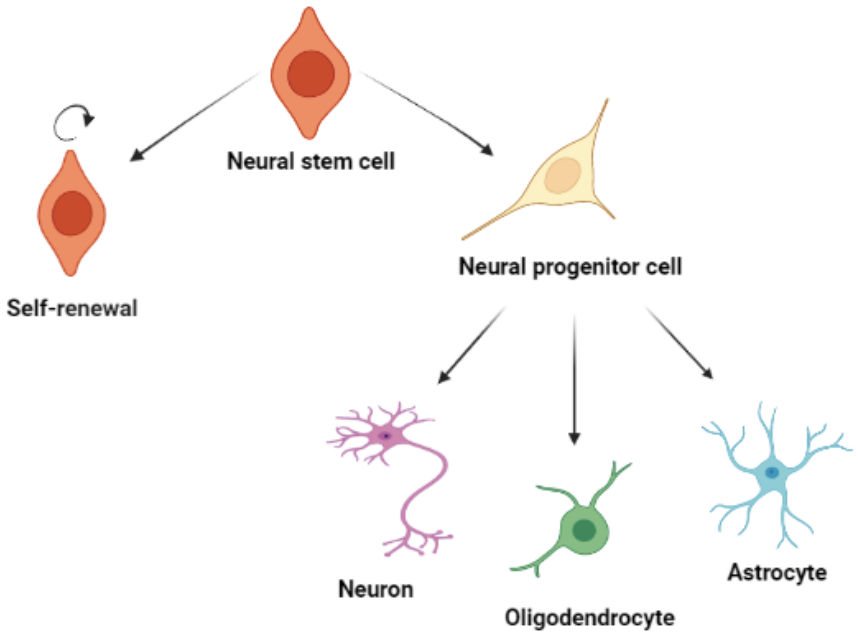


Figure 4. Differentiation of neural stem cells. Neural stem cells increase the accumulation of neural stem cells by self-renewal and differentiate into neural progenitor cells, produce neurons, oligodendrocytes and astrocytes.

Neural progenitor cells (NPCs) are CNS progenitor cells that give rise to many, if not all, types of glial cells and central nervous system neurons. NPCs do not produce non-neuronal cells that are also present in the CNS, such as those of the immune system. NPCs are present in the central nervous system of the developing embryo but are also found in the brains of infants and adults and are therefore not embryonic stem cells. "Embryonic NPCs" can eventually give rise to "adult NPCs", such as in the cerebral cortex (Martínez-Cerdeño and Noctor, 2018, Merkle et al., 2004).

NPCs are characterized by their location within the brain, morphology, gene expression profile, temporal distribution, and their function. In general, embryonic NPCs have additional potential than NPCs in the adult brain. NPCs may be generated in vitro by differentiating embryonic stem

cells or “induced pluripotent stem cells (iPSC).” iPSCs are derived from adult cells, most frequently from fibroblasts or blood cells, and programmed into an embryonic-like pluripotent state (fig.5) (Martínez-Cerdeño and Noctor, 2018).

In general NPCs divided to two main groups: 1- Embryonic Neural Progenitor Cells and 2- Adult Neural Progenitor Cells

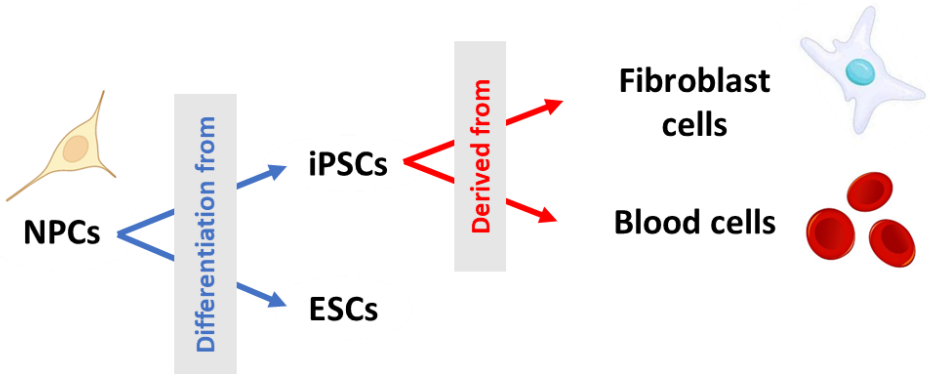


Figure 5. NPCs differentiation from iPSCs & ESCs in vitro.

8.1. Embryonic Neural progenitor cells

The first description of embryonic NPCs in the fetal spinal cord was done by Camillo Golgi in 1885. Nineteenth-century neuroanatomists began to recognize and describe the fundamental properties of NPCs and proliferative zones in the developing brain. It was discovered that cortical neurons were produced by mitotic germinal cells” which divide close to the telencephalic ventricle (Martínez-Cerdeño and Noctor, 2018). NPCs distribution developmental study in the growing cortex was carried out with Hamilton in 1901(Hamilton, 1901). It is shown that mitoses were localized in two basic locations such as in ventricle as ventricular mitosis and away from ventricle as extra ventricular mitosis. It is found that during development, the location of mitosis was changed. Most precursor cells during early growing stages divided at the ventricle and at the later growing stages the mainstream of precursor cells divided away from ventricle. Additionally,

it is reported that morphological differences of precursor cells were correlated with dividing cell position at the ventricle or away from ventricle (Hamilton, 1901). Ventricular zone (VZ) and subventricular zone (SVZ) are primary and secondary proliferative zones during first and late stages of development (Angevine Jr et al., 1970, Martínez-Cerdeño and Noctor, 2018).

The cells of VZ are preserved in many species from birth and organized in telencephalon, diencephalon, and spinal cord. Radial glial (RG) is the most common term that is used for primary NPCs in VZ (Rakic, 1971, Martínez-Cerdeño and Noctor, 2018).

Neuroepithelial cells are precursor cells that differentiate to RG cells. These cells produce neural tube walls. Neuroepithelial progenitor cells come from the ectoderm in the early stage of development and can be recognized by their radial orientation and bipolar morphology. In the early developmental phase, neuroepithelial cells have potential to self-renewing divisions which leads to increasing the precursor cell pool size when the neural plate is forming. When neural tube terminates, up regulation of glial specific factors initiated by neuroepithelial cells and transform to RG cells and obtain the ability to neurons and glia generation (Morest and Silver, 2003, Martínez-Cerdeño and Noctor, 2018).

It is demonstrated that division of asymmetric RG cells produce the majority of the neuronal daughter cells in the cerebral cortex by NPC daughter cell migration to SVZ (Noctor et al., 2008, Martínez-Cerdeño and Noctor, 2018). The various term is used for identification of mitotic NPCs which divided in SVZ, for instant extraventricular cells, subependymal cells, blood vessels (BVs), SVZ cells, non-surface progenitor cells and abventricular mitosis (Martínez-Cerdeño and Noctor, 2018).

8.2. Adult neural progenitor cells

Two positions in adult mammalian are well characterized for inhabiting NPCs, the first one is sub granular zone (SGZ) in dentate gyrus and second one is adult SVZ near the lateral ventricles in mature cerebral cortex (Martínez-Cerdeño and Noctor, 2018).

8.2.1. Adult NPCs in sub granular zone

Adult NPCs of sub granular zones are referred to as RG-like (RGL) cells or Type1 cells. Dentate gyrus adults NPCs share some basic properties with RG cells. RGL cells are found in SGZ and have an intricate radial procedure which expands via granular cell layer to the molecular layer where it ends on synapse and vasculature (Moss et al., 2016). Nestin, GFAP and SOX2 are expressed by Type 1 cells which produce granule neurons (Martínez-Cerdeño and Noctor, 2018).

Type 1 cells are inactive or proliferative and can divide symmetrically and asymmetrically when mitotically active. During neurogenic division, type 1 NPCs generate IP cells that are termed "type 2 cells", which, like in the developing cortex, express Tbr2, reveal multipolar morphology and undergo a limited series of divisions to produce newborn neurons which express doublecortin.

Newborn daughter cells mature to the Prox1+dentate granular neurons by migrating into the granular cell layer radially (Sun et al., 2015, Martínez-Cerdeño and Noctor, 2018). Adult dentate gyrus neurogenesis has been observed in all mammals like humans. The adult neurogenesis degree in dentate gyrus is associated with important emotional and cognitive behaviors such as learning and memory maintenance, recognition pattern and clearance of memory (Berg et al., 2018, Anacker and Hen, 2017, Martínez-Cerdeño and Noctor, 2018).

8.2.2. Adult NPCs in the Subventricular Zone

Glial cells are generated by adult NPCs in SVZ. Adult NPCs in SVZ are known as B1 cells. B1 cells are recognized by their position and GFAP, GLAST and BLBP expression and also through contact with blood vessels. B1 cells are inactive or proliferative. B1 cells in proliferative state go through asymmetric divisions for generating self-renewing B1 cells and passing progenitor cells which perform as a passage amplifying cell. These cells are called C cells. Consequently, C cells divide into a generation of daughter cells called A cells and transfer into the olfactory bulb. Ascl1 and

Dlx2 transcription factors are expressed by C cells, however DCX and PSA-CAM are expressed by A cells (Martínez-Cerdeño and Noctor, 2018, Lichtenwalner et al., 2001).

9. NPCs and hippocampal functions

Dentate gyrus of the hippocampus plays an important role in learning and memory formation (Spiers and Bendor, 2014, Kino, 2015). It is assumed that adult NPCs and granular cell neurons that are derived from NPCs contribute information about memory and learning activities in the brain. It is reported that learning tasks involved in hippocampal-mediated memory formation increases the granular cell number in dentate gyrus. It is suggested that learning is known as an effective and positive factor for NPCs that rescue its differentiated form from dead one (Quiroga et al., 2008, Kino, 2015). Accordingly, adult NPCs have positive and important roles in consolidation, preservation and organization of hippocampal-mediated memory(Sahay et al., 2011, Kino, 2015). The lineage of hippocampal progenitor cells preserves through self-renewal/proliferation when the process of differentiation and maturation to neurons occurs continuously. Additionally, hippocampal NPCs have the ability to develop apoptosis. Most hippocampal NPCs die in the differentiation process to the neuron. Therefore proliferation, differentiation and cell death/survival are three important and major activities of NPCs which are regulated through hippocampal neurogenesis(Zhao et al., 2008, Kino, 2015).

There is abundant evidence that the adult mammalian brain generates new neurons and integrates them into brain regions affected by disease processes (Eriksson et al., 1998, Oh et al., 2015). Hippocampal neurogenesis in the subventricular and subgranular zones in dentate gyrus act like an intrinsic and endogenous repair mechanism (Doetsch and Hen, 2005, Oh et al., 2015). Therefore, endogenous neurogenesis regulation has important effects on neurodegenerative disease such as AD therapeutic strategies(Mu and Gage, 2011, Oh et al., 2015). NPCs, multipotent progenitors known as type1 SGZ cells in the hippocampus have the ability to self-renew and generate neurons and glia. These progenitor cells are classified into three types of cells in the hippocampus; first is radial type 1 cells which show properties of astrocyte and are identified

through expression of glial fibrillary acidic protein and SOX2 and nestin as neuronal progenitor markers. Second one type 2 cells is related to transit-amplifying cells that come from type1 cells and third one is referred to type3 cells which are differentiated form of type 2 cells (von Bohlen und Halbach, 2011, Oh et al., 2015).

It is demonstrated that neurogenic activity of neurogenic in SGZ changes in AD early stage (Mu and Gage, 2011). There are several key molecules which are involved in pathogenesis of AD that appear to regulate adult neurogenesis. Concerning the mechanism of NPCs fate in AD, disruption of Wnt/b-catenin signaling through Aβ, plays an important role in regulating NPCs neurogenesis. According to various studies, it is revealed that Wnt3a, which is expressed in neurogenic niche, regulates the SGZ newborn neurons generation (Yoshinaga et al., 2010, Oh et al., 2015). Decreasing of Wnt/β-catenin signaling and expressing are reported in AD patients with inherited mutation (Lie et al., 2005, Oh et al., 2015). Therefore, neurogenesis in the AD brain is decreased by disrupted signaling and expression of these key molecules.

II. AIMS

Objective:

As mentioned, the current treatments for Alzheimer's disease have only helped in controlling the clinical symptoms, and all the drugs used in this disease slow down the progression of the disease and delay the development of symptoms, but none of them completely do not significantly improve cognitive function and do not cure the disease. It is believed that these drugs were effective in small amounts. Therefore, identifying the factors and solutions that can improve the amount of signaling and cognitive function through preserving brain energy and restoring/replacing the lost neurons can provide a clear perspective for the treatment of this disease. Beneficial effects of metformin have been reported as first-line treatment for type 2 diabetes and related diseases. Considering the role of increasing the effect of insulin on the body and the anti-resistance effect of insulin, the effects of metformin on modulating inflammatory responses, reducing oxidative stress and accelerating neurogenesis have been evaluated.

In this study, by using Streptozotocin as an inducer of type 2 diabetes in the brain, a model with sporadic Alzheimer's disease conditions was provided. In this model, the effect of metformin and the transplantation of neuronal progenitor cells in the hippocampus alone, as well as the treatment with metformin and the transplantation of progenitor cells with each other It has been investigated on the improvement of memorization and cognitive performance.

The general purpose of this thesis is to investigate the effect of reducing inflammation in the hippocampus and cerebral cortex by metformin, as well as to evaluate the recovery and replacement of lost neurons by stem cell transplantation in the process of improving cognitive function in sporadic AD models.

1. Evaluation of the effect of STZ (inducing type 2 diabetes) to obtain sporadic Alzheimer's mouse model.
2. Evaluation of the effect of metformin treatment in sporadic AD mice after 2 weeks
3. Evaluation of the effect of neural progenitor cells transplantation in the hippocampus of sporadic AD mice after 3 weeks
4. Evaluation of neurogenesis in sporadic AD mice by the simultaneous treatment of metformin injection and neural progenitor cells transplantation

III. MATERIALS AND METHODS

1. Animals and the interventions

In this study, 5-6 months old male mice (C57BL/6) were used. Mice have access to food and water ad libitum and are housed in a controlled environment with regards to light (12 hours light/12 hours dark), temperature (22°C ± 2°C), and humidity. Attempts were made to keep the number of animals utilized to a minimum and to alleviate their suffering. Animals were initially divided into 2 main groups: 1) eleven mice without any treatment as Control; 2) twenty-nine mice treated with Streptozotocin (STZ) as a sporadic AD model. These mice were treated with intracerebroventricular (ICV) injections of STZ + aCSF (0.5 mg/kg) at the first and third day of the experiment (Rostami et al., 2017). First, animals were anesthetized using administration of ketamine/xylazine (dose: 1 Ketamine-xylazine / 7 Distilled water) and placed in a stereotaxic device. The site of injection was 0.9 mm (lateral) either side of the midline (bregma) and the depth of the injection was 2.4 mm (dorsal ventricle). STZ powder (0.5 mg) was dissolved in aCSF (Ingredients of artificial cerebrospinal fluid: 147 mM NaCl, 2.9 mM KCL, 1.6 mM MgCl, 1.7 mM CaCl & 2.2 mM dextrose) and injected in the volume of 2 µl in each side (total volume: 4 µl). After 21 days, mice which showed SAD phenotype, underwent behavioral tests.

And then were divided into 4 subgroups; 11 mice were kept as a sporadic AD group with no further treatment, and 6 sporadic AD model mice which received metformin as treatment (STZ+MET group). Metformin, 200 mg/kg dissolved in PBS, was injected every day (100 µl in the morning and 100 µl in the afternoon (every 12 h)) for 2 weeks. Evaluations were performed at least 12 h after the last injection (Esmaeilnejad et al., 2021). 12 sporadic AD model mice received 100,000 human Neural Progenitor cells (NPCs) in each right and left hippocampus (The total number of NPCs injected into one brain is 200,000). The site of injection of NPCs was -1.9 mm (lateral) either side of the midline (bregma: -1.94 mm) and the depth of injection was -1.9 mm (dorsal ventricle). Coordinate of NPCs injection according to atlas coronal sections of mice (bregma coordinate: - 1.94mm, DV: -1.9, L: -1.9). These 12 transplanted groups divided into two groups, 6 of them after NPCs were transplanted, received IP 200 µl metformin (100 µl in the morning and 100 µl in the afternoon (every 12 h)) for 2 weeks. First group; 11 control mice (without any treatment), second group; 11 sporadic AD model (ICV-STZ, day1&3, after 21 days),

third group; 6-mice treatment group (STZ+MET, 200 µl metformin, 2 weeks), forth group; 6-mice treatment group (STZ+NPCs, 200,000 each side of hippocampus), fifth group; 6 mice-treatment group (STZ+NPCs+MET, 200,000 NPCs+200 µl metformin).

NPCs that were used in this project were induced from CAG-GFP hiPSCs cell lines which are derived from fibroblast cells (Royan Institute).

In the NPCs transplantation process of this project, Cyclosporin was used as a suppressor of the immune system to prevent NPCs transplantation rejection in transplanted treatment mice groups. Cyclosporin IP injections were done in 2 weeks after NPCs transplantation at a dose of 150 µl per mouse, every day.

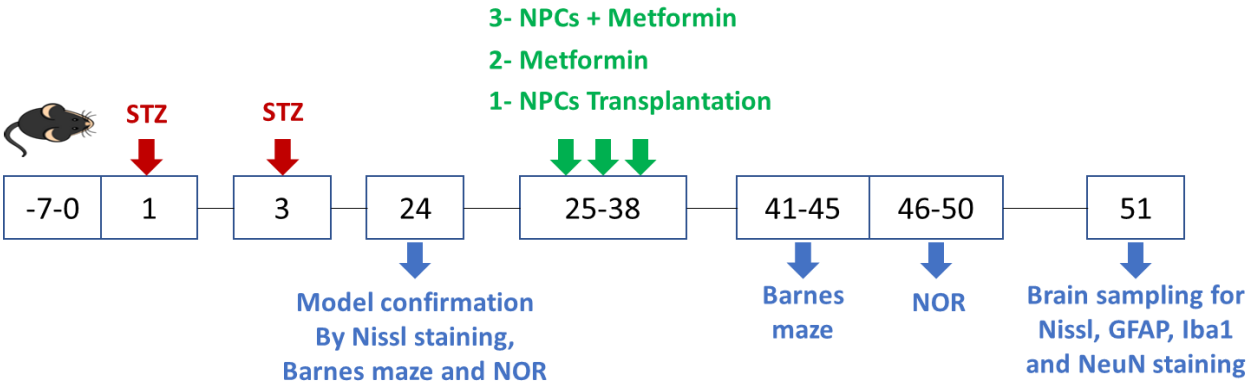


Figure 6. Schematic representation of experimental timeline. Streptozotocin (STZ) injection into mice ventricles caused cognitive impairment and neural damage as assessed 3 weeks post STZ injection. NPCs transplantation and metformin treatments alone and in combination improve cognition and learning in sporadic AD models by decreasing inflammation and by replacing lost neurons.

2. Behavioral tests

2.1. Cognitive assessment using novel objective recognition

The object recognition test (ORT) is a widely used behavioral assay in mice for studying different elements of learning and memory (Lueptow, 2017). The object recognition test (ORT), also known as the novel object recognition test (NOR), is a quick and easy way to assess different stages of learning and memory in mice. It was first developed in 1988 by Ennaceur and Delacour and was first employed in rats1; however, it has now been effectively modified for usage in mice. The exam can be completed in as little as three sessions: one for habituation, one for training, and one for testing. The training session consists of visual exploration of two similar things, whereas the test session consists of replacing one of the previously investigated objects with a novel one. Because rats have a natural affinity for novelty, a mouse who recalls a familiar thing will spend more time investigating the unfamiliar object. The ORT has a significant advantage over previous rodent memory tests in that it relies on rats' inherent tendency for novelty exploration. As a result, neither multiple training sessions nor positive or negative reinforcement are required to inspire behavior. This implies that the ORT is substantially less stressful than other memory tests and takes significantly less time to complete than the Morris water maze or Barnes maze, both of which may take up to a week or more to complete. As a result, the ORT's circumstances are more like those employed in human cognition research, enhancing the test's ecological validity above many other rat memory tests. ORT has also been effectively modified for use in a variety of species, including humans and non-human primates, to test distinct inter-species features of declarative memory, since it is a straightforward visual recall task. Finally, the ORT may be simply changed to investigate other periods of learning and memory (for example, acquisition, consolidation, or recall), different types of memory (for example, spatial memory), or different retention intervals (i.e., short-term vs long-term memory) (Lueptow, 2017). The memory of mice was assessed using the novel object recognition test (NOR). There were three steps in the task procedure: habituation, familiarization, and testing. During the habituation phase, mice investigated a square open-field arena with an inner (39×39×20 cm) and outer (40×40×20 cm) diameter without an item for three days, 10 minutes per session. Each mouse was placed in the arena with two identical items (A+A) in the middle of the field for 10 minutes on the fourth day (familiarization phase). For the test phase, mice were reintroduced to an open-field arena 24 hours later with two different objects; one that was identical to the day before and the other

that was novel (A+B) for 10 minutes. In all stages, the arena and objects were cleaned with 96 percent ethanol prior to animal introduction to remove smell signals. Exploration was described as an animal's snout pointing toward an object, smelling, or touching it. The percentage of discriminating index (DI %) was used to determine the difference in exploration time between familiar and unfamiliar objects. As a result, Novel object's exploration time was divided by the total exploration time, which was measured in seconds and expressed as a percentage (Ettcheto et al., 2016).

$$DI\% = \frac{new\ object\ exploration\ time}{total\ exploration\ time} \times 100$$

2.2. Cognitive assessment using Barnes maze test

Several well-known animal activities are used to assess spatial learning and memory in animals. These experiments presume that the animal learns to complete a maze by utilizing either positive or negative environmental stimuli (food, water, and shelter) (immersion in water, intense light, noise, or air blast). Researchers use the radial arm maze test, spontaneous alternation and win-shift tests in the T and Y mazes, as well as spatial versions of the new object identification test, Morris water maze, and Barnes maze (BM) test to assess spatial learning and memory (Gawel et al., 2019). The last test, detailed below, is predicated on the notion that an animal put on the surface of a platform would learn and remember where an escape box is situated (i.e., safe shelter, dark and located mostly below the surface of the platform). There are various stages to the test. There is a habituation phase (during which the animals are exposed to their surroundings), followed by an acquisition phase (during which the animals learn to find the location of the escape box). Because the test animals shift their applied strategy from random to spatial to resolve the labyrinth, decreased latencies to reach an escape box are predicted after a few trials (Gawel et al., 2019). Following the capture phase, capture probe attempts are made. This is done with the target hole closed and measures the time previously spent near the correct hole (or zone). This allows evaluation of spatial memory searches. The first part of the Barnes Maze task, for instance the acquisition phase and subsequent acquisition probe trials enable

spatial learning and evaluation of spatial memory. This part is believed to be related to hippocampal function. BM is not as popular a task as the Morris Water Maze or Radial Arm Maze, but it has some advantages that make it a very attractive alternative, especially for the former. In addition, the usefulness of this task is very wide, from pharmacologically and genetically induced Alzheimer's disease models to other disease / injury models (after traumatic brain injury, Parkinson's disease, lateral sclerosis, etc.), and There are even drugs (or drug therapies). May improve or worsen spatial learning and memory (Gawel et al., 2019). The Barnes maze was used in this study to measure the spatial memory and learning skills in all experimental groups.

The Barnes maze test was performed on a circular table with a diameter of 90 cm and a height of 90 cm from the floor. Twenty holes, each with a diameter of 5 cm, were regularly spaced around the table's circumference. The target hole (main hole) is the only one that leads to an escape chamber where the animal may hide. To enhance the animal's motivation to hunt for the target hole, illumination was increased and as measured at the middle of the table it was kept at 1350 lux. For evaluation of learning and memory of mice and help to mice for learning the place of scape box in trial days the environment around the test table should be marked with different and fixed signs (which don’t remove until the end of the test day). The animal was put in the start box placed in the maze's center for the habituation phase, at the first of each trail. The start box was removed after 30 seconds, and the mouse was given 300 seconds (5 minutes) to explore the area per experimental time. The procedure was based on four training days (every day 4 trials, each 5 minutes, between each trial in a day the mice should be rested for 20 minutes) and one test day. On the test day, the scape box was removed but the environmental test table signs were in place. First stage in test day is that the mouse was placed in the center of Barnes maze plate in the start box for 30 seconds, after 30 seconds the start box was removed and the mouse in increased illumination was on the plate without any escape box for 5 minutes. It is expected that according to the trend of 4 trial days the mice should know the place of scape box and according to the signs and their memory they should go toward the correct scape box place and stay around of it.The video tracking system Ethovision XT 11 (Noldus Company, Netherland) was used to collect and evaluate behavioral data (Cano et al., 2019).

3. Brain sectioning

After behavioral tests, mice in all groups (control, STZ AD model and treatment groups (Metformin - NPCs - NPCs+MET)) were intensely euthanized with 1-ml chloral hydrate 10% and then perfused transcardially with PBS followed by 4% paraformaldehyde (PFA) in PBS, PH 7.4. The all-group mice brains were extracted and fixed in the 4% PFA overnight. The fixed brain is stored in 30% sucrose solution (in PBS) for 48 hours at 4 degrees centigrade. For cryo-sectioning, the brain samples were fixed in optimal cutting medium (OCT; Bio-Optica) and were frozen at -80°C. Cryostat microtome (Histo-Line Laboratories, Italy) were used for preparing the coronal brain sections with 10 µm-diameter on superfrost form. For later evaluations the slides are stored at -20 °C (Rezaei et al., 2020).

4. Nissl staining

Nissl staining is used for detection of the Nissl body in the cytoplasm of neurons. In normal and alive neurons-stained Nissl body with Cresyl Violet appear in light purple and in dead neurons and impaired neurons after staining with Cresyl Violet appear dark purple. In this study 0.1 % Cresyl

Violet acetate (IHCworld, USA) was used to stain the coronal hippocampus sections. The protocol of Nissl staining in this study for Cryo coronal sections consists of 3 stages; stage 1) Ethanol passage (96% (1 min) - 80% (1 min)- 70% (1min))- running water for 2-5 min, stage 2) staining with Cresyl Fast Violet 0.1% (2-3 min), stage 3) Ethanol passage (70% (3-15 (S)) - 80% (3-15 (S))- 90% (1 min) - 96% (1 min) - 100% (1) (1-2 min) & 100% (2) (1-2 min) and Xylol (5-7 min). In this study, the duration of each ethanol passage was obtained experimentally. And the right time is selected based on the amount of alcohol bleaching in each step. Under an optical microscope (cellsense software, Germany), sections were photographed and later, the amount of brain injury was determined by counting the number of dark neurons in the hippocampal, dentate gyrus (DG),

CA1 and CA3 regions. Dead neurons were those with dark cytoplasmic Nissl staining, and not-prominent nucleoli (Lin et al., 2021).

5. Immunofluorescence staining

For immunofluorescence staining, mice were sacrificed under deep anesthesia and then perfused. The brains were extracted and post-fixed for an additional night; then cryoprotected in a 30 percent sucrose-PFA-PBS solution at 4˚C. The samples were kept frozen at -80°C. A cryostat was used to make 10µm thick coronal sections. Fixed sections collected on the positively charged slides were washed 3 times each for 5 minutes with PBS 1% in room temperature.

In this project immunofluorescence were followed by using some specific antibodies such as antibodies for reactive astrocyte (GFAP-Rabbit-red) and microglia (IBA1-Rabbit-red) for evaluate inflammations and also antibodies for neuronal nuclear proteins (NeuN-Rabbit-red) that using for neuronal studies and the other antibody which used in this study is fluorescence marker (GFP-mouse-green) for tracking transplanted cells. For starting the immunofluorescence staining the frozen brain coronal sections, the slides were kept at room temperature for drying by air for 30 minutes. When the temperature of the slides reaches room temperature, the first stage of immunofluorescence begins. Slides were washed three times with PBS (3 times × 5 min). In order to increase the permeability of brain cell membranes, sections were incubated with Triton 0.3% (sigma) for 20 minutes at room temperature. The next stage is incubating slides with blocking solution (10% normal goat serum (NGS) + 10 µl Triton100 X) for 1 hour at room temperature. After the 1h blocking stage the slides were washed with PBS 3 times for 5 minutes (3 times × 5 min). Afterward, samples were incubated with primary antibodies against Glial fibrillary acidic protein (GFAP- 1/500, the 1 µl GFAP antibody + 500 µl blocking solution) as a reactive astrocyte marker, Ionized calcium binding adaptor molecule 1 (IBA1- 1/1000, the 1 µl IBA1 antibody + 1000 µl blocking solution) as a stain-marker for reactive microglia, neuronal nuclear proteins (NeuN-1/1000, the 1 µl NeuN antibody + 1000 µl blocking solution) as a marker for mature neurons and GFP (1/200, the 1 µl GFP antibody + 200 µl blocking solution) for tracking transplanted NPCs at

4°C, overnight. (All primary antibodies were diluted with blocking solution). After 18-24 hours of incubation of primary antibodies, slides were washed three times with PBS for 5 minutes. Incubation with appropriate secondary antibodies was done in accordance with anti-rabbit or anti-mouse for GFAP, IBA1, NeuN (red) and GFP (green). Secondary primary incubation was done in a dark room for 1 hour at room temperature. Secondary antibodies (1/500; Invitrogen, Eugene, OR, USA) (Ettcheto et al., 2016, Seyedsadr et al., 2019, Rezaei et al., 2020). The slides were washed with PBS (3 times × 5 min) and finally incubated with 4', 6-diamidino-2- phenylindole (Dapi; Sigma-Aldrich; D-9542) as a counter stain of nuclear (blue) for 10-15 minutes. The list of primary and secondary antibodies which were used in this project is presented in Table 1. Immunofluorescence-stained slides were assessed by fluorescence microscope and data for this staining were averaged for three mice in each experimental group.

Table1. The primary and secondary antibodies characteristics in this study.

Antibody	Species isotype	Company	Dilution factor	Label
GFAP	Mouse polyclonal		1:1000	-
IBA1	Rabbit polyclonal		1:1000	-
NeuN	Rabbit polyclonal		1:1000	-
GFP	Mouse monoclonal		1:200	Green
Rabbit IgG	Goat anti-rabbit	ThermoFisher, A11008	1:1000	Alexa Fluor® 488 Green
Rabbit IgG	Goat anti-rabbit	ThermoFisher, A11036	1:1000	Alexa Fluor® 568
Mouse IgG	Goat anti-mouse	ThermoFisher, A11004	1:1000	Alexa Fluor® 568

6. Statistical analysis

Data are presented as means ± SEM, with minimum significance level of differences defined as $p < 0.05$. Student's *t*-test, 1-way and 2-way ANOVA with Tukey's post hoc test were used to assess differences between samples/animals. One-way ANOVA, conjointly referred to as one-factor ANOVA, determines however a response is affected by one issue. In NORT, Percentage of discrimination index between 5 experimental groups was analyzed by one-way ANOVA, in this subject (percentage of discrimination index) novel object detection as a factor led to choosing one-way ANOVA. Two-way ANOVA, also known as 2-factor ANOVA, determines however a response is affected by two issues. In this project the percentage of object exploration in NORT between 5 groups of experiment was analyzed by two-way ANOVA. Evaluation of two factors at the same time, such as exploration of Novel objects and exploration of familiar objects has led to the use of two-way ANOVA. An Unpaired t-test (also better-known as associate degree freelance t-test) may be a statistical method that compares the averages/means of 2 independent or unrelated teams to work out if there's a major distinction between the two. In general, in this study in behavioral test (NORT & BMT) prism analysis, percentage of discrimination index (NORT), novel object exploration (NORT), were analyzed by one-way ANOVA. Percentage of object exploration (NORT), Distance (BMT), Velocity (BMT), Primary latency (BMT), Escape latency (BMT), Primary error (BMT), Total error (BMT) and movement strategy (BMT) were analyzed by two-way ANOVA. Analyzing Nissl staining data and immunofluorescence staining (GFAP, IBA1 and NeuN), ordinary one-way ANOVA was used. The counting of GFP^+ cells and counting of GFP^+ /$NeuN^+$ cells was analyzed by unpaired T-test. GraphPad InStat V6.0 was used to produce both the statistical analysis and the graphics displayed here (GraphPad Software) (Ettcheto et al., 2016).

IV. RESULTS

1. AD phenotype was developed following STZ treatment

1.1. Novel Objective Recognition test results of control and STZ-AD model groups

Evaluation using NOR test showed that STZ caused significantly lower performance in exploring the novel object. Figure 7, the percentage of object exploration graph, shows the difference in the rate (%) of exploration and discovery of novel and familiar objects between the control and STZ AD model groups. The percentage of exploration and discovery of a novel object in a control mice group is almost two and a half times higher than the detection of a familiar object in test day (10 minutes), the percentage of novel object detection is about 250% when familiar object was 100%. In STZ AD model group, detection and poking the novel object in comparison with familiar object does not show much difference and is almost the same and is about 20% for novel and familiar objects. Significant differences, $^{***}P<0.001$ between novel and familiar object detection can be seen in the control group. And this graph shows the significant difference with $^{####}P<0.0001$ in novel detection between control and STZ group. Additionally, Control mice were more curious to discover the novel object than the STZ animals (Figure 7).

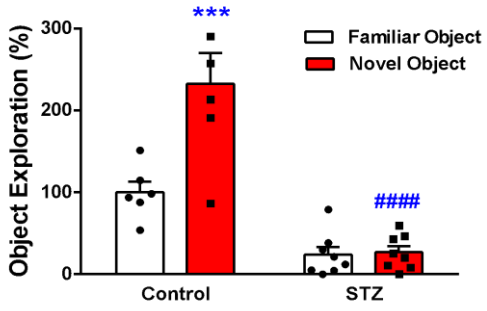


Figure 7. Novel Object Recognition test (NOR) results, demonstrating a significant memory loss in SAD mice model with STZ treated in comparison with control group. Object exploration

percentage was analyzed using two-way ANOVA, significant differences were demonstrated between Novel and Familiar objects in the control group. Novel object exploration between control and STZ model group demonstrated significant differences. ***P<0.001 vs old object in control and ####P<0.0001 vs novel object in control.

1.2. Barnes maze test results of control and STZ-AD model groups

The Barnes maze test results in the test day (5th day) showed that STZ-treated mice recognized the Goal sector (GS) or the target hole less effective than Control mice. Figure 8A shows the results of the number of correct poking on Barnes maze plate on the target hole that is linked to the escape box + right and left hole of the target hole on test day. These three holes that are blocked on test day are identified as correct areas for mice poking. To achieve Goal sector rate, the number of poking the correct holes in plate should be divided to 3. According to the graph of figure 8A, the GS of control mice is about 8 and STZ mice is about 6.5. The significant differences presented between control and STZ AD model with *P<0.05, that shows the control mice poking the correct area more than STZ AD group in 5 minutes of test day (figure 8A).

According to graph 8B, the number of Non-Goal Sector (NGS) between control and STZ group is evaluated on test day (5th day). NGS number is gained by dividing the poking number of incorrect holes to 17. The number of NGS for the control group is about 2.5 and for the STZ AD model group is about 3.5. The results also show that STZ animals appeared more frequently in non-target holes compared to Controls and spent more time in non-goal sectors. Although control mice poked non-correct areas compared to the STZ AD model group, no significant difference was seen in this evaluation. (Figure 8B).

According to figure 8C, the ratio of GS/NGS, correct poking on incorrect poking numbers, was significantly lower in STZ group in comparison with control group, the rate of ratio in control group is about 5 and in STZ AD group is about 2.5. Significant differences were seen with *P<0.05 between control and STZ AD group in 5 minutes of test day (5th day) (figure 8C).

Target seeking graph, shows no significant difference between control and STZ AD model, while the ratio of touching the entire holes/20 in 5 minutes of test day for control is about 3 and for STZ AD model group is about 4.5. And the target seeking of STZ AD model was more than control group (figure 8D).

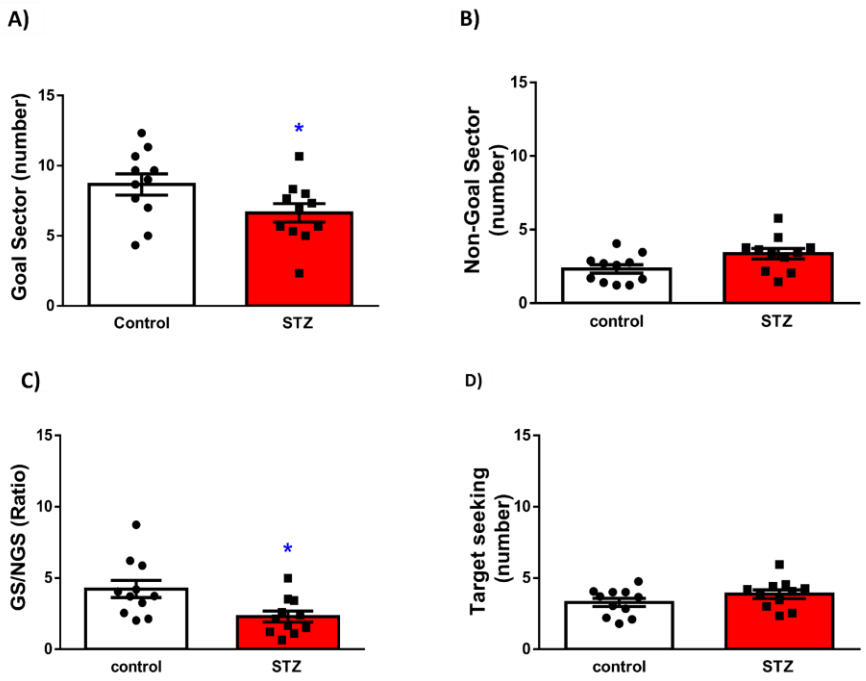


Figure 8. Barnes Maze test results. A) Goal Sector graph shows significant differences between control and STZ model group. B) Non-Goal Sector C) GS/NGS ratio graph demonstrated significant differences between the Control and STZ model group. D) Target seeking graph with no significant differentiation. *P<0.05, **P<0.01 vs control

1.3. Nissl staining results of control and STZ-AD model groups

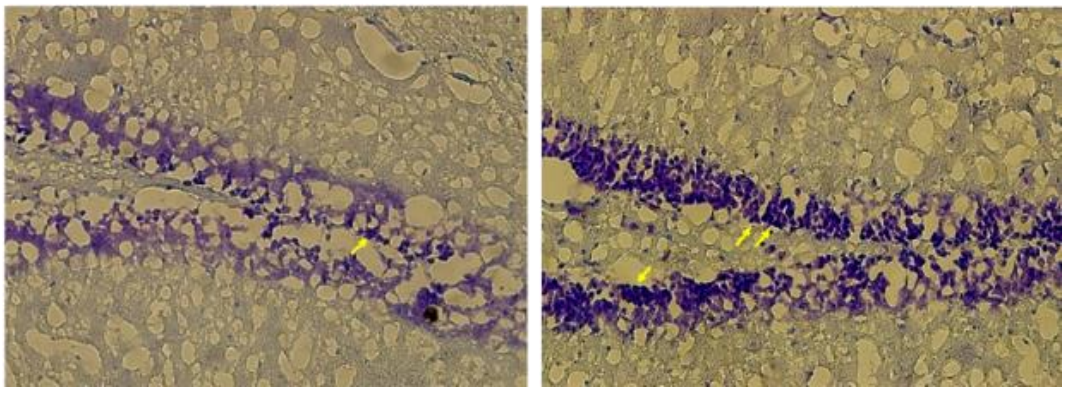
Nissl staining was used as a method for labeling the nucleus of neurons in three regions of the hippocampus. Figure 9 presented the Nissl staining results of three regions of hippocampus for control and STZ-AD model groups. In the first part, Figure 9A shows images of dental gyrus (DG), with yellow arrows that mark the dark purple cells (neurons) which are known as dead neurons (figure 9A).

According to graph 9B, the average number of dark neurons (dead neurons) in dental gyrus (DG-hippocampus) of three mice from per experimental groups, control and STZ-AD model shows significant increase in number of dead neurons in STZ AD model compared with control mice. The average number of dark neurons in DG control group is 45 and in STZ AD model group is 175 per field. Significant difference presented between these two groups with ** P<0.01 (figure 9B).

Graph 9C, compared the average number of dark neurons (dead neurons) in CA1-hippocampus of control and STZ AD model groups. The average number of dead neurons in control mice is about 10 and in STZ AD model mice is about 60. This graph demonstrated significant difference between control and STZ group with ***P<0.001 (figure 9C).

The average number of dead neurons in the CA3-hippocampus of control and STZ AD model group was evaluated in graph 9D. The average number of dark neurons in STZ mice was more than the control group. The rate of number of dark neurons in control mice measured 10 and in STZ mice measured 60 per field. Despite the higher number of dead neurons in AD mice compared to the control group, no significant difference was observed in this graph (figure 9D)

A)



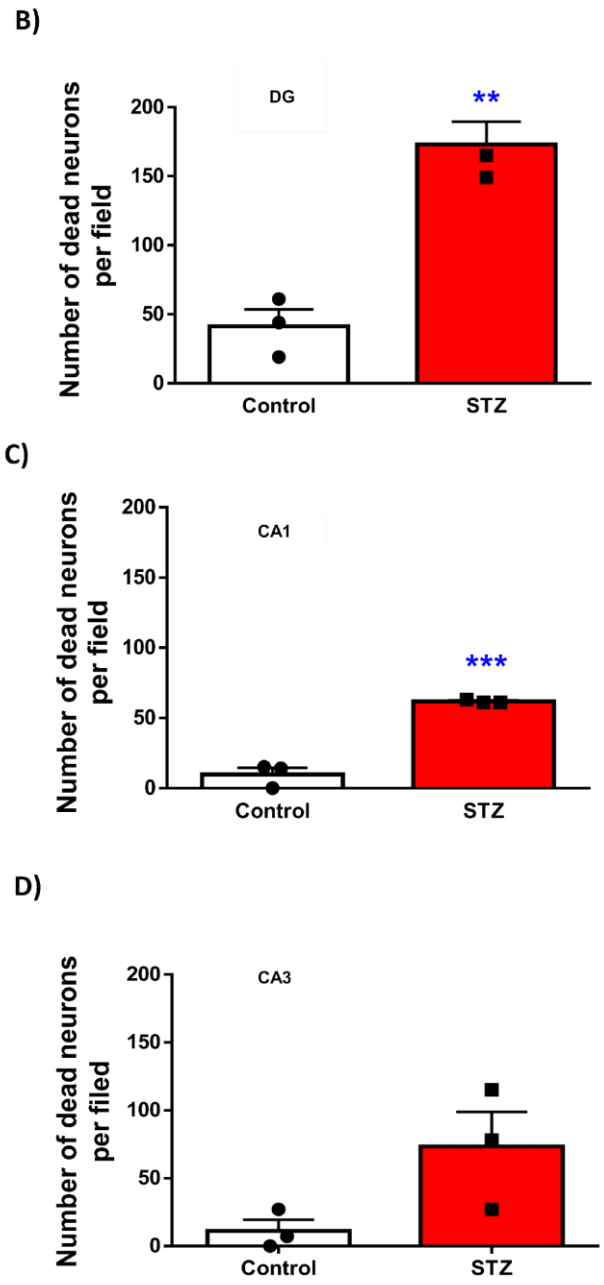


Figure 9. Nissl Staining results, A) Nissl-stained micrographs images of dental gyrus represent samples of dark neurons in two experimental groups B-D) the average number of dead neurons in DG, CA1 & CA3, and significant differences was shown between control and STZ model group in DG & CA1 region of hippocampus. **P<0.01, ***P<0.001 vs control.

2. Metformin, NPCs transplantation and metformin + NPCs transplantation treatment at the same time improved cognitive performance in SAD mice

2.1. Novel Object Recognition Test results (NORT)

In this part of the project, Novel Objective Recognition Test (NORT) was performed for 5 experimental groups of this study 1- Control, 2- STZ AD model and three treatment groups such as 3- STZ+MET group, the mice of this group received 200 µl metformin (IP) for two weeks. 4- STZ+NPCs group, transplantation of NPCs was performed in this group after 21 days passed from last injection of STZ. 5- And the last treatment group is STZ+NPCs+MET, in this group the STZ

AD model received NPCs transplantation accompanied with IP injection of metformin at the same time (figure 10).

2.1.1. Novel object exploration evaluation

Figure 10A shows the time (seconds) it takes to detect novel objects within 10 minutes of the NORT test day. According to Figure 10A, control mice spent an average of 9.2 seconds to discover novel objects. This rate is higher than other experimental groups such as the AD model group and treatment groups. There is significant difference in control vs STZ-AD model and STZ+MET groups with ****P value <0.0001. In addition, between control and STZ+NPCs+MET significant difference can be seen with *P value: 0.0246 (*P<0.05).

STZ-AD mice spent significantly less time recognizing the new object than the other groups. The AD mice model spent an average of 1.1 second to detect novel objects on test day of NORT. Between treatment groups, hippocampal transplantation of NPCs individually, increased the time of discovery more than other treatment groups and 1.1 (S) time of novel object discovery for STZ-

AD model reached to 5.76 (S) by hippocampal transplantation of NPCs treatment. This increasing time demonstrated significant difference in STZ-AD model vs STZ+NPCs with #P value: 0.0229 (#P<0.05).

In other treatment groups, STZ+MET the discovery time for novel objects did not change significantly however in STZ+NPCs+MET the novel object detection time increased from 1.1 to 4.77 (S). Between three treatment groups, a significant difference appears between STZ+MET group and STZ+NPCs groups with $P value: 0.0199 ($P<0.05) (Figure 10A).

2.1.2. Object exploration percentage evaluation

The results of object exploration percentage showed that the total time spent to poke the familiar and novel objects was significantly higher in the control group and lower in STZ AD model group between all five experimental groups of study (figure 10B).

Evaluation of object exploration percentage separately for each group, demonstrated in 10 minutes of test day of NORT, the control group mice poked the novel object significantly higher than familiar one (about two and half times more than familiar). In this graph, the percentage of familiar objects for the control group is considered 100 and this percentage for novel objects in the control group measured about 230% (figure 10B). According to the rate of percentage of novel exploration in all groups there is significant difference in control group vs other groups. Control vs STZ_AD model and STZ+MET treatment group with **** P<0.0001 and vs STZ+NPCs and STZ+NPCs+MET groups with *P<0.05 and **P <0.01 respectively.

In the STZ AD model group, there was no attempt to discover novel objects compared to familiar ones. The percentage of object exploration in STZ AD model group was measured about 30 % for two novel and familiar objects. Novel exploration percentage in STZ_AD model demonstrated significant difference vs transplant treatment groups, STZ+NPCs with ###P<0.001 and STZ+NPCs+MET with ##P<0.01 (figure 10B).

In the STZ+MET treatment group the percentage of total exploration for both novel and familiar objects increased about 10 % in comparison with STZ AD model group. The percentage for Novel and familiar objects was measured about 40% for both. According to Figure 4, the low percentage of exploration in metformin treatment group (STZ+MET), especially the discovery of novel object, has created a significant difference compared to transplant treatment groups STZ+NPCs with $$$P<0.001 and STZ+NPCs+MET with $P<0.05 (figure 10B).

The second treatment group, STZ+NPCs transplantation, object exploration percentage significantly increased for both novel and familiar objects in comparison with STZ AD model and metformin treatment groups. The exploration percentage after treatment with NPCs transplantation reached about 160% for novels and 60% for familiar objects. Novel object exploration in this group increased more than two times that of familiar one (figure 10B).

The total object exploration percentage in the last treatment group, STZ+NPCS+MET, increased and reached 50% for familiar and 110% for novel objects in comparison with STZ AD model and metformin treatment groups (figure 10B).

General evaluation of object exploration percentage graph demonstrated significant difference between control group and STZ, STZ+MET and STZ+NPCs+MET groups and NPCs transplantation individually or accompany with IP injection of metformin increased the exploration time for both novel and familiar object compare with STZ-AD model group (figure 10B).

2.1.3. Discrimination Index percentage evaluation

The percentage of discrimination index was significantly higher in the control group (70%) and was the lowest in STZ-AD model group (50%) (Figure 10C). A significant difference was observed between Control and STZ-AD model with **P<0.01. Among treatment groups STZ+NPCs demonstrated the highest percentage of index, which was about 68%. Treatment with metformin increased the percentage of discrimination index to 63%. In both NPCs, transplant treatment groups increased to 68% and 65% for STZ+NPCs and STZ+NPCs+MET groups

respectively (figure 10C). Increasing the index percentage in both transplanted groups demonstrated significant difference vs STZ-AD model group with #P<0.05 (figure 10C).

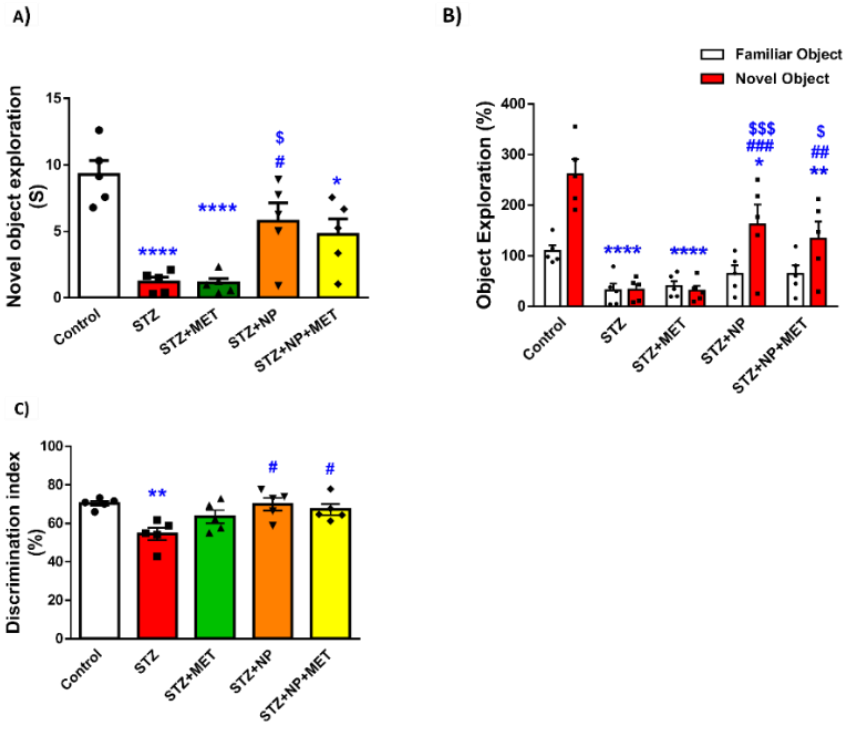


Figure 10. Novel Object Recognition test (NOR) results in control, STZ, STZ+MET, STZ+NP & STZ+NP+MET groups demonstrating a significant memory loss in SAD mice model with STZ treated in compare with control group and relative improvement in treated groups such as STZ+MET, STZ+NP & STZ+NP+MET. A) Novel object exploration (S) was analyzed using one-way ANOVA, significant differences were demonstrated between control and STZ groups. And, in the treated groups significant improvement can be seen in comparison with STZ group. B) Object exploration percentage was analyzed using two-way ANOVA, significant differences were demonstrated between Novel and Familiar objects in the control group. Novel object exploration

percentage between control and STZ model group demonstrated significant differences and treated groups show significant increase in exploration for novel objects. C) Discrimination index percentage shows the high ability to discriminate between familiar and novel objects between the control group and treatment groups in comparison with STZ_AD model group. There is a significant difference between control groups Vs STZ group. Significant differences can be seen between STZ group and transplant treatment groups. *P<0.05, **P<0.01, ***P<0.001 vs control, #P<0.05, ####p<0.0001 vs STZ group. $P<0.05 Vs STZ+MET

2.2. Barnes Maze test results (BMT)

2.2.1. Trial day's results

Figure 5 shows the results of four trial days of Barnes Maze test process. Figure 11A, demonstrated distance traveled to find the escape box for all five experimental groups in four trial days. The distance traveled in four days of learning was evaluated in centimeters (CM) for all five groups. In four days of learning, per mouse was tested 4 times for 5 minutes per day on a Barnes plate. Vary distances in different groups depend on how the location of the escape box is learned. According to the distance results graph in figure 11A, distance traveled to find the escape box was reduced in control, STZ AD model and STZ+MET treatment groups. For control group 2500 CM in day 1 reduced to 100 CM in day4, STZ AD model distance traveled reduced from 5500 CM to 2000 CM during 4 days and STZ+MET mice distance decreased from 9000 CM to 4500 CM during trial days. In other treatment groups such as STZ+NPCs and STZ+NPCs+MET mice there was not much change in the distance traveled during trial days and both groups traveled 100 CM in 4 days (figure 11A). In the last day of trial days, the distance traveled of control and two transplanted treatment groups reached 100 CM. but STZ-AD model and STZ+MET treatment groups traveled 2000 and 4500 CM on the last trial day respectively. Significant difference was appeared in day one in control group vs STZ+MET group with ***P<0.001(0.0006) and in STZ-AD model vs two transplanted treatment groups with #P< 0.05 (0.0123) and $^{\#\#}$P<0.01 (0.0079). On the first trial day, variation in distance traveled between three treatment groups

made a significant difference in STZ+MET vs transplanted groups with $^{\$\$\$\$}$P<0.0001. In day two between control and STZ+MET groups significant difference appeared with *P<0.05 (0.0359) and in STZ+MET group vs transplanted groups STZ+NPCs and STZ+NPCs+MET with $^{\$}$P<0.05 (0.0298 and 0.0255) respectively. There is no significant difference demonstrated in day three and four between all groups (figure 11A).

Figure 11B shows the velocity results of 5 experimental groups in four trial days. In this graph the velocity is evaluated in centimeters on second (CM/S). Consistent with measurement, the velocity during 4 days has been decreasing in STZ+MET treatment group from 50 to 40 CM/S. The velocity in the control group was almost constant in the same range (20-25 CM/S) and about 5 CM/S were added on the last day. STZ-AD model mice had variable velocities every 4 days, day one 25, day two 30, day three 40 and last day 25 CM/S. velocity in two transplanted treatment groups was not changed significantly, and all trial days it was 5 CM/S. 5 unite (CM/S) were added on last day in STZ+NPCs mice. Significant difference appears between control and STZ+MET groups in day 1 with *P<0.05 (0.0304) and Between STZ+MET and transplanted groups there is a significant difference about $^{\$\$\$\$}$P<0.0001. In second trial day, velocity measurement demonstrated significant difference between control and STZ+MET groups with *P<0.05 (0.0207). STZ vs two transplanted treatment groups show the significant difference with $^{\#}$P<0.05 for both 0.0274 for STZ+NPCs and 0.0369 for STZ+NPCs+MET. The velocity difference between STZ+MET and two transplanted groups was significant with $^{\$\$\$}$P<0.0001, 0.0003 and 0.0004 for NPCs and NPCs+MET respectively.

In the third day STZ-ADS model and STZ+MET groups demonstrated significant difference with two transplanted groups $^{\#\#\#}$P<0.001 and $^{\$\$\$\$}$P<0.0001 respectively. And velocity in fourth day of trial shows the significant difference just between STZ+MET group and two transplanted groups with $$P<0.01 for STZ+NPCs and $$$P<0.001 for STZ+NPCs+MET (0.0025 and 0.0004) (figure 11B).

Figure 11C demonstrated primary latency results of 5 experimental groups in 4 trial days. Primary latency was defined as the time (S) it took to locate the target hole for the first time because the mouse did not always enter the hole when it was first identified. This time in the control group

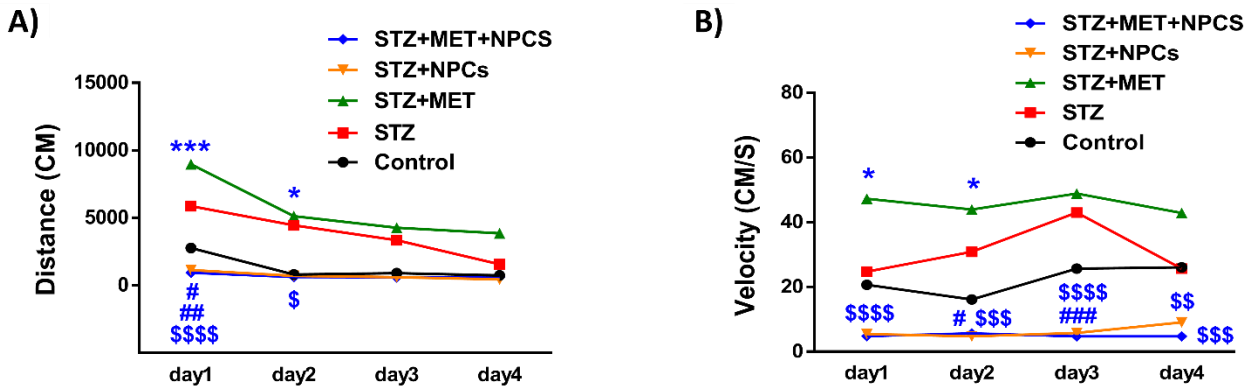
from day 1 to 4 was decreased from 50 to 20 (S). Although primary latency in STZ AD model and STZ+MET treatment groups in day one for both was 55 (S) but in AD model mice this time unexpectedly decreased to 20 on last day and in STZ+MET treatment mice was decreased 22 (S) on day 3 but again increased on last day close to day 1 measurement value. Reduction of primary latency was demonstrated for both transplanted treatment groups from 120 and 80 (S) to 60 (S) STZ+NPCs and STZ+NPCs+MET respectively. On the last trial day, primary latency was shortest for the control and STZ AD model groups and most of the time was related to the transplanted treatment groups. There was no significant difference between 5 experimental groups (figure 11C). Figure 11D, presents results of escape latency evaluation in 4 trial days of BMT. Escape latency measurement is defined as the time (S) it takes for the mice to find the escape box and enter it completely. The escape latency time (S) has been decreasing in all groups except the STZ+NPCs+MET treatment group duration of 4 days. In day 1 control mice spent less time finding the escape box than other groups that evaluated about 160 (S). And after that STZ AD model, STZ+MET and STZ+NPCs+MET 200 (S) were spent on day 1. In STZ+NPCs escape latency was more than the other groups and it was about 250 (S). On the last trial day, the least time was related to control mice about 50 (S) and most was related to treatment groups around 150-200 (S). No significant difference was shown between 5 experimental groups (figure 11D).

Figure 11E shows the primary error results of 5 experimental groups on 4 trial days of BMT. The primary error is defined as a poking number to find the escape box for the first time. The number of primary errors decreased during the 4 trial days in all experimental groups differently. On the first day of trial, the lowest primary error number (6) is linked to STZ+MET treatment mice and on the last day this number reached 3 close to the control rate. The lowest primary error number (3) is related to control mice at the last day of trial day and the largest primary error number (6) belongs to the STZ+NPCs+MET treatment group. Primary error number in STZ+NPCs+MET treatment group on day 2, 3 and 4 was not changed and measured 6. There is no significant difference between the 5 groups (figure 11E).

Figure 11F, demonstrates the total error number of 5 experimental groups in 4 trial days of BMT. Total error in barnes maze shows the wrong poking number of mice to find and enter the escape box completely. The number of total errors in control mice decreased from 28 on day 1 to 5 on

day 4. This number of STZ AD model mice decreased from 32 on day 1 to 14 on day 4. All three treatment groups demonstrated a reduction in total error number from day1 to day 4. The lowest number of total errors belonged to control mice and most number is related to STZ+NPCs+MET mice with a difference of 1 compared to the STZ+MET treatment group. No significant difference was shown in this evaluation (figure 11F).

Figure 11G, shows the movement strategy of 5 experimental groups during 4 trial days of BMT. The movement strategy of animals was analyzed as direct, serial, and random types. At the initial trials animals in different groups mostly used the random strategy while it was gradually reduced in the next trials. Control animals showed the highest amount of reduction in random strategy and replaced it mainly by serial strategy and in lower extent by the direct strategy. STZ AD model mice showed lower amounts of reduction in random strategy. STZ+MET treatment group mice presented a prominent decline in using random strategy by replacing it mainly with the serial strategy and some direct. STZ+NPCs and STZ+NPCs+MET transplanted treatment groups mice analyzing showed the most replacing of serial strategy instead of random strategy. Direct strategy to find the escape box mainly observed in control by time compared to STZ AD Model and treatment groups. The highest rate of serial strategy substitution instead of random was created in the treatment groups specially transplanted treatment groups (figure 11G).



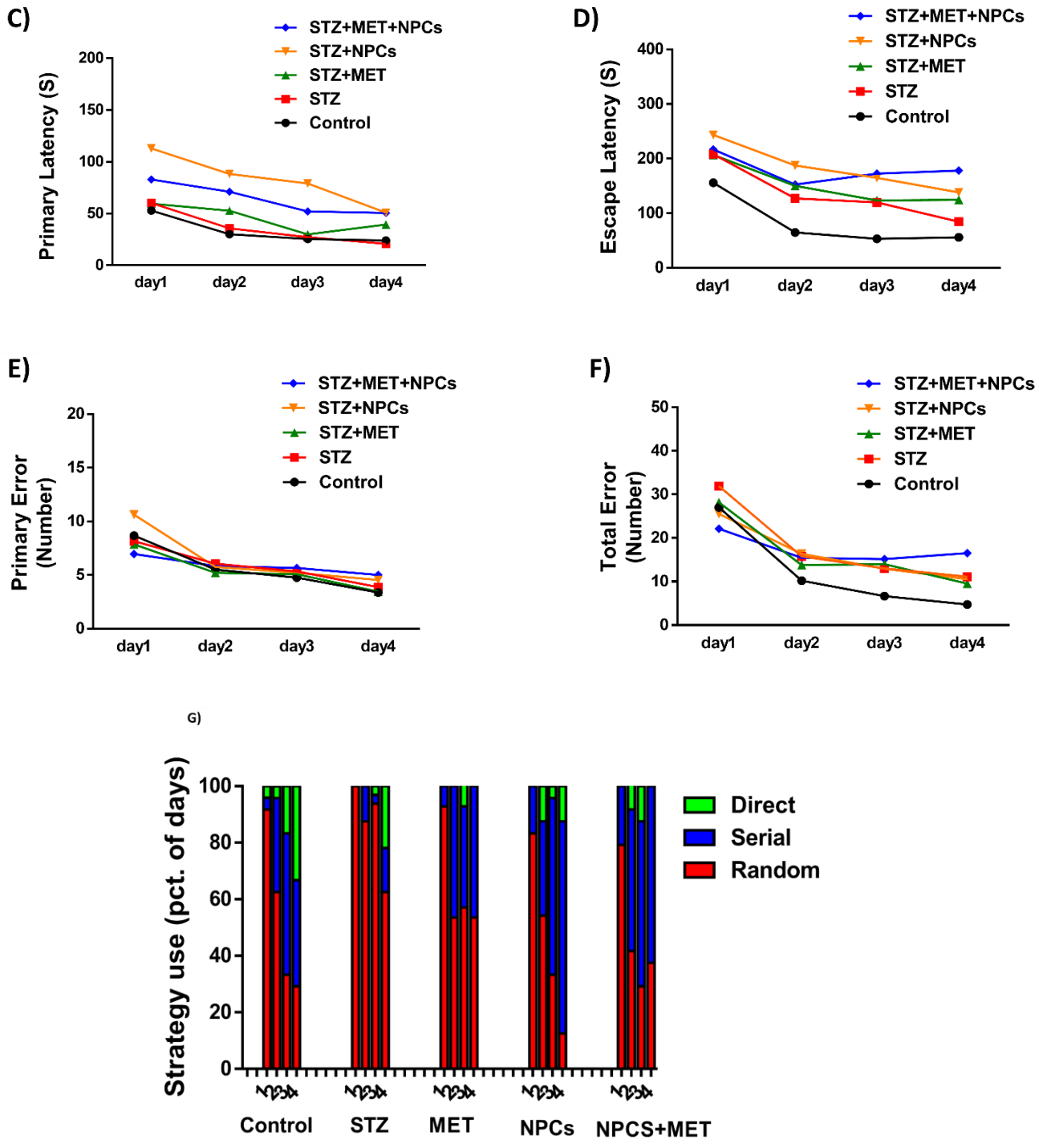


Figure 11. Barnes Maze test results in trial days. A) Distance measurement in 4 trial days demonstrated no significant difference between the 5 groups. B) Velocity measurement analyzed using two-way ANOVA, demonstrated significant differentiation between control and treated groups in day 1 and 2. And significant differences can be seen in the treatment group in

comparison to the STZ group. C-D) Primary latency & Escape latency graphs calculated duration time in seconds until escape box was found for first time and whole test time; there is no significant difference between 5 groups. E-F) Primary & Total error graphs evaluated between 5 groups show no significant difference. G) Demonstrated movement strategy to find a escape box in trial days. *P<0.05, ***P<0.001 vs Control, ##P<0.01, ###p<0.0001, ####P<0.0001 vs STZ, $P<0.05, $$P<0.01, $$$P<0.001 and $$$$P<0.0001 vs STZ+MET.

2.2.2. Test (probe) day results

Figure 12 A-D shows the BMT test day evaluation results of 5 experimental groups.

Figure 12A demonstrates Goal sector (GS) numbers calculation for all 5 experimental groups. Measurement of GS defined as poking the correct holes (main, right & left of the hole) /3 within 5 min of test day. GS number in test day was highest in STZ+MET treatment group calculated 11 and demonstrated significant difference with STZ AD model group with $^{\#\#}$P<0.01. Control group was about 9 vs. STZ group that was about 4.5 with **P<0.001, significantly different. GS number in transplanted treatment groups increased about 4 units and reached 8 approximately. A significant difference can be seen between STZ AD model and STZ+NPCs treatment groups with $^{\#}$P<0.05. The lowest GS number was related to STZ AD model mice and the highest one belonged to STZ+MET treatment group (figure 12A).

Figure 12B, shows the Non-goal Sector (NGS) evaluation results on test day of BMT. The number of poking the rest of the holes divided by 17 is defined as NGS on test day. The highest NGS was found in STZ AD model mice and the lowest was related to treatment groups. No significant difference was observed between all experimental groups (figure 12B).

Figure 12C, demonstrates GS/NGS ratio in test day in BMT for all groups. The highest ratio was found in STZ+MET treatment group and lowest one belonged to STZ AD model group. Control GS/NGS was 5 and in STZ AD mice was 2. Treatment with metformin and NPCs transplantation increased this number from 2 to 9 for STZ+MET, to 6 for STZ+NPCs and to 4 for STZ+NPCs+MET. A significant difference was shown between control and lowest one in STZ AD model with

*P<0.05 and highest one in STZ+MET treatment group with *P<0.05. In this evaluation significant difference was presented between STZ AD model group and two treatment groups one STZ+MET with $^{\#\#\#\#}$P<0.0001 and second one STZ+NPCs transplanted treatment group with $^{\#\#}$P<0.01. The other significant difference is related to STZ+MET and STZ+NPCs+MET treatment groups with $^{\$}$P<0.05 (figure 12C).

Figure 12D, Target seeking parameter (the number of poking of both correct and incorrect holes/20), on the test day was higher in the Control group however there was no significant difference between the other groups. Target seeking number in treatment groups was calculated 2.5 for all. This number for STZ AD model group was the lowest one with lowest differentiation was about 2.2 and in control group target seeking was highest 3 (figure 12D).

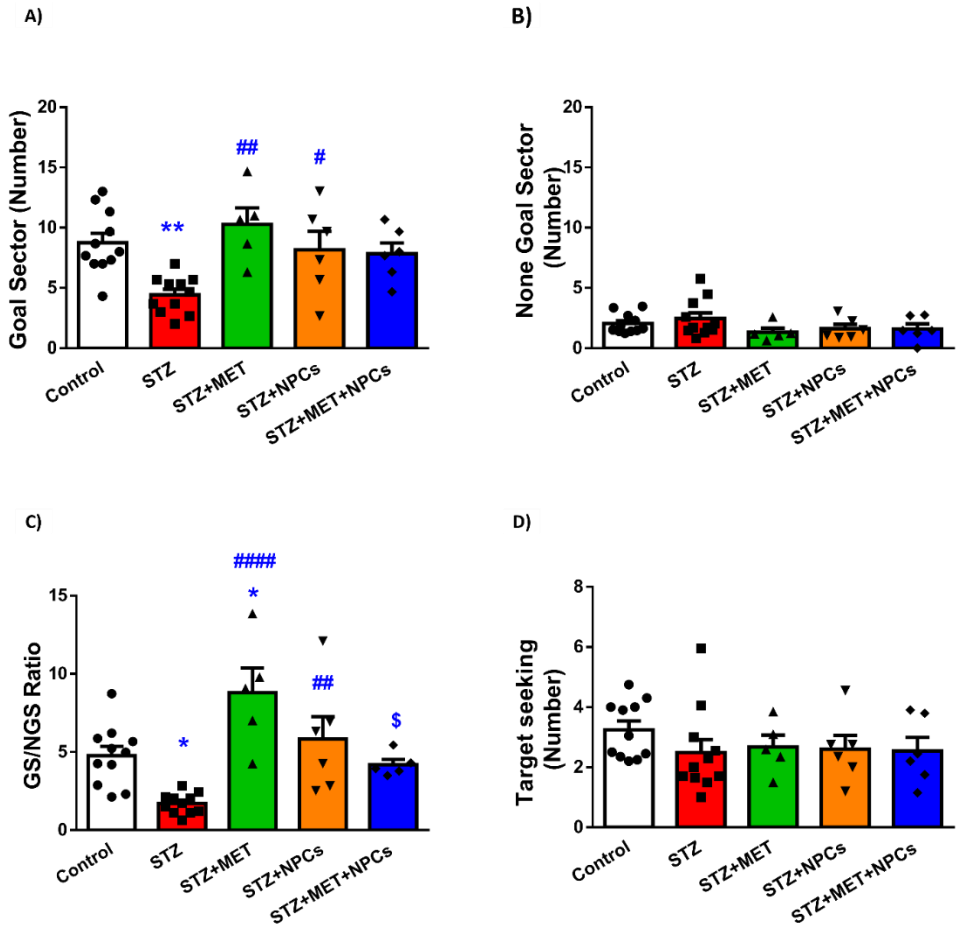


Figure 12. Barnes Maze test results on test days. A) The Goal Sector graph shows significant difference between control and STZ group and between STZ and treatment groups (STZ+MET, STZ+NP &STZ+NP+MET). B) The Non-Goal Sector shows no significant difference between 5 groups. C) GS/NGS ratio demonstrated significant difference between control in comparison with STZ and STZ+MET treatment group. Between STZ and treatment groups (STZ+MET and STZ+NP) significant differences can be seen. H) Target seeking graph shows no significant difference between 5 groups. *P<0.05, **P<0.01, ***P<0.001 vs Control, ###p<0.0001 vs STZ and $P<0.05 vs STZ+MET.

3. Treatment with Metformin, NPCs transplantation and both at same time reduced dark neurons in SAD mice.

3.1. Nissl staining results

The number of dark (dead) neurons was evaluated within the DG, CA1 and CA3 using Nissl staining in 5 groups of experiment (figure 13 A-D).

Figure 13 A presents the Nissl-stained hippocampus of control, STZ-AD model and three treatment groups STZ+MET, STZ+NPCs and STZ+NPCs+MET.

Figure 13 B shows quantified data showing the average number of dark neurons in DG was significantly increased following the STZ AD model group compared to control and treatment groups. The average number of dark neurons in STZ AD model mice was near 140 however this number in the control group was about 50 in DG. The lowest average number of dead neurons was related to the control group. Metformin treatment reduced the number of dead neurons to near control level of about 60. In the other NPCs transplanted treatment group STZ+NPCs and STZ+NPCs+MET the average number of dead neurons decreased to 80 in both. STZ AD model and transplanted treatment groups made significant difference vs control group with ****P<0.0001

and *P<0.05. Alternatively, treatment IP injection of metformin and transplanted NPCs and both together significantly reduced the average number of dark neurons in compare with STZ AD model group with $^{###}$P<0.001 and $^{#}$P<0.05 (figure 13B).

Figure 13C, shows CA1 dark neurons average number in 5 groups. STZ AD model group CA1 demonstrated the highest average number of dark neurons, about 55 in comparison with control and treatment groups. The lowest one is related to control by about 10. Treatment in this study significantly decreased the number of dark neurons in CA1 of treatment groups. In the treatment group's lowest level of dark neurons belonged to treatments with metformin and NPCs transplantation separately. Reduction of dark neurons in treatment groups made significant difference in treatment groups vs STZ AD model group with $^{#}$P<0.05. Additionally, all treatment groups and STZ AD model group have significant difference vs control group with ****P<0.0001, **P<0.01 and *P<0.05 (figure 13C).

Figure 13D shows the dark neurons in CA3 of 5 groups. The highest level of dark neurons was related to the STZ AD model group with an average number 90. The lowest one is 15 in the control group. Treatment groups all presented a low average number of dark neurons in comparison with STZ AD model group. Metformin decreased dark neurons level in CA3 by about 50 units and transplanted NPCs reduced about 60 for STZ+NPCs and 40 units for STZ+NPCs+MET. Significant difference was shown in STZ AD model and STZ+NPCs+MET groups in comparison with control group in level of ****P<0.0001 and **P<0.01. Metformin and NPCs transplantation and both together reduced the level of dark neurons in CA3 and shown significant differences vs STZ AD model group with $^{####}$P<0.0001 and $^{##}$P<0.01(figure 13D).

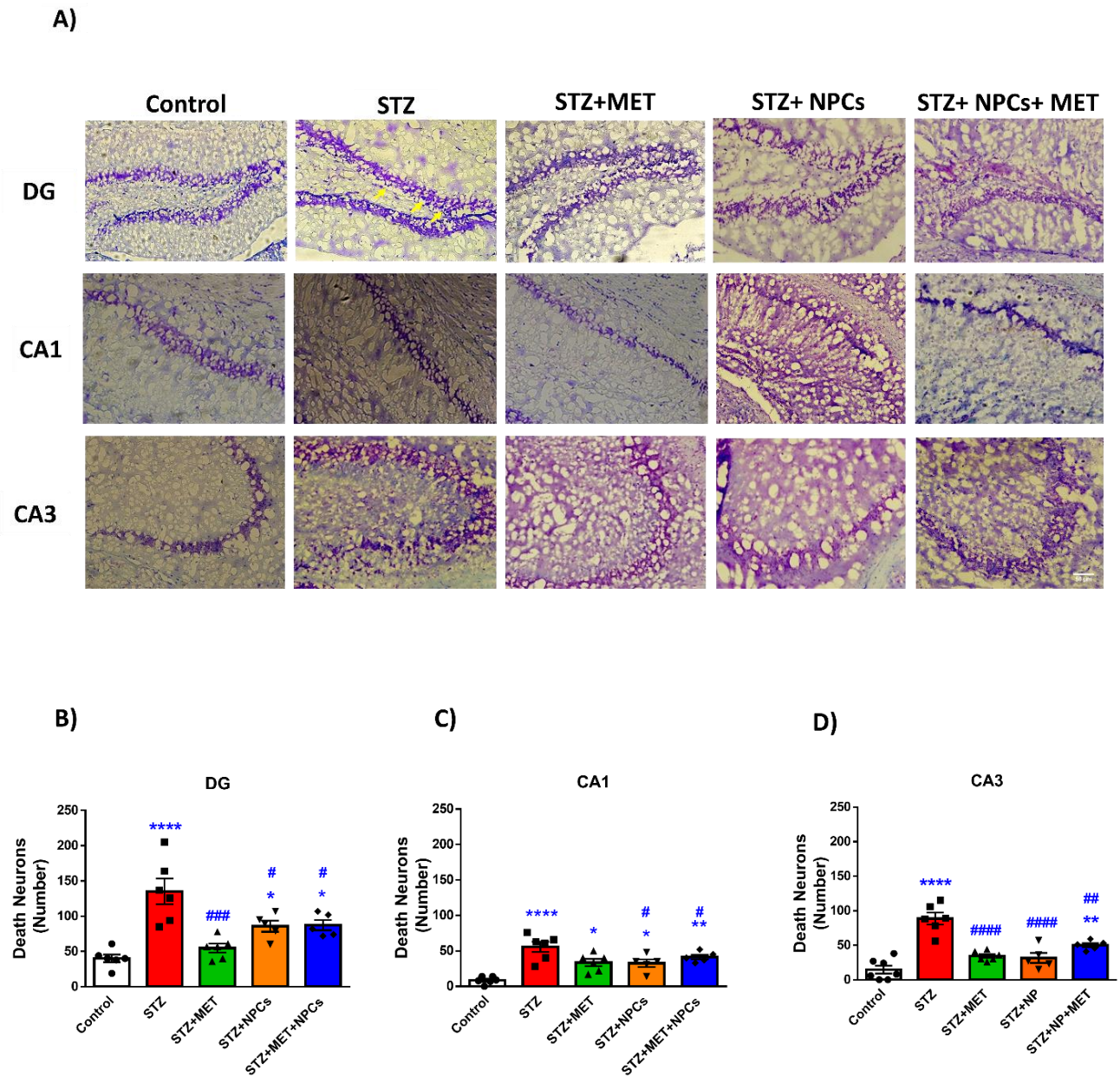


Figure 13. Nissl Staining demonstrated the average number of dark neurons in the hippocampus in control, STZ & treatment groups. A) Nissl-stained images of DG, CA1 and cortex. B) In this graph the average number of dead neurons in DG is calculated and a significant difference is shown between control Vs. STZ, and transplanted groups. In addition, significant differences between STZ and treatment groups can be seen. C) This graph presents the number of dark neurons in CA1 and shows a significant difference in control Vs. STZ and treatment groups. A significant difference can be seen between STZ and transplanted groups. D) This graph shows the average

number of death neurons in CA3 and a significant difference can be seen between control and STZ and STZ+NPCs+MET. And STZ vs. STZ+MET and the transplanted group significantly demonstrated differences. *P<0.05, **P<0.01, ****P<0.0001 vs Control, #P<0.05, ##p<0.01, ####P<0.0001 vs STZ. Scale bar: 50µm

4. Metformin, NPCs transplantation and both together (Metformin +NPCs) ameliorated gliosis in SAD mice

4.1. Glial fibrillary acidic protein (GFAP) fluorescence staining

To measure the extent of gliosis following STZ-AD induction and the treatments, the intensity of GFAP staining was measured in different areas of the brain section including DA/CA1, CA3 and brain cortex.

Figure 14A shows the immunofluorescence images of GFAP staining in DG/CA1. Based on immunofluorescence studies, GFAP intensity in DG/CA1 was significantly increased in STZ-AD model group compared to Control group. The intensity of GFAP in the control group measured 4.66 a.u. and in the STZ-AD model group measured 11.43 a.u. In DG/CA1 hippocampal area significant difference was observed in control vs STZ-AD model with *P< 0.05 (0.0261). Treatment with metformin individually measured 7.93 a.u. and there are no significant observed vs other experimental groups. Additionally in DG/CA1 increasing inflammation in STZ-AD model made significant difference vs NPCs transplanted groups with $^{\#}$P<0.05 (0.0237) for STZ+NPCs and $^{\#}$<P0.05 (0.0145) for STZ+NPCs+MET (Figure 14B). The measurement of GFAP intensity for both transplanted groups was 4.55 and 3.96 for STZ+NPCs and STZ+NPCs+MET respectively.

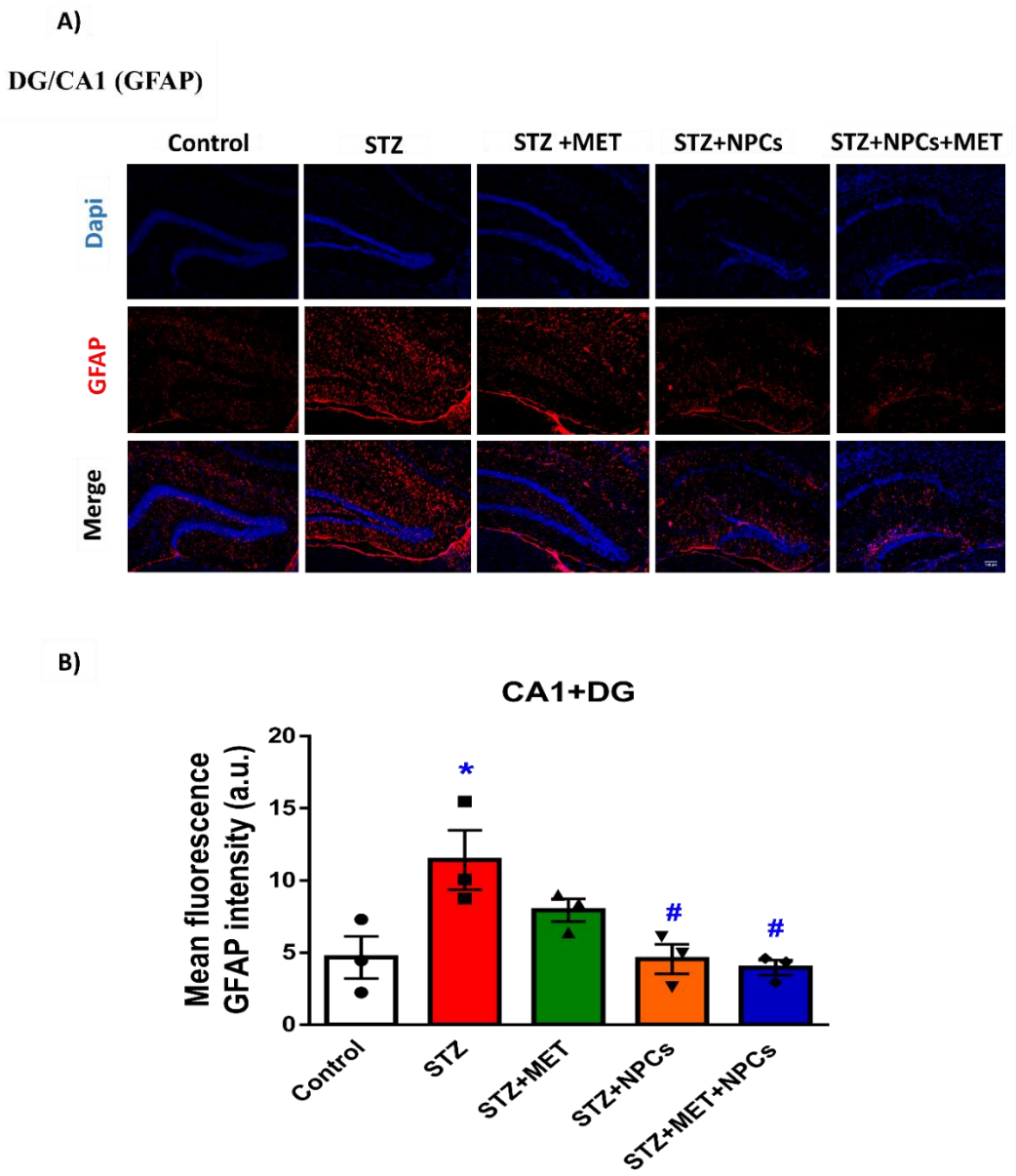


Figure 14. A) Immunofluorescence representative of GFAP staining and intensity measurement on DG/CA1. B) Quantification of GFAP immunofluorescence of DG/CA1 regions of hippocampus and mean fluorescence GFAP intensity in DG/CA1 that show significant difference between control and STZ group. Between treatment groups STZ+MET and transplanted groups significant difference is demonstrated. *P<0.05 vs Control, #P<0.05, ##P<0.01 vs STZ. Scale bar of DG: 100µm

Figure 15 A-B shows mean fluorescence GFAP intensity in CA3 of 5 experimental groups. Part A demonstrated immunofluorescence images of GFAP staining in the CA3 *arena* of hippocampus. Part B evaluated the mean fluorescence GFAP intensity in 5 groups. In control, STZ-AD model, STZ+MET, STZ+NPCs and STZ+NPCs+MET groups intensity of GFAP measured 4.73, 17.57, 4.67, 4.20 and 2.89 respectively. Mean fluorescence GFAP intensity was significantly highest in CA3 of STZ-AD model group. Control vs STZ-AD model group demonstrated significant difference with *** P <0.001(0.0004) and significant decreasing of intensity in all treated groups can be seen vs STZ-AD model group. There are significant differences between STZ-AD model and STZ+MET treatment group with $^{###}$p<0.001(0.0003), STZ+NPCs transplanted treatment group with ###P<0.001(0.0003) and STZ+NPCs+MET with ###P<0.001 (0.0001) (Figure 15B).

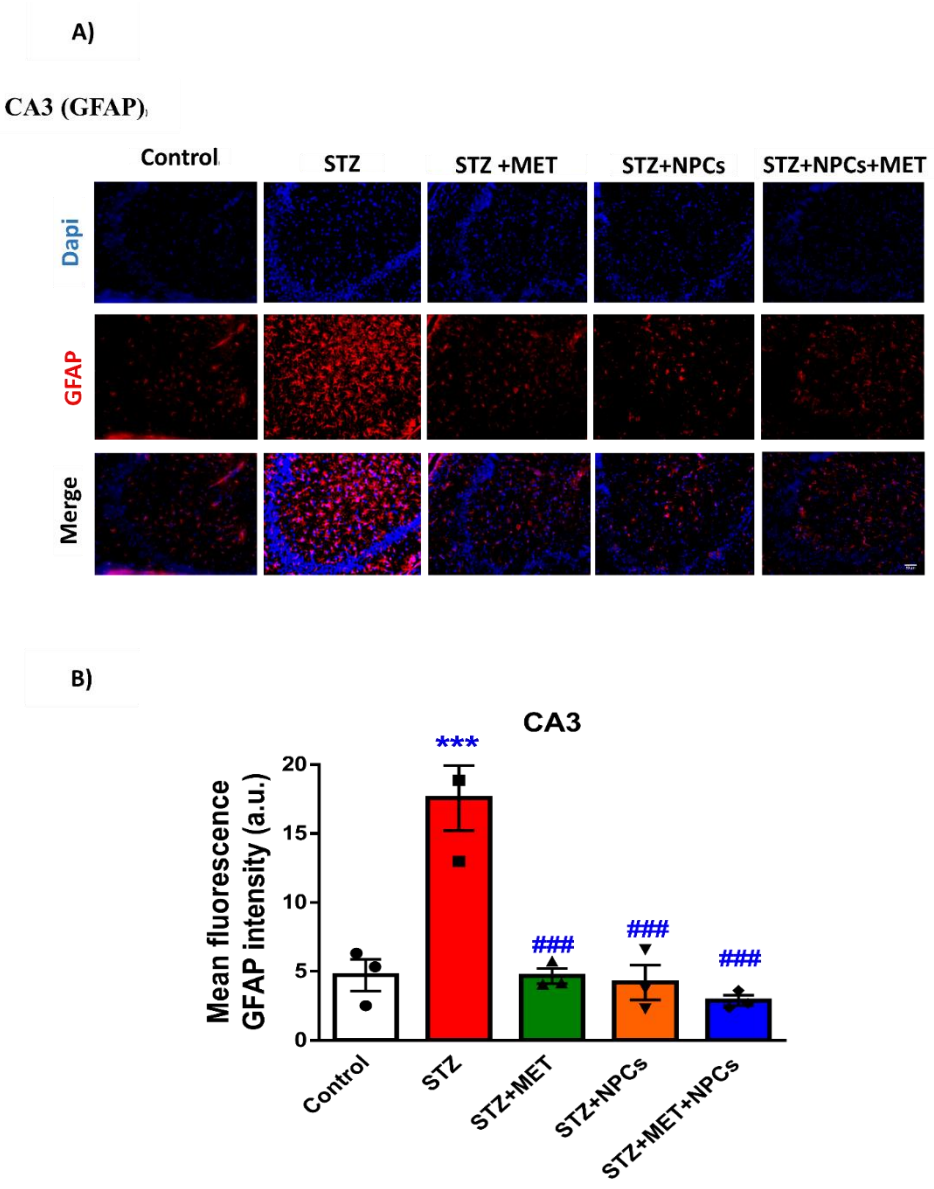


Figure 15. A) Immunofluorescence representative of GFAP staining and intensity measurement on CA3. B) Quantification of GFAP immunofluorescence of the CA3 region of hippocampus and Mean intensity of GFAP in CA3 in five experimental groups demonstrated a significant difference between control and STZ group and between STZ and treatment groups. ***P<0.001 vs Control, ###P<0.001 vs STZ. Scale bar of CA3: 50µm

Figure 16 A-B shows mean fluorescence GFAP intensity in the cortex of 3 experimental groups (control, STZ-AD model and STZ+MET treatment groups). Part A demonstrated immunofluorescence images of GFAP staining in the cortex arena.

Figure 16 B shows the highest inflammation in the brain cortex of STZ-AD model group. The intensity of GFAP in cortex was measured for control, STZ-AD model and STZ+MET treatment groups 2.80, 9.90 and 6.15 a.u. respectively. In cortex there is significant difference between control and STZ-AD model groups with *P<0.05 (0.0196). According to the evaluation results of GFAP immunofluorescence staining, STZ injection significantly increased reactive astrocytes in DG/CA1, CA3 and brain cortex in comparison with the control group. However, the treatment with metformin, NPCs transplantation and both together considerably reduced the inflammation in the hippocampal arena. Metformin was more effective in CA3 in comparison with DG/CA1 and cortex. NPCs transplanted in both transplantation groups were effective in all hippocampal regions especially in CA3 (figure 16B).

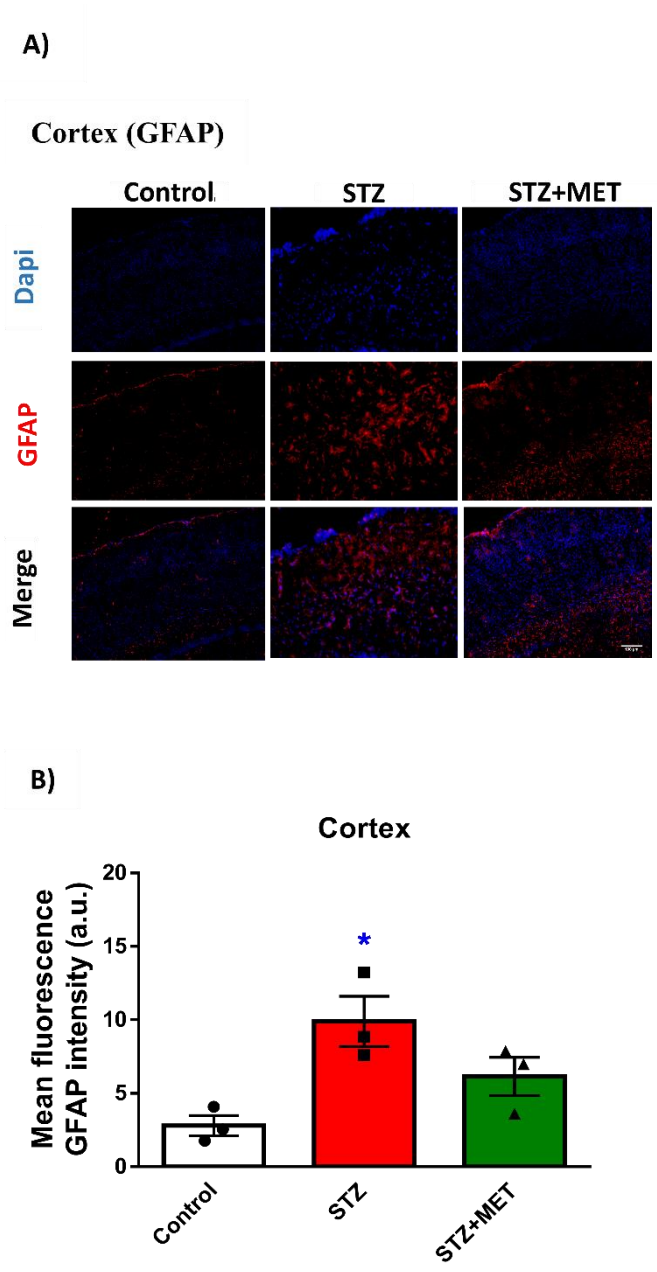


Figure 16. A) Immunofluorescence representative of GFAP staining and intensity measurement on Cortex. B) Quantifications of GFAP immunofluorescence of Cortex and mean fluorescence GFAP intensity in cortex of five groups of study that significantly difference presented in control Vs. STZ group and STZ and STZ+NP+MET group. *P<0.05 vs Control. Scale bar of &cortex: 100µm

4.2. Ionized calcium-binding adaptor molecule 1 (IBA1) protein fluorescence staining

To evaluate the neuroinflammation extent, we measured the intensity of IBA1 staining in the selected arena. Figure 17 shows the mean fluorescence IBA1 in DG/CA1 of 5 experimental groups. Figure 17 A demonstrates IBA1 fluorescence staining images in DG/CA1 hippocampal areas. Figure 17B, IBA1 intensity in DG/CA1, shows the higher reactive microglia in STZ-AD group compared to control, while the difference between control and STZ AD model group was not statistically significant. The mean intensity of IBA1in DG/CA1 measured in control, STZ-AD model, STZ+MET, STZ+NPCs and STZ+NPCs+MET 4.58, 10.89, 10.40, 3.22 and 3.44 a.u. respectively. Transplanted NPCs significantly decreased the intensity of IBA1 in DG/CA1, the reduction of reactive microglia in STZ+NPCs group compared to STZ AD model group, demonstrated no significant difference. According to graph 17B, Metformin was not an effective treatment to decrease the level of reactive microglia in DG/CA1 compared with levels of reactive microglia in STZ-AD model mice. However, neural progenitor cells transplantation alone and in combination with metformin injection had a positive effect on reducing the level of reactive microglia (figure 17B).

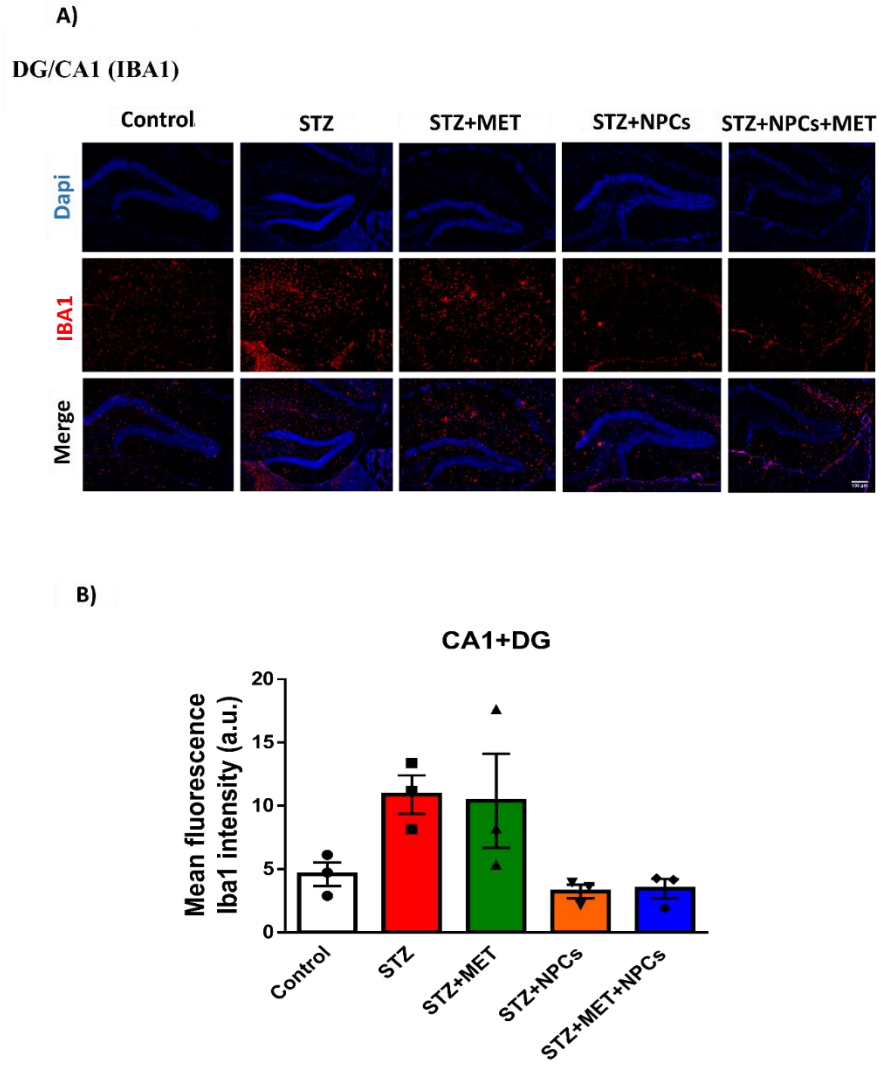


Figure 17. A) Immunofluorescence representative of IBA1 staining and intensity measurement on DG/CA1. B) Quantification of IBA1 immunofluorescence in DG/CA1 and IBA1 intensity show no significant difference in this area. Scale bar: 100µm

Figure 18, shows the mean fluorescence IBA1 in CA3 of 5 experimental groups. Figure 18A, demonstrates IBA1 fluorescence staining images in CA3 hippocampal areas. The mean fluorescence IBA1 intensity was measured for control, STZ-AD model, STZ+MET, STZ+NPCs and STZ+NPCs+MET 4.90, 14.94, 7.44, 3 and 2.90 a.u. respectively. In compared to Control,

Intensity of IBA1 in CA3 was significantly increased by STZ-AD model with ****P< 0.0001. Between control and STZ+MET groups significant difference observed with *P<0.05 (0.0459). According to graph B, significant differences were observed between STZ-AD model groups and all three treatment groups. STZ-AD model vs STZ+MET, STZ+NPCs and STZ+NPCs+MET with $^{\#\#\#\#}$P<0. 0001 based on the measurement of IBA1 intensity and the evaluation of reactivated microglia in the CA3, metformin was able to considerably reduce and improve the inflammation in CA3 compared to STZ-AD model group. In two other transplanted treatment groups STZ+NPCs and STZ+NPCs+MET, IBA1 intensity significantly decreased even less than the control group. This evaluation demonstrated significant difference between STZ+MET vs STZ+NPCs and STZ+NPCs+MET with $^{\$\$}$P<0.01(0.0012) and $^{\$\$\$}$P<0.001 (0.00103) respectively. Between treatment groups, although all three treatments were effective, the rate of inflammation reduction in transplant groups was greater than metformin treatment group with significant differences (Figure 18 B).

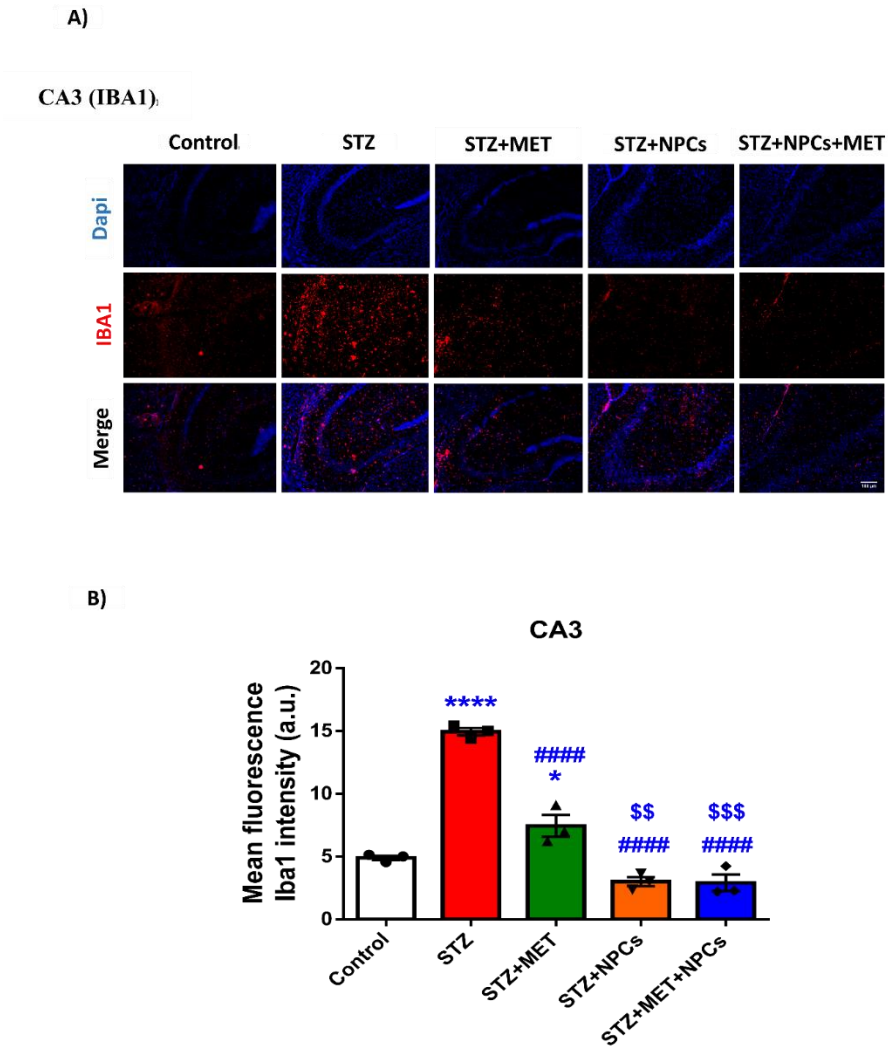


Figure 18. A) Immunofluorescence representative of IBA1 staining and intensity measurement on CA3. B) Quantification of IBA1 immunofluorescence of CA3 that demonstrated significant difference between control and STZ and STZ+MET, between STZ and treatment groups, and between STZ+MET and STZ+NP+MET group. *P<0.05, ****P<0.0001 vs Control, ####p<0.0001 vs STZ, $$P<0.01 and $$$P<0.001 vs STZ+MET. Scale bar: 100µm

Figure 19 A-B shows mean fluorescence IBA1 intensity in cortex of 3 experimental groups (control, STZ-AD model and STZ+MET treatment groups). Part A demonstrated immunofluorescence images of IBA1 staining in the cortex arena. Figure 19B shows the highest inflammation in the brain cortex of the STZ-AD model group. The intensity of IBA1 in cortex was measured for control, STZ-AD model and STZ+MET treatment groups 5.33, 17.42 and 7.83 a.u. respectively. In cortex there is significant difference between control and STZ-AD model groups with ***P<0.001 (0.0001). According to evaluation results of IBA1 immunofluorescence staining, STZ injection significantly increased reactive astrocytes in cortex in comparison with the control group. According to evaluation of IBA1 intensity after treatment with metformin, there is significant difference between STZ-AD model and STZ+MET with $^{###}$P<0.001 (0.0004). Treatment with metformin significantly decreased the inflammation and reactive astrocyte in cortex compared with STZ-AD model group (figure 19B).

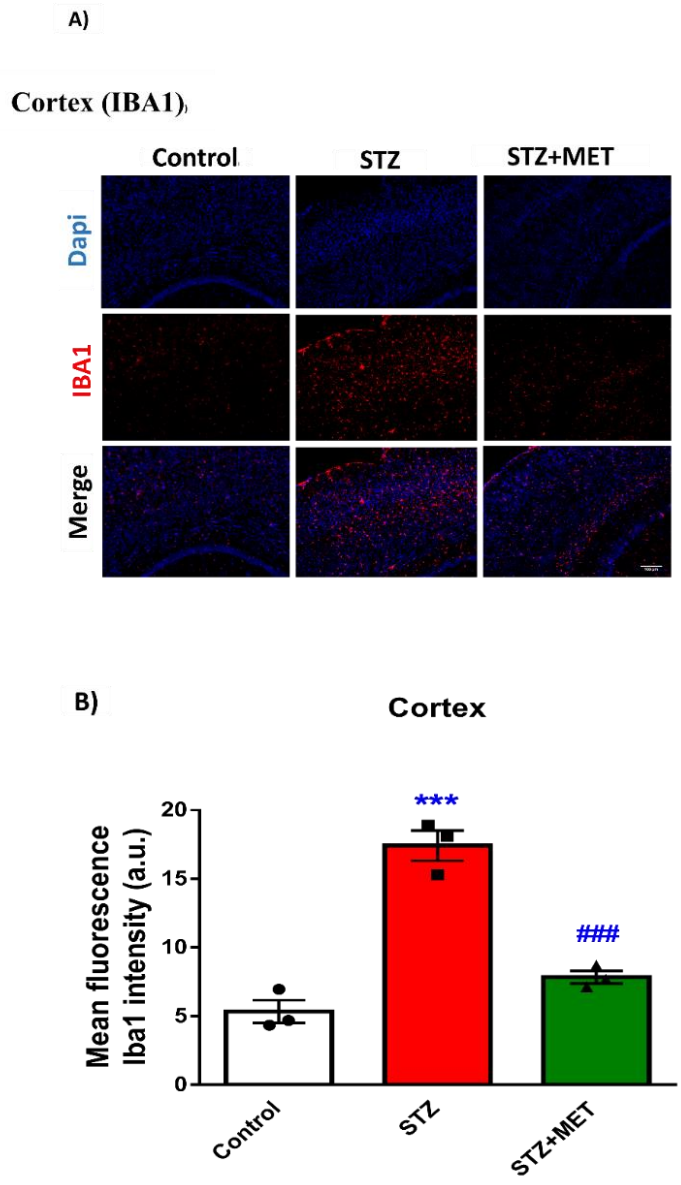


Figure 19. A) Immunofluorescence representative of IBA1 staining and intensity measurement on Cortex. B) Quantification of IBA1 immunofluorescence of Cortex, significant differences are shown between control and STZ group, between STZ and treatment groups and between STZ+MET and STZ+NP+MET group. ***P<0.001 vs Control, ###p<0.001 vs STZ. Scale bar: 100µm

4.3. NeuN (neuronal nuclei) protein fluorescence staining

NeuN as a mature neuronal marker stains the nuclei was used to evaluate changes in the number of neurons in different areas of the brain such as DG/CA1, CA3 of hippocampus and cortex.

Figure 20A, present NeuN immunofluorescence staining images of hippocampus. Part B demonstrated two cut out regions of DG/CA1 for counting the NeuN+cells. Immunofluorescence findings and $NeuN^+$ cells counting in DG/CA1 presented significant reduction following the Sporadic AD induction by ICV STZ compared to the control group, which declined from $3500/mm^{2\ to}\ 2500/mm^2$). Compared to STZ AD model group, the number of $NeuN^+$ cells were increased in treatment groups in DG/CA1 area, showing that metformin was able ameliorate neuronal loss, but no significant difference was shown between STZ AD model and STZ+MET. NPCs transplantation significantly increased the level of NeuN+ cells in DG/CA1 compared to STZ AD model group ($^{\#}P<0.05$ and $^{\#\#}P<0.01$). Among treatment groups, STZ+NP mice demonstrated highest NeuN+ cells number in DG/CA1and made significant difference vs metformin treatment group ($^{\$}P<0.05$) (figure 20C).

Figure 21 A, present NeuN immunofluorescence staining images of hippocampus. Part B demonstrated three cut out regions of CA3 for counting the NeuN+ cells. In CA3 area of STZ AD model mice, neuronal loss significantly increased compared to control level. Treatment with transplantation of NPCs increased the neurons in CA3 remarkably and reversed toward the control even more than control in STZ+NPCs group. Significantly difference presented between control vs STZ AD model and transplanted treatment groups and STZ AD model group with $^{*}P<0.05$, $^{\#}P<0.05$ and $^{\#\#}P<0.01$ (figure 21 C).

Figure 22 A) Immunofluorescence representative of NeuN+ cells counting on Cortex. Part B presented three cut out regions of cortex for counting the NeuN+ cells. Part C demonstrated the number of Neu N+ cells counted in the cortex of three experimental groups (control, STZ AD model and STZ+MET). However, the number of NeuN positive cells in STZ+MET treatment group reached to control and increasing in compare with STZ AD model group, but significant differentiation cannot be seen between these three experimental groups (control, STZ, STZ+MET) (Figure 22 C).

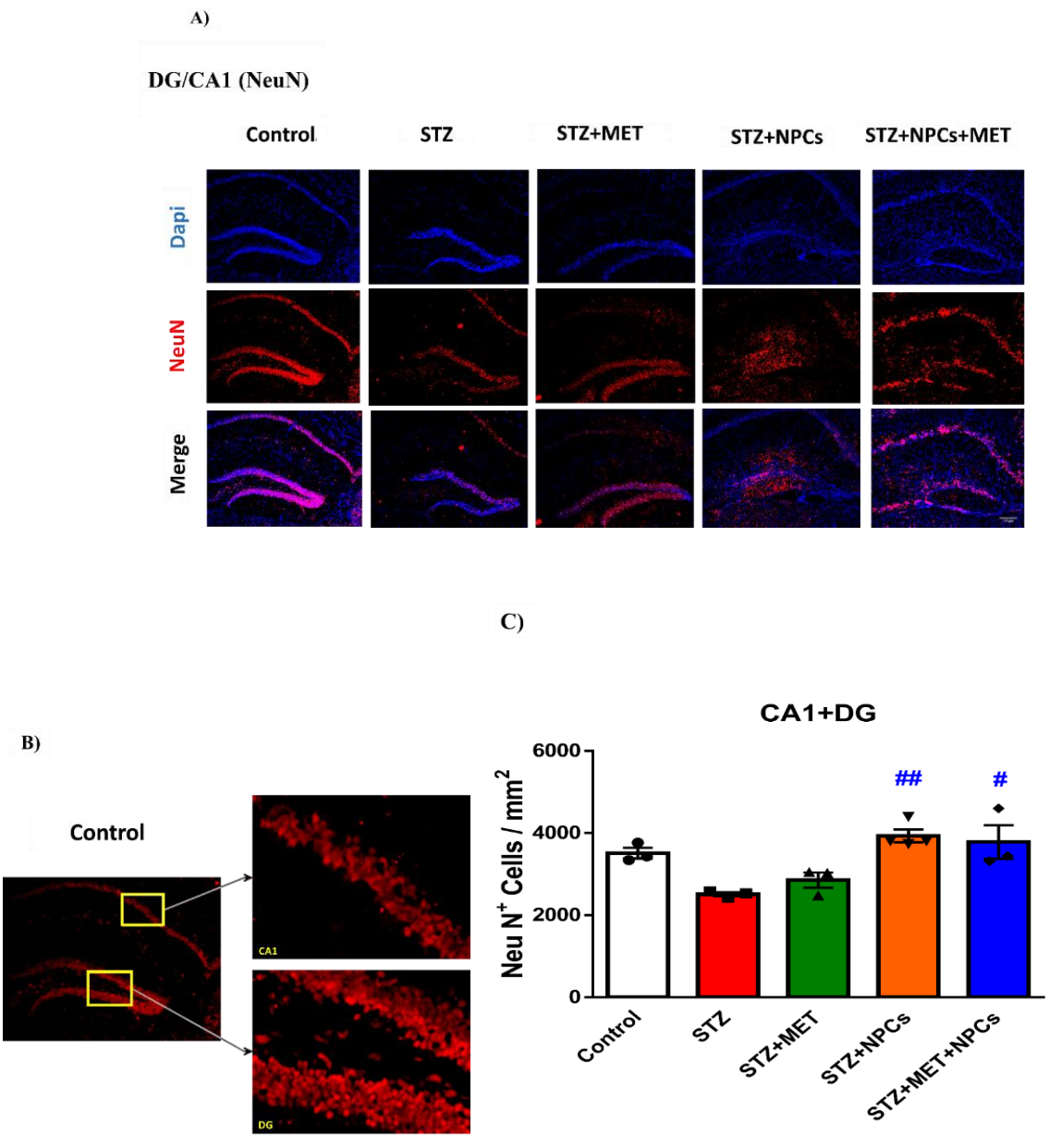


Figure 20. A) Immunofluorescence representative of NeuN+ cells counting on DG /CA1. B) Part B demonstrated two cut out regions of DG/CA1 for counting the NeuN+ cells.C) Number of Neu N+ cells of DG/CA1 hippocampus region in control, STZ and treatment groups in (mm2). The

graph shows significant differences between STZ and transplanted treatment groups. And between STZ+MET and STZ+NP groups. #p<0.05, ##P<0.01 vs STZ. Scale bar: 100µm.

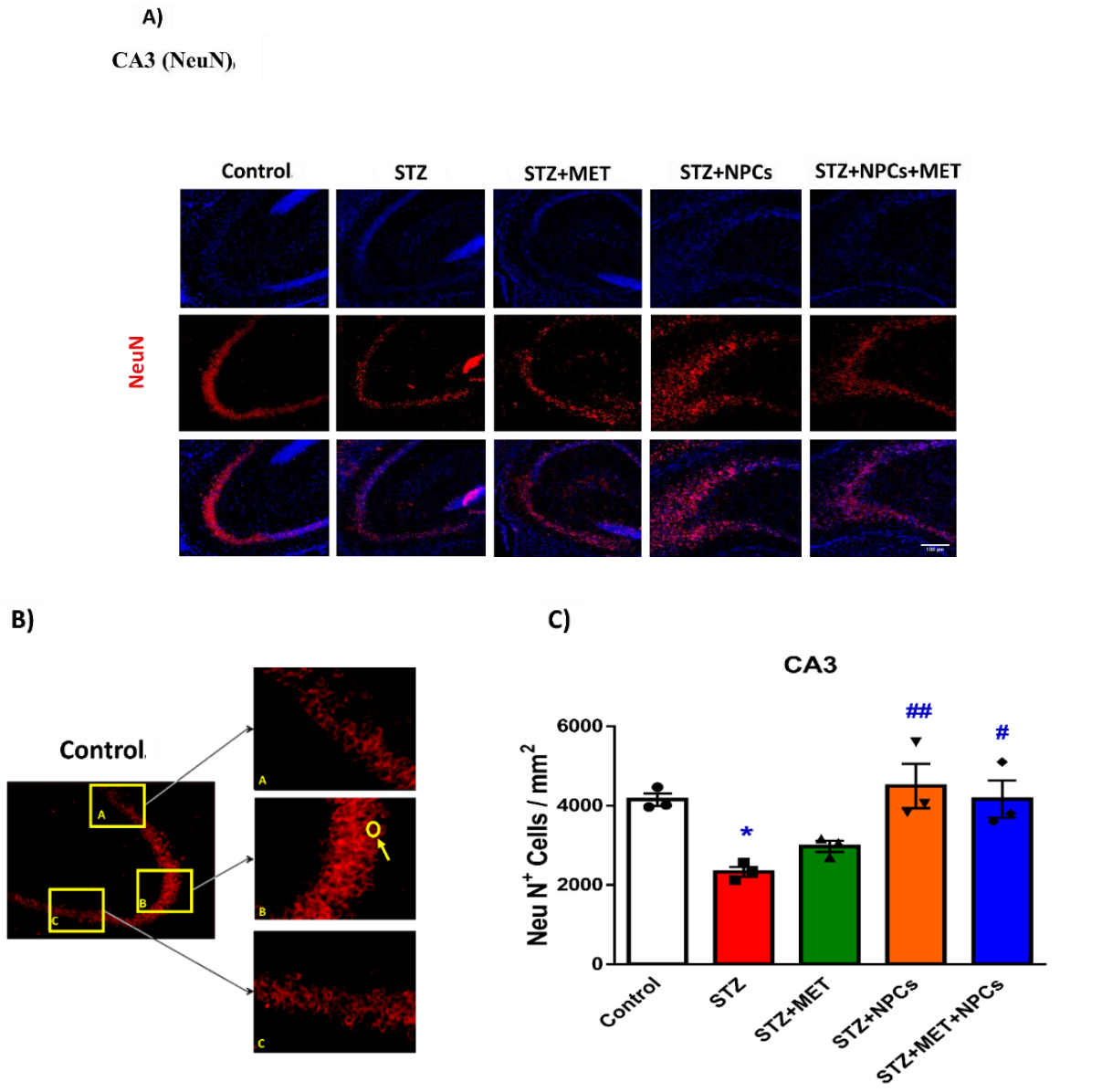


Figure 21. A) Immunofluorescence representative of NeuN+ cells counting on CA3 of 5 experimental groups, control, STZ and treatment groups such as STZ+MET, STZ+NPCs and STZ+NPCs+MET. B) Part B demonstrated three cut out regions of CA3 for counting the NeuN+ cells. C) The Neu N+ cells number of the CA3 hippocampus region in control, STZ and treatment groups. The graph demonstrated significant differences between control and STZ group, between

STZ and transplanted treatment groups. *P<0.05 vs control, #p<0.05, ##P<0.01 vs STZ. Scale bar: 100µm

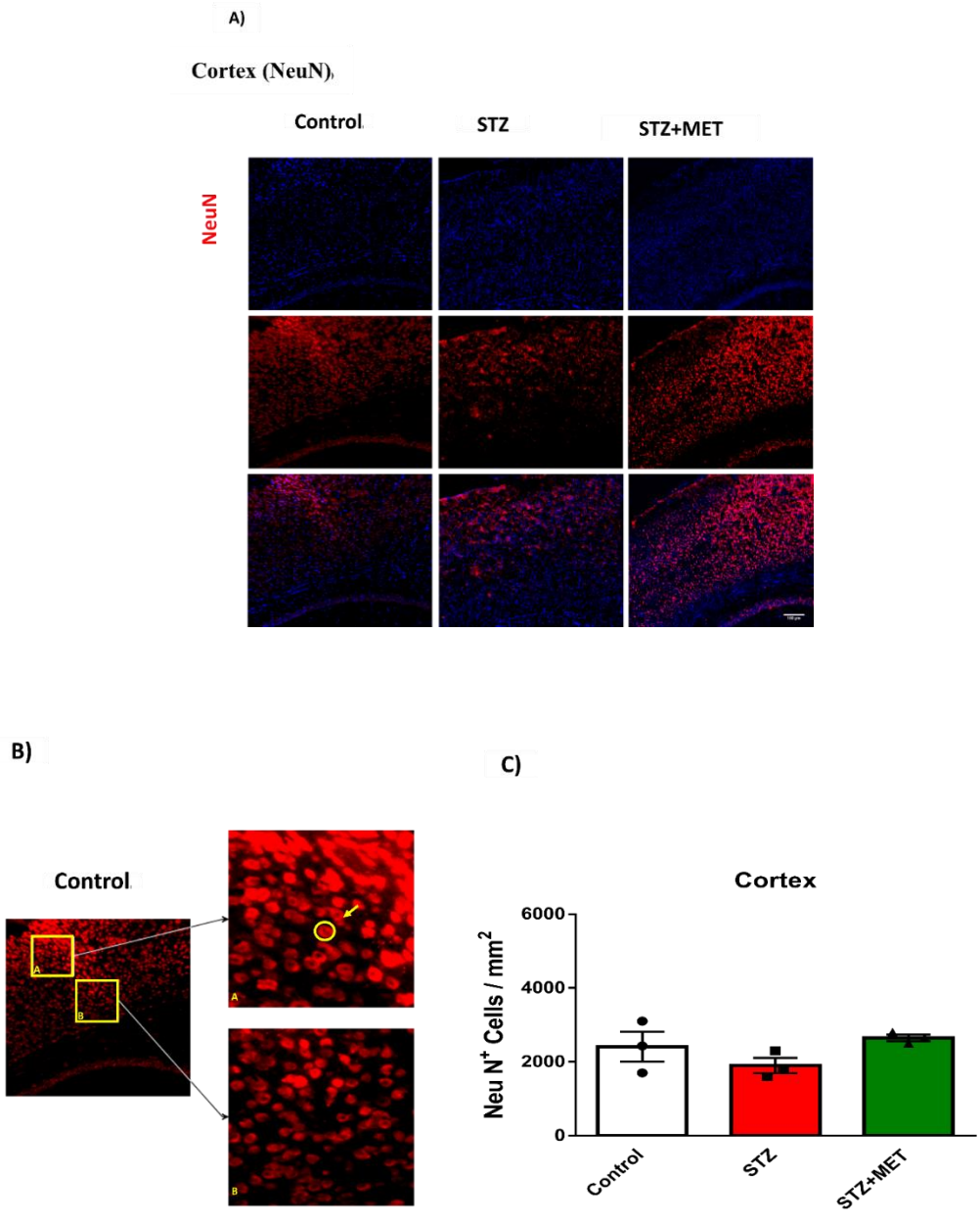


Figure 22. A) Immunofluorescence representative of NeuN+ cells counting on Cortex. B) Part B demonstrated three cut out regions of the cortex for counting the NeuN+ cells. C) The number of Neu N+ cells counted in the cortex of three experimental groups. There is no significant differentiation can be seen between control, STZ, STZ+MET groups. Scale bar: 100µm

4.4. GFP$^+$cells counting (neuronal nuclei)

GFP is constantly used as a tag in protein. The neural progenitor cells (NPCs) which were transplanted in the hippocampus in two groups of STZ+NPCs and STZ+NPCS+MET groups were marked with GFP (green fluorescence). Figure 23 A-C demonstrates the number of GFP$^+$ NPCs that sit on the hippocampus. Figure 23 A shows the GFP$^+$ cells in hippocampus, DG and CA3 separately.

Figure 23 B presents the number of GFP$^+$ NPCs in DG. In both transplanted treatment groups STZ+NPCs and STZ+NPCs+ the number of GFP$^+$cells were around 70. In STZ+NPCs+MET level of this number was more than STZ+NPCs with little difference and no significance was shown between these two groups (figure 23 B).

Measurement of GFP+ cells in CA3 demonstrated a significantly increased number in STZ+NPCs+MET compared to STZ+NPCs. GFP$^+$ cells in STZ+NPCs+MET was about 60 and in other transplanted groups which just received NPCs was about 40. A significant difference was shown between two transplanted groups with *P<0.05. Metformin was effective to increase the GFP$^+$ cell in CA3 of STZ+NPCs+MET group (figure 23 C).

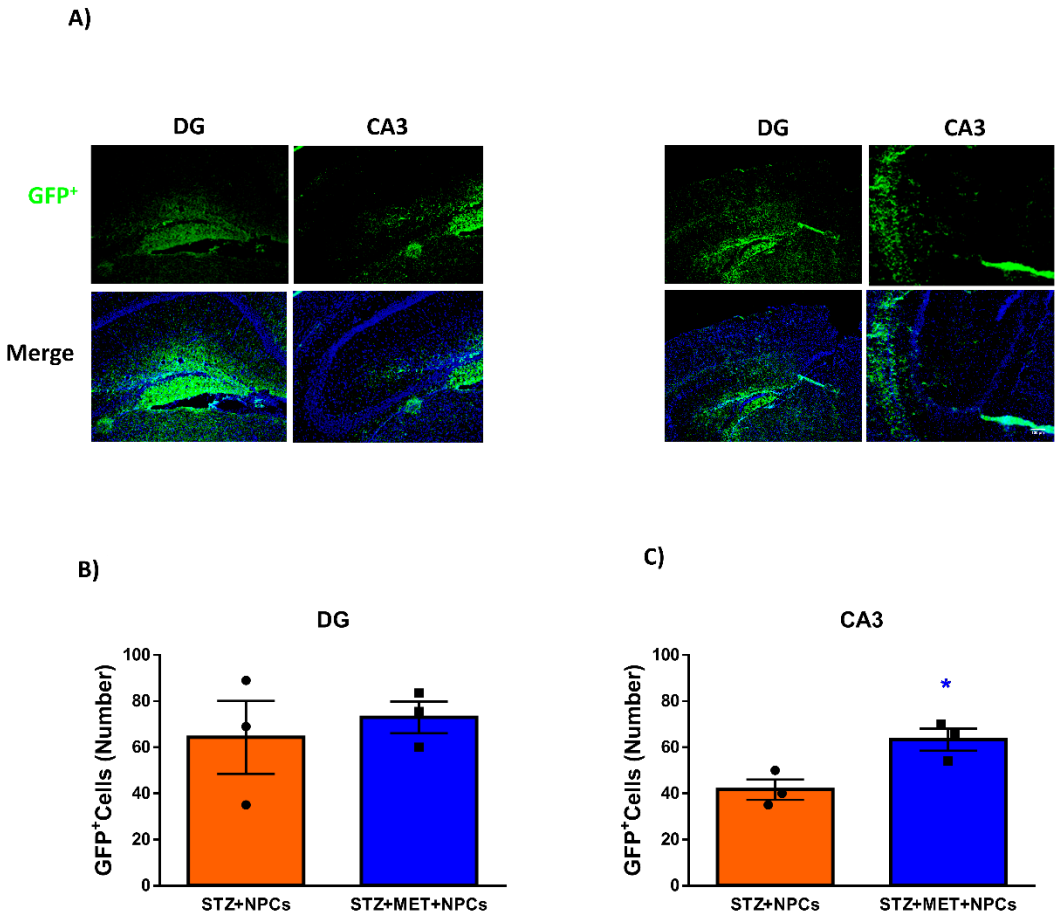
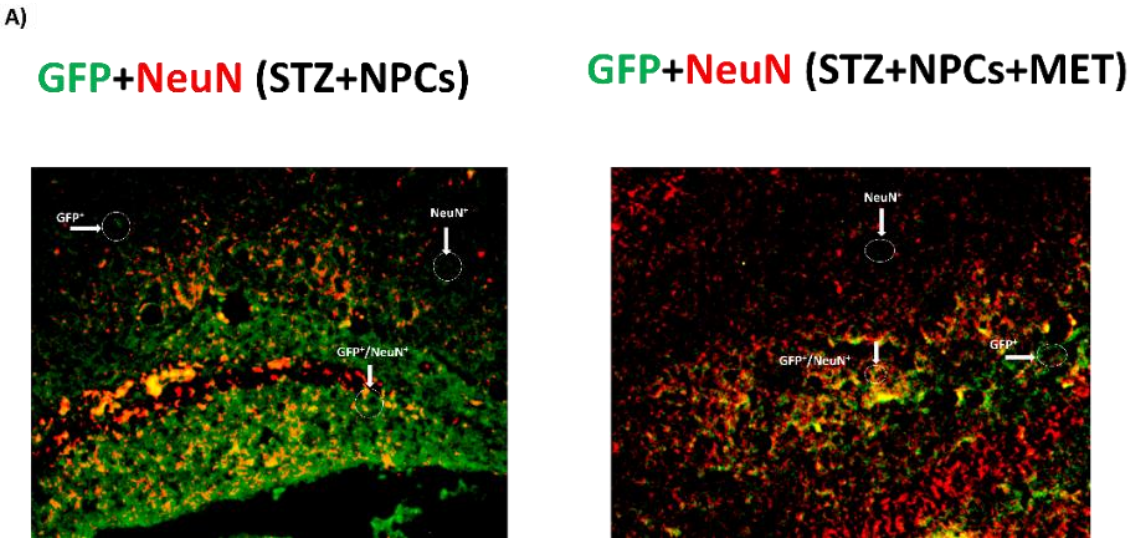


Figure 23. A) Immunofluorescence representative of GFP+ cells counting on DG, CA3 & Cortex in transplanted treatment groups 1- STZ+NPCs and 2- STZ+NPCs+Metformin. A) The immunofluorescence GFP images in DG, CA3 and cortex of STZ+NP & STZ+NP+MET groups. B) The number of GFP + cells of DG hippocampus region in STZ+NP and STZ+NP+METtreatment groups. There is no significant difference between transplanted groups in DG. C) The number of GFP + cells of CA3 hippocampus region in STZ+NP and STZ+NP+METtreatment groups. In CA3 Significant difference is seen between transplanted groups. *P<0.05, scale of HPC images: 200µm, scale of DG, CA3: 100µm

4.5. GFP⁺/ NeuN ⁺cells counting

It is expected that the NPCs which transplanted in hippocampus after 2 weeks differentiate to neurons. As a result, figure 18 evaluates the number of GFP+ cell (green) with is stained with NeuN+ (red) at the same time. Figure 24 A shows the images of hippocampus with GFP⁺ / NeuN ⁺cells in DG. The images consisted of GFP+ cells (green), NeuN +cells (red), merge for GFP and NeuN. Figure 24 B demonstrates the number of GFP⁺ / NeuN ⁺cells in DG, the number was higher in STZ+NPCs+MET than STZ+NPCs but no significant were found between them. GFP⁺ / NeuN ⁺cells number in STZ+NPCs was measured around 40 and STZ+NPCs+MET around 50 (figure 21 B). According to figure 18 C, the level of GFP⁺ / NeuN ⁺cells in CA3 was higher in STZ+NPCs+MET than STZ+NPCs. The p value between two groups was near to 0.05 so it can be considered there is significant difference in CA3 number of GFP⁺ / NeuN ⁺cells between two transplanted groups (figure 24 C).

A)



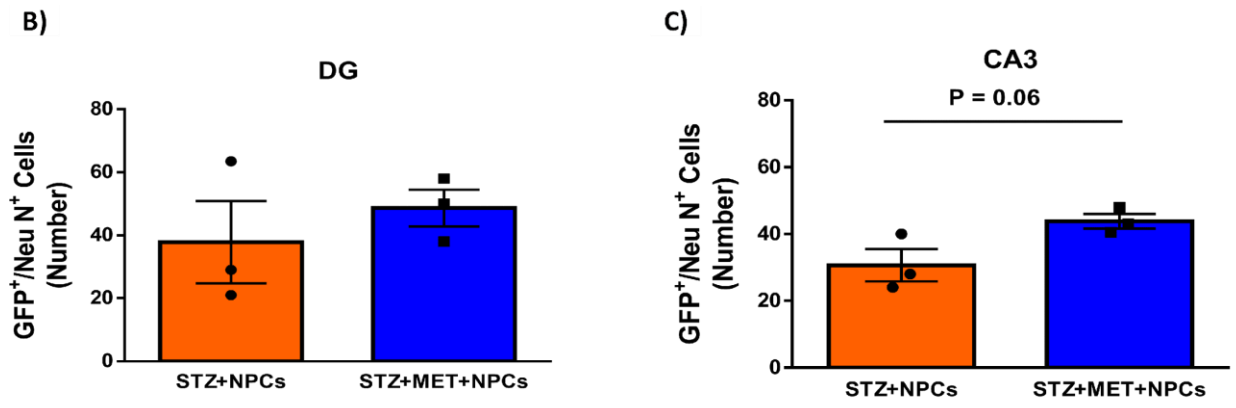


Figure 24. Immunofluorescence representative of GFP+ /Neu N + cells counting on DG and CA3 in transplanted treatment groups 1- STZ+NPCs and 2- STZ+NPCs+Met. A) The immunofluorescence GFP+ /Neu N + cells images in DG and CA3 of STZ+NP and STZ+NP+MET groups. B) The number of GFP+ /Neu N + cells of DG region in STZ+NP and STZ+NP+MET treatment groups. There is no significant difference between transplanted groups in DG. F) The number of GFP + cells of CA3 region in STZ+NP and STZ+NP+MET treatment groups. In CA3 Significant difference is seen between transplanted groups

4.6. GFP^{+}cells tracking:

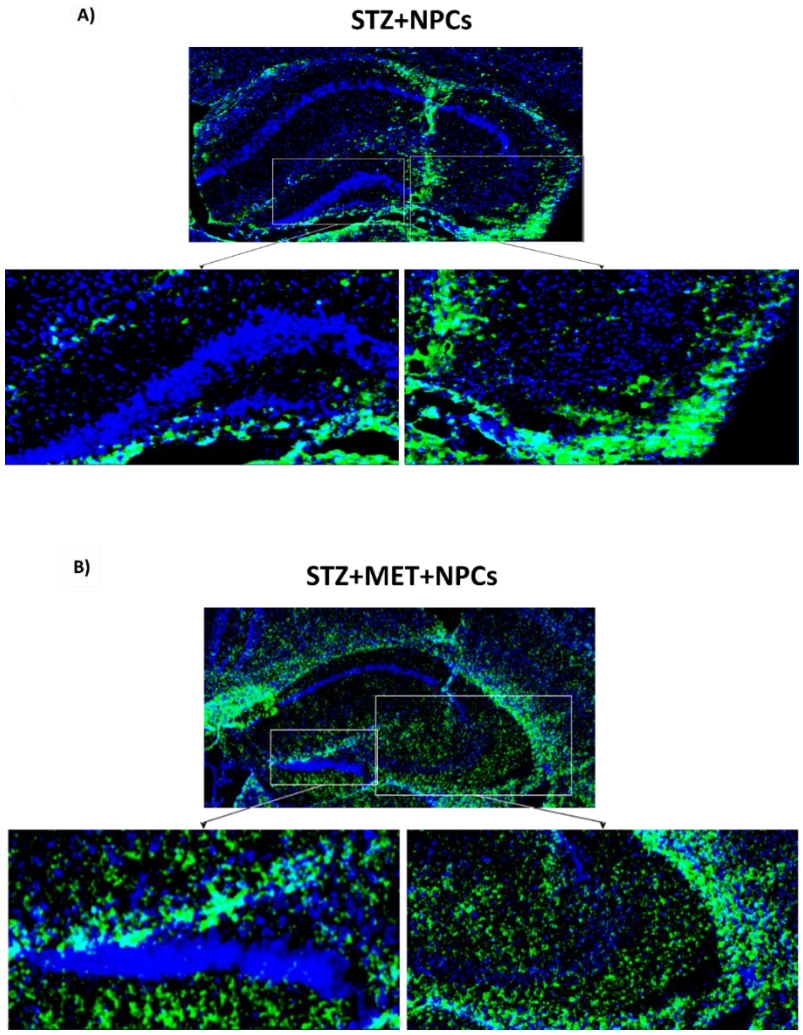


Figure 25. GFP + cell expansion in DG, CA1 and cortex of transplanted treatment groups STZ+NPCs, STZ+MET+NPCs.

V. DISCUSSION

AD is a multifactorial disease in which older age is the strongest risk factor, suggesting that age-related biological processes may be involved in the pathogenesis of the disease. In addition, various factors can cause the progression of this disease (Qiu et al., 2022). Studies have pointed out several mechanisms for the occurrence of Alzheimer's disease and subsequent dementia. The order of these events does not have a neat series but interaction of Aβ oligomers with the glial cells and neurons results in various pathological and physiological anomalies. This pathologic hallmark comprises mitochondrial dysfunction, stimulation of pro-inflammatory cascades, increased tau phosphorylation and oxidative stress, deregulation of calcium metabolism, enhanced glycogen synthase kinase (GSK)-3b activity, stimulation of cell death and neuronal apoptosis in the brain regions that involved in the learning and memory formation (Ghoweri et al., 2020, Rao et al., 2020).

On the other hand, insulin plays an important role in the brain's healthy function. Generally, it is responsible for the regulation of food intake, body weight, eating habits, and homeostasis of energy (Kleinridders et al., 2014, Banks et al., 2012). In the CNS, it plays a crucial role in the activity of neurotransmitters, in particular, its role can be indicated in both long-term potentiation (LTP) and long-term depression (LTD) as most important modulator functions of memory formation. The concept of insulin resistance can be summed up in the simultaneous injection of glucose and insulin in diabetic patients. Some diabetics have had or have low blood sugar levels due to this challenge. These were called insulin sensitivity. In some people, the challenge significantly raises blood sugar. These were not considered insulin sensitive (Himsworth, 1936). We now know that in insulin-resistant patients, at normal plasma insulin levels, target tissues are unable to produce a natural coordinated response to glucose depletion and cannot keep glucose homeostasis in the all vital organism including CNS (Kahn and Jeffrey, 2000, Huang et al., 2002). Deficiency occurs in insulin-target tissues and β cells to cause fasting hyperglycemia and T2DM. Also, numerous bioactive agents are able to disrupt insulin sensitivity (Khan, 2003, Samuel and Shulman, 2012).

Numerous experimental studies provided important evidence of the underlying mechanisms by which T2DM and AD may be related, whether AD and T2DM are both diseases that involve the formation of amyloid in different types of tissue in the human body (Bharadwaj et al., 2017). Neuroinflammation also plays an important role in AD progression (Calsolaro and Edison, 2016).

Activation of microglial leading to release of pro-inflammatory factors that play an important role in nerve injury (Varnum and Ikezu, 2012). Moreover, proinflammatory cytokines, including TNF-α and IL-6, were detected at high levels in the serum and brain of AD patients compared to controls. In addition to proinflammatory cytokines, chemokines are crucial players in neuroinflammation, which are reported to increase in the brain and induce microglial chemotaxis. Accordingly, neuroinflammation in AD reflects abnormal hyperactivation of microglia, overexpression of proinflammatory factors, and neuronal death (Fillit et al., 1991, Strauss et al., 1992). On the other hand, insulin resistance may lead to decreased IGF-1 (insulin-like growth factor) and insulin uptake into the brain, which initiates the inflammatory process (Suzanne, 2009). The brains of rats receiving STZ had significant pathological, biochemical, and neuromolecular abnormalities that overlapped with AD pathological features. STZ injected brains were atrophic due to the neurons and oligodendroglial cells loss because of neuroinflammation and oxidative stress (Lester-Coll et al., 2006). As a consequence, increased beta-amyloid levels interfere with the binding of insulin to the IGF-1 receptor, leading to the release of inflammatory agents (Emmerling et al., 1997). The triggered inflammatory cascade in combination with oxidative stress, toxicity, and β-amyloid accumulation cause a pathological feedback cycle (Blasko et al., 2004). Finally, the dysregulation of insulin signaling can affect the metabolism and function of pro-inflammatory factors and cause neuroinflammation and the activation of inflammatory cytokines, playing an important role in the onset of AD (Thakur et al., 2022, Suzanne, 2009). So, in the early stages of AD, neuroinflammation occurs and its manifestations include the increase of inflammatory cytokines and the infiltration of microglial cells and astrocytes, which initiates some pro-inflammatory cascades (Eikelenboom et al., 2010, Tuppo and Arias, 2005). Besides oxidative damage, tau protein hyperphosphorylation, beta-amyloid accumulation, and cholinergic dysfunction that are the pathways influenced by neuroinflammation in AD (Markesbery and Carney, 1999, Laurent et al., 2018, Giovannini et al., 2002), high levels of inflammatory cytokines such as interleukin-1β (IL-1β), IL-6, and interferon-gamma in the vicinity of beta-amyloid plaques and macrophage cells supports the important role of neuroinflammation in the pathology of AD (D'Andrea et al., 2001).

1. PART 1 – STZ injected mice mimics both behavioral and histopathological hallmarks of AD

In the first step of present study, we validated STZ injection in mice as a SAD model with behavioral and histological evaluation of injected mice. The main mechanism of action reported for STZ is entering pancreatic beta cells via a transporter called the glucose transporter 2 and finally causing DNA damage. Studies have shown that STZ administration leads to brain insulin resistance as a central disorder in the diabetic brain. After STZ metabolization, N-nitrosoureido is released and causes DNA damage and cell death through the production of reactive oxygen species and increases the activity of inflammatory cytokines. Animal experiment using STZ indicated that i.c.v. injection of STZ impairs oxidative metabolism, brain glucose utilization, insulin receptor function, and spatial learning and memory (Lester-Coll et al., 2006). So this animal model is considered an appropriate experimental model for assessment of physiological changes in AD (Gao et al., 2014).

Our investigation revealed that twice STZ injection in the brain i.c.v. caused memory impairment. The injected mice were evaluated via Novel Object Recognition and Barnes Maze test at day 32 and 37 of experiment, respectively. It has been confirmed by several studies that brain injection of STZ may alter insulin receptor function, and spatial learning and memory (de la Monte et al., 2006). Also, Fine and colleagues demonstrated an impairment of long-term memory after the administration of STZ in the lateral ventricle, while the rats were still capable for learning (Fine et al., 2017). Similar studies have reported the same results of memory dysfunctions that observed in different behavioral test assessing memory such as the Morris water maze and Novel object recognition (Huang et al., 2022). According to Novel object recognition test we found consistent results with other's experiments. STZ injection reduced both familiar and novel objects in exposed group. The memory dysfunction in STZ injected mice was proved by significantly reduced novel object exploration in this group. Also, decreasing in the goal sector reaching of STZ group in the Barnes maze was another behavioral data for indicating the memory impairment.

So, both behavioral tests used in the current study approved SAD model development with mentioned dose, route of administration and experimental timeline in mice.

In addition to behavioral results, we need more confirmation data for developing SAD models in mice. In the next step, using brain tissue of STZ group that compared with normal mice, we measured the count of dark cells in the Nissl staining method as an index showing dead neurons. Our results revealed the level of cell death following STZ injection in different areas of the hippocampus that are involved in learning and memory formation. Nissl staining data showed that STZ exposure increased the average number of dead neurons in the dentate gyrus and CA1 regions of the hippocampus. Although no significant changes were observed in the CA3 region, the elevation in the number of dark cells in this region was considerable as well. As STZ treated mice represent an induction model, it could bypass the production of genetically modified animals. Tracking the onset, development, and progression of AD-like pathology including behaviorally and histologically in this model may help elucidate the etiopathogenesis of SAD because its early stages can follow in this model. As mentioned, one of the main histopathological symptoms of AD model is the initiation of inflammatory processes due to STZ which followed by the activity of inflammatory cytokines and as a main consequence the death of neurons is occurred (Thakur et al., 2022). Observations of acute-phase inflammatory proteins alongside cytokines and chemokines associated with AD have been reported, suggesting multiple ways of interaction between these inflammatory mediators (Akiyama et al., 2000). All these changes could trigger cell death in affected neurons.

Overall, the findings of the current study confirmed SAD development after STZ injection in both behavioral and histological. Twice injection of STZ via i.c.v. route can lead to the appearance of AD like behavior 21 days after injection. This phenomenon is caused by neuronal death in the different areas of the hippocampus.

2. PART 2 – Metformin administration and NPCs transplantation ameliorated memory impairment of STZ injected mice but had no synergic effects

In the present study, we used two different treatments alone and in combination. Both metformin administration and NPCs transplantation showed promising effects on the cognitive performance in both Novel Object Recognition and Barnes Maze tests, however simultaneous use of both treatments didn't lead to better results. Currently, metformin is used as the first line of treatment for T2DM (Bendlin, 2022). A link between diabetes and neurodegenerative diseases is for the most accepted, although data is not clear, and the exact mechanisms are unknown. Several experiments reported improvement in cognitive functions following its consumption (Lv et al., 2012). Metformin reduces insulin resistance through gluconeogenesis in the liver and ultimately reduces plasma glucose levels. It should be noted that metformin has the ability to cross the blood-brain barrier (Markowicz-Piasecka et al., 2017). This ability and FDA approval of metformin make it a good candidate for treatment of neurodegenerative disease. The molecular mechanism involved in the neuroprotective action of metformin was multifaceted. This drug revealed remarkable antioxidant and anti-inflammatory activity together in the user's body. It is known that the metformin inhibits mitochondrial complex I of the electron transport chain that is necessary for mitochondrial respiration. This inhibition leads to an energy deficit and indirectly activates the AMPK pathway as a consequence (Stephenne et al., 2011). Thus, stimulation of the AMPK pathway can be seen as a key mechanism of action during metformin administration and explaining many of the known effects of the drug. AMPK is a highly conserved sensor of cellular energy status, which its activation via increasing of AMP levels (in conditions of energy deprivation) consequently inhibits energy consumption and stimulates catabolic pathways. Numerous effects have been attributed to activation of AMPK, including inhibition of mTor and PI3K-Akt signaling. Interestingly, dysregulation of AMPK pathway is associated with insulin resistance and T2DM (Xu et al., 2012) and neuroinflammation (Meares et al., 2013). AMPK signaling plays a crucial role in AD disease progression since AMPK has been shown to regulate neuroinflammation, Aβ generation and tau phosphorylation.

On the other hand, the prevention of neuronal loss has become a more common area of research to find effective treatments against AD (Donev et al., 2009). Even though previous studies suggested some therapeutic strategies for neuronal loss include inhibition of neuronal apoptosis and stem cell therapy, protecting neurons against apoptosis simply does not work for cognitive

impairment in some AD animal models (Kim et al., 2020, He et al., 2020). Interestingly, adult hippocampal neurogenesis is responsible for regulating cognitive functions including learning and memory. Some evidence supports the hypothesis that newly born neurons from NPCs may play a key role in long-term and short-term spatial memory and in object recognition memory (Deng et al., 2010, Piatti et al., 2013). Therefore, enhancing adult hippocampal neurogenesis by stimulating NPCs has been proposed as a suitable strategy to enhance cognitive functions. Also, using the NPCs transplantation to the hippocampal area could be used as another treatment strategy to prevent cognitive performance.

In addition to both mentioned treatment strategies, we evaluated the third strategy to understand the synergist effects of metformin and NPCs transplantation in the SAD. Some evidence highlighted the relation between metformin and NPCs' fate. Metformin has multiple molecular actions, and it is still not clear which ones are important for its neural effects. it is demonstrated that metformin regulate gluconeogenic gene expression in liver cells by activating atypical protein kinase C (aPKC)-mediated CREB-binding protein (CBP) phosphorylation and enhance embryonic murine and human NPC differentiation (Wang et al., 2012). Besides, metformin elevates the levels of the p53 family member transcription factor TAp73 (Engelmann et al., 2015)as an essential element for adult NPC self-renewal and proliferation (Alexandrova et al., 2013), suggesting that this protein might also be important for metformin’s effects in the brain.

In the present study, we demonstrated memory impairment in the STZ receiving group via behavioral experiments. The results of the Novel Object Recognition test showed that both parameters of novel object exploration and familiar object exploration decreased in the STZ group. Treatment with metformin exerts a slight improvement in the treated group. However, the discrimination index results indicated no significant differences between the metformin and STZ group, the index level was like the control group and the difference was non-significant.

Moreover, an assessment of Barnes' maze experiment revealed that the random movement strategy to escape the box increased dramatically in the STZ group compared to control mice. Based on these results, there was no clear strategy for learning and finding the escape box in the

mentioned group. On the other hand, the random strategy decreased in metformin treated mice, while serial strategy increased slightly in this group compared to SAD mice. However, both changes were very narrow and insignificant. An elevated random strategy could explain the existence of strategy to learn, and these mice serially pock in each hole to find the escape box. Besides, the number of mice reaching the goal sector in metformin received mice increased significantly compared to STZ injected animals. Evaluation of GS/NGS in Barnes maze showed a considerable healing effect of metformin in memory restoration.

The various studies give support to our data, as metformin treatment in different routes has led to ameliorate memory function (Chen et al., 2021c, Ou et al., 2018, Plaschke et al., 2010, Katila et al., 2020). Kazkayasi and colleagues showed that intranasal metformin treatment improved cognitive dysfunctions via insulin signaling pathway in ICV-STZ-induced mice model of AD (Kazkayasi et al., 2022). Although various studies reported the improvement effect of metformin on learning and memory, as well as our behavioral results, the responsible mechanism that metformin causes ameliorative effects on memory and learning is not fully understood yet. Several mechanistic pathways have been proposed for the ameliorative effects of metformin.

In 2016, Mustafa and his colleagues studied the scopolamine-amnesic mouse model with impaired learning and memory skills. They reported memory improvement after acute administration of metformin for about two weeks as recovery effects of metformin (Mostafa et al., 2016). Mustafa et al., also have shown that the protective effect of metformin against scopolamine-induced cognitive impairment is probably through the Akt/GSK3 beta signaling pathway and inhibition of tau protein phosphorylation. Importantly, metformin treatment successfully restored pAMPK and CREB transcription factor levels in the hippocampus, while scopolamine treatment reduced pAMPK and CREB levels, these results support the hypothesis that metformin can be used as a preventive drug potentially against cognitive and memory disorders (Mostafa et al., 2016, Ma et al., 2021).

One of the other keys signaling pathways that are regulated via metformin administration and play an important role in memory dysfunction of AD subjects is neuroinflammation. Several pieces of evidence demonstrated that metformin promotes neuroprotection in diabetic mice by

dampening inflammatory responses through its inhibitory effects on various signaling pathways. The main mechanism reported for metformin inhibitory action is regulating the expression of the upstream controller NLRP3, related cytokines and NF-κB signaling pathway (Docrat et al., 2021). There are controversial results for the anti-inflammatory mechanism of metformin suggesting that it suppresses inflammatory response by inhibition of NFκB via AMPK-dependent and independent pathways. Metformin increases nitric oxide (NO) production and inhibits the poly [ADP ribose] polymerase 1 (PARP-1) pathway through AMPK activation, leading to suppression of inflammatory response. In addition, it suppresses inflammatory response through inhibition of advanced glycation endproducts (AGEs) formation and receptor for AGE (RAGE) expression (Saisho, 2015).

The observed ameliorative effects of metformin on learning and memory in our study may be because of the mentioned neuroprotective effect of it. As we briefly discussed the role of AMPK activity, there are several reports on the effect of AMPK activation via different mechanisms on AD (Cao et al., 2017). Even in other experimental models of AD that involve a non diabetic pathway to cause dementia, metformin could influence sharply on Aβ metabolism and showed reduction of brain Aβ levels (Gupta et al., 2011). Interestingly, a recent study by Chen et al. demonstrated that metformin reduced the Aβ burden by increasing AMPK-induced autophagy. This was confirmed by a genetic study in which neurons from AMPKα2 knockout mice showed increased Aβ production (Chen et al., 2019).

According to another study, 6-week metformin treatment significantly improved rat memory impairment, including recovery of long-term potentiation (LTP) and normalization of several brain molecular changes such as RAGE and NF-kB (Chen et al., 2016). Zhao et al. (Zhao et al., 2014) evaluated the ameliorative effects of metformin on seizures, cognitive impairment, and markers of brain oxidative stress observed in PTZ-induced kindling animals. The authors confirmed that metformin suppresses the development of kindling, improves cognitive impairment, and reduces brain oxidative stress. These results led to the conclusion that metformin may be a potential preventive agent against cognitive impairment (Zhao et al., 2014). Similarly, in the chronic L-methionine model of memory impairment, metformin was shown to prevent pathology possibly by normalizing oxidative stress in the hippocampus (Alzoubi et al.,

2014). In another study, metformin was shown to prevent spatial reference memory impairment associated with high-fat diets in rats (Allard et al., 2016). These results were also confirmed by Ashrostaghi (Ashrostaghi et al., 2015), who showed that metformin administered orally for 36 days had a positive effect on spatial memory performance in the Morris Water Maze. Also, McNeely (McNeilly et al., 2012) investigated whether metformin treatment could reduce cognitive deficits induced by a high fat diet by improving insulin sensitivity. It acts as a modulator to reduce insulin resistance and weight gain associated with high fat feeding but does not affect performance in matching and mismatching-to-position tasks (MTP/NMTP task) (McNeilly et al., 2012). In the most recent study, Allard et al. investigated the effect of long-term administration of metformin on brain neurotrophins and cognition in aged male C57Bl/6 mice. Metformin has been reported to prevent spatial reference memory impairment associated with a high fat diet in this experiment as well. Analysis of brain homogenates showed decreased transcription of BDNF (brain-derived neurotrophic factor), NGF (nerve growth factor), and neurotrophin 3 (Nrf3), but protein levels were unchanged. Also, they reported downregulation in the expression of the antioxidant pathway regulator Nrf2. This study highlights the need for further research on long-term metformin treatment and its potential role in altering brain biochemistry (Allard et al., 2016). In contrast with our results, metformin-treated high-fat fed rats showed no significant effect on recognition memory in the studies conducted by Lennox (Lennox et al., 2014).

Conversely, the results of some clinical studies show that long-term treatment with metformin can reduce the risk of cognitive decline. Although, Ng et al. found no significant interaction effect of metformin use with APOE-ε4 and depression (Ng et al., 2014), in a study carried out by Guo et al., it was confirmed that a 24-week intervention with metformin improved cognitive function in depressed patients with T2 diabetes. The authors emphasized that metformin significantly improved depressive performance and altered glucose metabolism in depressed patients with diabetes (Guo et al., 2014). Another clinical study that examined the effect of diabetes treatment on specific cognitive domains over 4 years found that only participants using metformin alone had better cognitive performance (verbal learning, working memory, and executive functions) compared to participants using other antidiabetic drugs (Herath et al., 2016). Interestingly, Moore et al. stated in 2013 that the effects of metformin on patients' cognitive function may be

influenced by its dependence on vitamin B12 deficiency, which is widely accepted as one of the main causes of cognitive decline. The authors highlighted that vitamin B12 and calcium supplements may reduce metformin-induced vitamin B12 deficiency (Moore et al., 2013). However, a recent study by Khater et al. showed that although vitamin B12 levels were deficient in diabetic patients taking metformin, this was not the cause of cognitive impairment (Khattar et al., 2016).

Consistent with some of the mentioned studies, behavioral results showed that besides metformin healing effects in some parameters, no improvement was observed in cognitive performance in some other parameters. Some studies show evidence that metformin may have beneficial effects on cognitive impairment and memory loss, while others suggest that metformin may be harmful to neuronal survival. The results of our data showed that following metformin injection in the STZ model, the memory loss that occurred in the novel object recognition test was reversed. In the spatial memory (Barnes test) we observed that there was no difference in learning with metformin injection in the STZ model and that the learning strategy didn't change. However, the memory has improved following the disorder of the injection of STZ. Several other studies have suggested an association between chronic metformin administration and beta-amyloid accumulation (Li et al., 2012, Picone et al., 2016, Picone et al., 2015). For example, in 2015 Picone and colleagues (Picone et al., 2015) found that metformin increased the metabolism of Aβ and APP. In vitro evaluation approved that higher concentrations of metformin were associated with increased APP expression and, consequently, the formation of Aβ fragments and aggregates. In addition, they showed that this drug regulates APP and presenilin 1 gene expression in the mouse brain. Scientists have suggested that this drug exerts a similar effect in vitro, ex vivo, and in vivo (Picone et al., 2015). Another same report assessing the effects of AMPK activation on neuronal function (Potter et al., 2010) showed that metformin administration reduced long-term late potentiation in hippocampal slices, an electrophysiological correlate of memory. These studies predict that metformin treatment may be harmful to patients with AD by worsening their memory performance (Potter et al., 2010). One of the most recent studies showed that metformin increases the processing and accumulation of Aβ, mainly in the cerebral cortex (Picone et al., 2016). The authors treated C57B6/J mice with metformin for seven days or

three months. They noted that this drug stimulates APP processing, especially in chronic administration. In addition, they found that metformin also increased the accumulation of Aβ aggregates in the cortical region. In contrast, they did not observe the presence of Aβ granules in the hippocampus. According to some experiments, this antidiabetic drug induces molecular mechanisms leading to neurodegeneration in the rat brain (Picone et al., 2016).

Aligned with mentioned evidence our data showed that the amount of motor activity increases in metformin treatment. As mentioned, harmful effects of metformin on cognitive activities have been reported in some studies. In line with this hypothesis, Arabmoazzen et al showed that in the rats of multiple sclerosis disease model, the amount of movement and speed, which are the parameters of motor activity, increased in metformin treatment (Arabmoazzen and Mirshekar, 2021). Also, Lei et al. have shown that chronic metformin treatment had complex effects on locomotor and cognitive function in non-diabetic mice. Metformin enhanced locomotor and balance performance. On the other hand, metformin treatment induced an anxiolytic effect and impaired cognitive function upon chronic treatment (Li et al., 2019). Taken together, our results demonstrated a slight reversal effect of metformin on memory impairment in STZ exposed mice, with no considerable impact on the learning process. In the molecular evaluation part of our data, we will discuss pathways that may involve in the mentioned behavioral alterations via metformin treatment.

In the next part of behavioral data, we highlighted the effect of NPCs transplantation in the hippocampus on the learning and memory performance. In the group receiving NPCs, the time of novel object exploring elevated remarkably. The percentage of both novel object and familiar object exploration increased in this group compared with STZ injected mice. Also, the discrimination index influenced significantly via NPCs transplantation.

In addition, Barnes maze experiment evaluation showed that the random movement strategy to escape the box was reduced in NPCs transplanted mice. Reciprocally, serial, and direct strategy elevated in these mice when compared to SAD group. Also, measuring the number of reaching to the goal sector in NPCs received mice increased significantly compared to STZ injected animals

and calculation of GS/NGS approved an ameliorative effect of NPCs transplantation on inhibition of memory impairment.

Transplant regeneration and endogenous regeneration are the most common methods used for stem cell therapy in relevant research studies (Vasic et al., 2019). Several studies have shown the positive effect of in vivo transplantation using specific cell types in animal models of AD. A variety of transplantation protocols existed. In some cases, mouse and human embryonic stem cells were first induced into mature basal forebrain cholinergic neurons and then transplanted into a mouse model of AD (Moghadam et al., 2009). In addition, human neural stem cells from the fetal telencephalon were transplanted into AD mouse brains (Lee et al., 2015). In another study, by treating with specific protein extracts, transplantation of induced pluripotent stem cells derived from mouse skin fibroblasts was performed in an AD 5XFAD transgenic mouse model (Cha et al., 2017). Following the transplanted cells, it has been demonstrated that after transplantation into the hippocampus of AD mice, neural progenitors were successfully differentiated into cholinergic neurons (Fujiwara et al., 2013). As another NPC related strategy for AD treatment, previous research has shown that stimulating the proliferation of NPCs can be an effective therapeutic option in ameliorating cognitive decline in several AD mouse models (Huang et al., 2017, Morello et al., 2018). These investigations showed that the number of new neuroblasts and immature neurons significantly increased after induction of NPC proliferation, which has functional implications for the hippocampal network. On the other hand, decreased proliferation of NPCs is related to their cell cycle dysregulation, leading to cognitive dysfunction. However, the mechanism involved in causing cell cycle arrest of NPCs in AD needs to be investigated (Choi et al., 2018).

As mentioned, the induction of NPCs proliferation improves cognitive functions, although we transplanted NPCs into the hippocampus, aiming to determine whether their direct influence on the hippocampus affects cognitive performance or not. In our study, the animals were evaluated 3 weeks after cell transplantation and showed performance enhancement in novel object recognition. Ngwenya et al assessed the model of traumatic brain injury and they found that NPCs injection in the hippocampus region improves performance in new object recognition in the NOR test after one week, which was in line with our study (Ngwenya et al., 2018).

The results of the Barnes maze in the learning phase showed that the transplantation of NPCs did not exert a meaningful influence on learning parameters. Although the NPCs group showed a significant reduction in distance and velocity when compared with STZ exposed mice. These results confirmed that NPCs receiving mice spent less time finding the escape box. The data explained that NPCs transplantation could inhibit learning distractions caused by STZ injection. Also, lower level of velocity and speed in the cell transplanted group may be because of stress decrease in mentioned mice. In contrast with our findings, Doeppner et al showed that the speed parameter in the Morris water maze increased in ischemia models by injecting NPCs in the brain, insignificantly (Doeppner et al., 2014).

The mechanism of action for NPCs' effect in improving cognitive activities is not fully understood. One of the most crucial roles of NPC could be related to anti-inflammatory features of these cells. Ryu et al have shown that NPCs transplantation significantly inhibits the inflammatory response and provides neuroprotection in the hippocampus of mice injected with Aβ1-42. Their data showed a correlation between inflammatory reactivity and neuronal viability supports the possibility that NPC actions to reduce inflammatory responses may be beneficial in reducing neuronal damage in the inflamed AD brain (Ryu et al., 2009). In addition, some studies attributed the effect of NPCs on preventing the activity of tau proteins. Transplantation of these cells into 2-month-old transgenic mice with a mutation in tau (human P301S) protected neuronal networks influenced by tau pathology (Lee et al., 2015). Altogether, our findings approved the potential of learning and memory improvement in NPCs transplanted mice of AD model.

Finally, we investigated the combination of metformin and NPC cells as a third treatment strategy in the STZ injected mice and evaluated the effect of both therapeutic strategies in the AD model. Our behavioral data in this group showed that the combination of these two treatments in the NOR test had positive effects and memory improvement. However, the improvement level of memory metformin plus NPCs group, considering novel object exploration, familiar object exploration and discrimination index, did not increase compared to NPCs mice. These observations may have been due to the small ameliorative effect of metformin on cognitive performance in NOR test. Furthermore, the Barnes Maze results confirmed previous behavioral results, and there was no synergistic effect when using both treatments together.

Most studies show the beneficial role of metformin on neural stem cells (Chiang et al., 2016, Scarpello and Howlett, 2008, Chung et al., 2015). The predicted improvement of memory following the combination use of these two could be related to the effect of metformin on nerve cells. Metformin drug has been shown to activate neural stem cells and promote differentiation. Ruddy et al have indicated that metformin activated NPCs and consequently reported an improvement in cognitive functions following an ischemia model (Ruddy et al., 2019). Also, metformin increased NPC-derived neurogenesis and oligogenesis and elevated the size of neural stem cells in the SVZ of postnatal mice (Wang et al., 2012, Fatt et al., 2015). Metformin as an AMPK activator rescues PGC1α, NRF1, and Tfam gene expression levels in human neural stem cells that are necessary for mitochondrial mass, cell respiration and viability in presence of Aβ. These gene expressions were blocked by simultaneous treatment with an AMPK antagonist (Chiang et al., 2016).

Achieving the mentioned results may be since both metformin and NPCs therapeutic options are effective by their anti-inflammatory influence. So, their simultaneous use could not increase the response. To find the reason for these observations, molecular studies are needed, which we have discussed in the next part of the study.

3. PART 3 – Metformin administration and NP transplantation inhibited neuronal cell death and inflammation in the hippocampus of STZ injected mice

In the next step of our experiment, immunohistofluorescence assay and Nissl staining on the brain tissue sections of mice receiving 2 weeks treatment with metformin, NP transplantation and/or both of treatment are dedicated to detecting underlying molecular mechanisms that lead to SAD model, and also protective effect of metformin and NP transplantation.

There are several studies that investigated the effect of STZ injection on the cognitive impairment caused by neuronal loss in the hippocampus. Here, using Nissl staining, we assessed the rate of dark cells in the different regions of the hippocampus as an indicator of dead neurons. Our result

showed an increase of the neuronal cell death in the STZ exposed mice. Consistent with our results, Li and colleagues showed a decrease in cell count of hippocampus in mice received STZ. Although a wide range of doses (usually higher than 0.5 mg/kg) and duration used for mentioned experiments, our findings showed that even low doses of STZ could affect neuronal cell viability (Li et al., 2016). Another experiment using Nissl staining in STZ model by Knezovic et al., demonstrated that morphologic changes after STZ treatment are dose-dependent showing more dark cells with a higher dose. They also indicated dose-dependent pathological changes in the brain of STZ treated animals particularly as a reduction of cortical thickness (Knezovic et al., 2015). In addition to the mice model of SAD, other studies on rats injected with STZ demonstrated the same effect on the hippocampus region of induced animals. They pointed DG and after that CA3 as most affected areas, respectively, in the hippocampus as our data indicated (Li et al., 2016, Dehghan-Shasaltaneh et al., 2016).

Based on staining results, treatment with metformin reduced the generation of dark cells in the DG and CA3 compared to SAD mice. Although there was a decrease in the number of Nissl bodies in the CA1 region of the hippocampus, the reduction was not significant. On the other hand, alterations in both DG and CA3 were remarkable.

Gorgich et al., in a recent study revealed that metformin significantly reduced the lipid peroxidation level and increased the total antioxidant capacity in the hippocampus of the metformin group. They showed that survival of hippocampal neurons was significantly higher in the metformin group as compared to the control group, while the number of TUNEL-positive neurons decreased significantly (Gorgich et al., 2021). Other studies showed that metformin can significantly reduce neuro-inflammation, decrease the consequence loss of neurons in the hippocampus of diabetic animals, and prevent diabetes-induced memory loss in rats (Sangi and Al Jalaud, 2019).

Besides, several investigations indicated that metformin could significantly prevent the neuronal tissue damage in all areas of the brain of the animals in different animal models presenting neuronal dysfunction and damage (Patil et al., 2014, Tang et al., 2017, Akinola et al., 2012). A study investigating the neuroprotective effect of metformin with respect to Parkinson's disease

demonstrated that long-term metformin treatment led to significant protection of neuronal damage and improvement of the locomotor and muscular activities in MPTP-treated mice. They attributed metformin treatment effectiveness to the antioxidant activity improvement as compared to the MPTP-treated group (Patil et al., 2014). On the other hand, Tang et al, showed that metformin can exert a neuroprotective effect by increasing survival percentage for neuronal cells, decrease brain edema, preserve the BBB and improve cognitive function. Also, they reported that metformin treatment reduced the neuronal apoptosis of the cerebral cortex, striatum, and hippocampus in a septic brain mice model (Tang et al., 2017).

Our assessment in comparison to the STZ receiving mice, NP transplanted animals showed considerable reduction in Nissl positive neuronal cells in all three regions of the hippocampus. In line with the previous part of the result, the CA3 area received the most protective effect of treatment.

Cell survival evaluation on the hippocampus by Villar and coworkers indicated that STZ promotes neuronal loss in the CA1 field and decreases neurogenesis in the dentate gyrus. Also, they showed that STZ induced a reduction in hippocampal volume and presynaptic protein levels and an exacerbated microgliosis, relevant AD features. The stem cell transplantation rescued CA1 neurodegeneration but did not reverse the decrease of immature neurons, suggesting that the therapy effect varied among hippocampal neuronal populations (Zappa Villar et al., 2020). Another study carried out by Oh et al., reported that the neuronal stem cell transplants play a role in protecting cholinergic neurons (Oh et al., 2015).

The third treated group in our study using metformin and NP transplantation via Nissl staining showed that cell survival was not improved in comparison with the NP group. These mice received metformin along with cell transplantation, but the protective effect of transplanted cells was not improved. In contrast with our findings, several studies used combination therapy with stem cell transplantation to achieve better performance and protection in the CNS. Soleimani Asl and coworkers revealed that the administration of Chitosan-coated Selenium nanoparticles enhances the efficiency of transplanted stem cells in decreasing the neurotoxicity induced by STZ through an increase in the antioxidant capacity (Asl et al., 2021). Also, another study

demonstrated that metformin represents an optimal candidate neuro-regenerative agent that is capable of not only expanding the adult NPC population but also subsequently driving them toward neuronal differentiation by activating different molecular pathways including aPKC-CBP and AMPK. Although in the mentioned experiment metformin has been reported as an enhancer for neural precursor proliferation/self-renewal and differentiation, inhibition of apoptosis was not confirmed a major contributor to the elevation in the number and size of adult neurospheres induced by metformin treatment (Fatt et al., 2015).

In the continuation of the experiment, to find the mechanisms involved in the observed changes, we investigated the markers related to inflammatory pathways including glial fibrillary acidic protein (GFAP) and ionized calcium-binding adapter molecule 1 (IBA1).

In physiological conditions astrocyte cells play a crucial role in memory consolidation. Astrocyte dysregulation led to the release of GFAP, which has been linked with memory impairments in animal studies. During normal physiological conditions, these cells are involved in memory consolidation and retrieval. In contrast, after CNS damage, pathology, and/or immune cell activation, astrocytes tolerate a series of changes that this process is called astrogliosis. Experiments demonstrated that inhibition of this process results in harmful effects on brain health. Activated astrocytes release GFAP, an intermediate cytoskeletal protein that is a downstream biomarker of astrogliosis and its release triggers several different mechanisms leading to neuronal and synaptic dysfunction. Recent experiments reported the modulatory role of GFAP in astrocytic regulation of neurogenesis. They demonstrated that GFAP ablation decreases reactive gliosis processes and increases hippocampal neurogenesis in animal models. The mentioned result is achieved in both normal conditions and after a sustained injury. Based on astrocytes' role in regulation of synaptic transmission, glial dysfunction, and dysregulation of GFAP levels are also implicated in AD pathogenesis and progression.

In addition to animal model studies, in human investigations for neurodegenerative disease, GFAP level alteration has been reported in different specimen types. Studies have indicated increased levels of GFAP in the CSF, plasma, serum, and brain tissue (GFAP+ astrocytes) of AD patients relative to controls. Despite numerous evidence showing astrogliosis' relation with AD

pathology, little is known about the effect of astrocytic biomarkers changes on cognitive function and brain structure in aging adults and AD patients. Epidemiological studies have demonstrated a negative association between overall cognitive performance later in life and higher levels of GFAP measured in different regions of cortices during autopsy (Bettcher et al., 2021, Pereira et al., 2021).

Our result related to GFAP measurement using immunohistofluorescence assay revealed the increased level of this marker in different brain regions of SAD mice including dentate gyrus and CA1, CA3 and cortex. The fluorescent intensity in all evaluated regions increased significantly compared to control mice. Although, the elevation level of intensity was not the same in all three measured areas and the level of GFAP intensity was remarkable in CA3 compared with other parts.

Recent study by Tiwari et al, on hippocampus and cortex of STZ model indicated an increase in the level of GFAP protein expression in both regions of the brain via western blotting. They also confirmed western blot data with counting GFAP positive cells in cortex and hippocampus using immunohistofluorescence assay. Both counting positive cells and protein assessments showed that elevation in GFAP level of the hippocampus was more considerable compared to cortex area (Tiwari et al., 2021). Moreover, Dos Santos and co-workers investigated the level of GFAP in the hippocampus of STZ injected animals (3 mg/kg) during one and four weeks after injection via enzyme-linked immunosorbent assay (ELISA). They showed that GFAP level increased in STZ group during both time points. Although, this elevation in 1 week was more than 4 weeks changes (Dos Santos et al., 2020). Another experiment, confirming our results, evaluated GFAP level representing astrocyte activation in different brain areas including CA1, CA3, dentate gyrus and cortex of STZ injected mice. They showed that GFAP increased in all mentioned regions but CA3 elevation was more than others compared to control mice (Zhang et al., 2019). In addition to brain areas, there were several studies that measured GFAP level in other regions of interest like retina tissue of SAD animal models. They also indicated an elevation in the level of GFAP expression in mentioned tissue (Canovai et al., 2022).

Based on our observations, metformin treatment could reduce the intensity of GFAP in the CA3 area remarkably, while the decrease in the marker intensity of dentate gyrus & CA1 and cortex was not significant compared to STZ group. In line with our findings, several investigations confirmed the effect of metformin on astrogliosis and GFAP levels in STZ animal models. They showed that metformin treatment with different doses and timelines decreased the level of GFAP positive cells in the hippocampus of treated group compared to STZ group (Canovai et al., 2022, Oliveira et al., 2021). Wei et al., proposed a mechanism for reduction of GFAP positive cells via metformin. They demonstrated that metformin rescued insulin resistance leading to decrease in the GFAP level (Wei et al., 2022a). Also, metformin treatment significantly decreased GFAP expression to nearly the same distribution of the control group in a diabetic retinopathy animal model induced by STZ (Hassan et al., 2019).

NPCs transplantation in STZ mice showed a significant reduction of GFAP in dentate gyrus & CA1 and CA3. In this treatment group also CA3 influenced more than other areas. This result confirmed that engrafted NPCs didn't differentiate into $GFAP^+$ astrocytes, and almost all of them may differentiate into neurons. We assessed this possibility in the next part. Armijo and colleagues evaluated reactive astrocyte levels by GFAP staining. Quantification of GFAP in the hippocampus and fornix area revealed a reduction of this marker in both brain regions of transgenic AD mice receiving induced pluripotent stem cells (iPSCs)-NPC injection in comparison with non-injected animals. Furthermore, they noticed a decrease in the cell body hypertrophy and thickening of the processes of reactive astrocytes in the iPSC-NPCs transplanted group (Armijo et al., 2021). Zhao et al., examined whether intranasally transplanted human neural stem cells (hNSCs) attenuate neuroinflammation in the brains of AD mice model by quantifying the density of microglia and astrocytes as the glial cells responsible in initiating neuroinflammation in the brain. The coronal sections of transgenic mice were immunostained with GFAP and AD mice model exhibited a significantly increased density of astrocytes in both the hippocampus and cortex compared with WT mice, while hNSCs transplantation reduced the density of astrocytes to a level comparable to that of WT mice (Lu et al., 2021). Also, Park and co-workers evaluated neural precursor cells differentiate into multiple cell types to delay disease progression in a neurodegenerative mice model. Their results suggested that functional recovery of astrocytes

and reduced GFAP positive cells is one of the mechanisms by which the NPCs restore neuronal function in animal models of Huntington's disease (Park et al., 2021).

Measurement of GFAP intensity in the dentate gyrus & CA1 and CA3 regions of third treated group (metformin+NPCs) demonstrated a reduction in GFAP level compared with SAD mice. Our result indicated that the combination of treatments in this group had the same result as the NPCs group. Although, the observed changes in this group were more remarkable compared to the NPCs group. Studies demonstrated metformin can inhibit stem–cell aging and promotes the regeneration and development of neurons. It has been shown to expand the endogenous neural stem cell (NSC) pool and promote neurogenesis under physiological conditions and in response to brain injury (Derkach et al., 2021).

Iba-1 was known as a microglia/macrophage-specific marker that was widely used for microglial detection. Moreover, microglial activation mostly relied on the immunostaining of Iba-1 to characterize its morphology and distribution and quantify its numbers. Evidence demonstrated that the expression of Iba-1 is increased in activated microglia, suggesting that the increased expression of Iba-1 can be used as a marker for microglial activation. However, some investigations have found that the activation of microglia in brain tissue is not always accompanied by increased expression of Iba-1. It is believed that Iba-1 can only be labeled microglia and its expression level may not relate to microglia activation in brain tissue. Iba-1 protein expression detected by Western blot was increased slightly in diabetic rats compared with age-matched normal control. There was statistically significant between two groups at 2wk after diabetes onset (Shi et al., 2021).

We evaluated Iba-1 expression by immunostaining as a marker showing neuro-inflammation in different regions of the experimental group's brain. Based on our results, the level of Iba-1 positive cells increased in the CA3 and cortex of STZ injected mice, however there was an insignificant elevation of Iba-1 positive cells in the CA1 and dentate gyrus regions. Almeida dos Santos et al., in 2020 investigated microglial activation (based on IBA-1 content) in the icv STZ-induced inflammation in the hippocampus. They also found an increase in hippocampal IBA-1 content at 1 week and 4 weeks after STZ treatment (Dos Santos et al., 2020). In addition, other

study assessing the STZ-induced mice (STZ was microinjected bilaterally into the dorsal hippocampus of *C57*BL*/6J* mice) reported an increase in fluorescence intensity of IBA1 in the hippocampal CA1 region(Wei et al., 2022b). Besides, consistent with our findings, some evidence showed the same increased level of Iba-1 positive cells in the cortex of STZ-injected animals (Hai-Na et al., 2020). Metformin treatment affects the level of Iba-1 expression in the hippocampus and cortex of treated mice compared to STZ group. We observed a significant reduction in the level of Iba-1 of both CA3 and cortex areas of treated mice. Studies showed that treatment with metformin improved the spatial memory scores of diabetic animals align with reduced expression of Iba-1 (Oliveira et al., 2021). In addition, Li and colleagues demonstrated the same effect of metformin in diabetic retinopathy animal models. Based on their report, metformin treatment for 12 weeks moderately reduced the number of Iba1-positive retinal microglia when compared with saline treatment STZ-injected animals (Li et al., 2020b).

Our investigation related to 2 other treated groups including NPCs transplantation and NPCs plus metformin treatment indicated the inhibitory role of both treatment options on microglia activation and Iba-1 positive cell levels. Other studies also reported that stem cell transplantation like MSCs decreased Iba-1 total cells in the hippocampus of STZ injected animals (Zappa Villar et al., 2020). Also, it has been demonstrated that intranasally transplanted hNSCs attenuate neuroinflammation in the brains of APP/PS1 mice showing by quantifying the density of microglia and astrocytes, the glial cells responsible in initiating neuroinflammation in the brain. The researchers evaluated coronal sections of transgenic mice that were immunostained with antibodies of Iba1 (marker of microglia) and GFAP (marker of astrocytes), respectively. As they expected, saline-injected APP/PS1 mice exhibited a significantly increased density of astrocytes and microglia in both the hippocampus and cortex compared with WT mice. Whereas hNSCs transplantation reduced the density of astrocytes and microglia to a level comparable to that of WT mice. These findings demonstrate that transplantation of stem cells rescues neuroinflammation in the brains of AD mice models (Lu et al., 2021).

In the final step of this part, we evaluated the neuronal count in the different regions of the mice brain using NeuN immunostaining. The result of this part showed that treatment with metformin,

NPCs and metformin plus NPCs had an impact on improvement of neuronal survival. Besides, these results approved those transplanted cells differentiated to neuronal cells dominantly.

The NeuN protein is localized in nuclei and perinuclear cytoplasm of most of the neurons in the central nervous system of mammals. Monoclonal antibodies to the NeuN protein have been actively used in immunohistochemical research of neuronal differentiation to assess the functional state of neurons in normal and pathology. As mentioned, our results indicated that NeuN positive cells decreased in the hippocampus of STZ-induced animals and the reduction was significant in the CA3 area. As NeuN reduction represents decrease in cell viability and neuronal count, this finding supported our behavioral results and cognitive impairment in STZ group. An in vitro study confirmed the STZ effect on neuronal loss in HT-22 cells with NeuN staining. Results showed that the expression level of NeuN was reduced in a time-dependent manner (Park et al., 2020). Another study evaluated neuronal cell death in the diabetic retina. They immunostained the cryosections of the retina of STZ mice at 1, 3, 6 months for Cleaved caspase-3 and NeuN and showed NeuN immunoreactivity of ganglion cells progressively decreased at 3 and 6 months, which was also confirmed by counting of NeuN-positive cells (Madrakhimov et al., 2021). Also, align with our result Verma et al., revealed that STZ injection caused neuronal loss in the hippocampus (Verma and Singh, 2020).

Our observation showed that treatment with metformin couldn't affect NeuN level significantly. Interestingly, this result may prove the lower effect of metformin treatment on learning and memory performance compared to NPCs transplanted groups. There was some evidence approving the slight effect of metformin on brain cell death following stroke in a type-2 diabetes mouse model of stroke (Kumari et al., 2022). However, other studies showed the significant effect of metformin on neuronal restoration in the hippocampus of a cerebral ischemia/reperfusion rat model (Yuan et al., 2019).

NPCs transplanted group results for NeuN staining also were aligned with the memory state of animals that demonstrated improvement in memory while NeuN positive cells increased in the hippocampus of animals. This data approved that most portions of transplanted NPCs differentiated to Neuronal cells. Also, studies indicated that stem cell transplantation could

increase NeuN cell count in the hippocampus of STZ model (Zappa Villar et al., 2020). In addition, the third treated group with NPCs and metformin had the same results in NeuN positive cells count in the hippocampus compared to the NPCs group that also approved behavioral findings. As we explained before it may be a sign of the anti-inflammatory effect of both treatment options. They act in the same manner and couldn't have a synergistic effect when used together.

Overall, STZ injection could mimic the astrogliosis and microgliosis of the AD pathology, while reducing neuronal viability in the hippocampus of animals. Metformin, NPCs transplantation and NPCs transplantation plus metformin treatment had a remarkable impact on inflammation and reduced inflammatory response in STZ models. In addition, all the treatment increased neuronal cell count and viability in the different regions of STZ injected mice. All three groups showed ameliorative influence differently that present the same manner to cognitive performance of each group. In contrast with our hypothesis, metformin treatment couldn't increase the protective effect of NPCs in the third treatment group, and we reported the effect of metformin administration on the viability and the fate of transplanted NPCs in the hippocampus of STZ mice in the next part.

4. PART 4 – Metformin administration had no significant influence on the fate of NP transplanted cells in the hippocampus of STZ injected mice

In the final part of our investigation, we tracked the transplanted cells in the dentate gyrus, CA3 and cortex of treated mice using their GFP reporter. Since the transplanted cells concentrated in the dentate gyrus and CA3 dominantly, we assessed these regions to find cell destination and level of differentiation to neurons.

GFP positive cell counting in comparison between STZ+NPCs group and STZ+NPCs+Metformin group revealed no significant change in dentate gyrus, whereas the level of GFP positive cells increased significantly in CA3 group. So, metformin treatment had a slight effect on the NPCs migration in the CA3 area of the recipient brain. This slight effect of metformin on NPCs

distribution of the CA3 region could be a reason for insignificant improvement of some behavioral parameters in the STZ+NPCs+Metformin group. Also, we guessed that more distribution of GFP positive NPCs may result in more differentiated to NeuN positive cells in this region.

In line with the previous section, our findings indicated that the number of GFP/NeuN positive cells of both dentate gyrus and CA3 area increased in metformin treated group, slightly. Although this elevation was more in CA3, neither region showed any significant increase. Several experiments confirmed that metformin can inhibit inflammation and stem–cell aging, and promotes the neuroprotection, regeneration, and development of neurons.

Chiang and colleagues demonstrated that metformin as an anti-metabolic disease drug stimulated AMPK, a critical regulator of energy homeostasis and a major player in lipid and glucose metabolism, and potentially implied in the mitochondrial deficiency of AD. They showed that metformin significantly rescued human NSCs from Aβ-mediated mitochondrial deficiency (lower D-loop level, mitochondrial mass, maximal respiratory function, COX activity, and mitochondrial membrane potential). Moreover, treatment with metformin significantly restored fragmented mitochondria to almost normal morphology in the human NSCs in an AD model (Chiang et al., 2016). Also, Tanokashira et al. investigated the age-related reduction in adult hippocampal neurogenesis and its correlation with cognitive impairment. They reported that diabetes is a chronic systemic disease that negatively affects adult neural stem cells and memory functions in the hippocampus and showed that the combination of aging and diabetes in mice causes a marked decrease in hippocampal neurogenesis along with memory impairment and elevated neuroinflammation. They used treatment with metformin that promoted cell proliferation and neuronal differentiation and inhibited aging- and diabetes-associated microglial activation, which is related to homeostatic neurogenesis, leading to enhanced hippocampal neurogenesis in middle-aged diabetic mice. The used treatment strategy improved hippocampal-dependent spatial memory functions accompanied by increased phosphorylation of AMPK that contributed to neuroprotective effects on hippocampal neurogenesis and cognitive function independent of a hypoglycemic effect (Tanokashira et al., 2018).

In addition to the mentioned studies, there are several experiments in different contexts with consistent conclusions. Since increased neurogenesis elicits ant depressive-like effects, Zhang et al., used metformin to promote hippocampal neurogenesis, which ameliorates spatial memory deficits and depression-like behaviors. However, they couldn't show precise molecular mechanisms underpinning metformin induced neuronal differentiation of neural stem cells, showed that metformin enhanced neuronal differentiation of neural stem cells via a protein named Growth arrest and DNA-damage-inducible protein 45g (Gadd45g) but not Gadd45a and Gadd45b. They further found that Gadd45g increased demethylation of some master regulator genes and active DNA demethylation enzymes in metformin treated neural stem cells. After that they approved genetic deficiency of Gadd45g decreased hippocampal neurogenesis, which could contribute to spatial memory decline, and depression-like behaviors in the adult mice, whereas forced expression of Gadd45g alleviated the depressive-like behaviors (Zhang et al., 2022).

On the other hand, resident NPCs, neural stem, and progenitor cells, reside in a well-defined neurogenic niche in the subventricular zone (SVZ) and contribute to ongoing postnatal neurogenesis and NPCs activation is a promising therapeutic strategy for brain repair. Another experiment revealed that metformin has been shown to activate neural stem cells, promote differentiation, and lead to functional motor recovery in a neonatal stroke model. They demonstrated metformin-induced NPCs expansion, functional recovery and increased the size of the NPCs pool, and promoted cognitive recovery in a model of brain injury which has important implications for neural repair (Ruddy et al., 2019). Besides, the development of cell replacement strategies to repair the injured brain has gained considerable attention, with a particular interest in mobilizing endogenous NPCs to promote brain repair. A work by Dadwal et al., in 2015 demonstrated metformin as neurogenesis promoter determined its role in neural repair following brain injury. They indicated that metformin administration activates endogenous NPCs, expanding the size of the NPCs pool and promoting NPCs migration and differentiation in the injured neonatal brain in a hypoxia-ischemia injury model (Dadwal et al., 2015).

Also, other studies demonstrated that differentiation of metformin treated MSCs in the neuronal induction media resulted in an increase in the number of differentiated cells in a metformin concentration dependent manner. The differentiation rate reached its maximum at 3 H after the

initial treatment with neuronal induction media approving via increased neurite length and expression of neuronal-specific marker genes confirmed by immunoblotting. Based on their experiments this effect was abolished upon treatment with the AMPK inhibitor as evident by different molecular tests. Thus, they concluded that metformin treatment promotes neuronal differentiation and neurite outgrowth in MSCs through AMPK activation (Ahn and Cho, 2017).

Overall, all these results may be related to metformin dose and treatment timeline and could support the behavioral and immunohistofluorescence findings. In all parts of our research, we didn't see any remarkable difference between STZ+NPCs and STZ+NPCs+Metformin group, and it was because of the same level of cell differentiation in transplanted cells. Metformin was not able to influence the fate of transplanted cells, so it could not show better treatment results.

5. Integrated discussion

Current study confirmed SAD development after STZ injection in both behavioral and histological. Twice injection of STZ via i.c.v. route can lead to the appearance of AD like behavior 21 days after injection. This phenomenon is caused by neuronal death in the different areas of the hippocampus. In the next step of study, we used two different treatments alone and in combination. Both metformin administration and NPCs transplantation showed promising effects on the cognitive performance in both Novel Object Recognition and Barnes Maze tests, however simultaneous use of both treatments didn't lead to better results. Achieving the mentioned results may be due to the fact that both metformin and NPCs therapeutic options are effective by their anti-inflammatory influence. So, their simultaneous use could not increase the response. Also, STZ injection could mimic the astrogliosis and microgliosis of the AD pathology, while reducing neuronal viability in the hippocampus of animals. Metformin, NPCs transplantation and NPCs transplantation plus metformin treatment had a remarkable impact on

inflammation and reduced inflammatory response in STZ models. In addition, all the treatment increased neuronal cell count and viability in the different regions of STZ injected mice. All three groups showed ameliorative influence differently that present the same manner to cognitive performance of each group. In contrast with our hypothesis, metformin treatment couldn't increase protective effect of NPCs in the third treatment group, and we reported the effect that metformin administration had not remarkable impact on the viability and the fate of transplanted NPCs in the hippocampus of STZ mice in the next part. All these results may be related to metformin dose and treatment timeline and could support the behavioral and immunohistofluorescence findings. In all parts of our research, we didn't see any remarkable difference between STZ+NPCs and STZ+NPCs+Metformin group, and it was because of the same level of cell differentiation in transplanted cells. Metformin was not able to influence the fate of transplanted cells, so it could not show better treatment results.

In conclusion, 2 weeks treatment with metformin and/or NPCs transplantation on day 21 of experiment possessed neuroprotective activity and provides preclinical support for therapeutic perspective of this compound in the treatment of SAD. However, transplantation with NPCs exerts more neuroprotective and anti-inflammatory effects, using both treatments at the same time couldn't have any synergic effect (figure26).

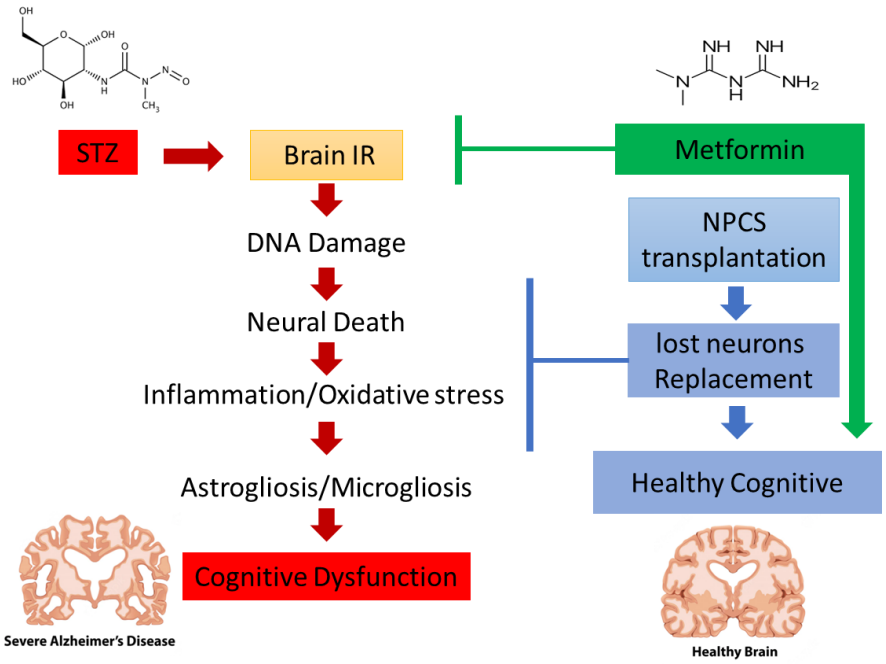


Figure 26. STZ injection in the brain leads to the appearance of AD caused by inflammation and neuronal death in the different areas of the hippocampus. Metformin administration and NPCs transplantation showed promising effects on cognitive performance.

VI.CONCLUSIONS

In general, in this study it was shown that the STZ was able to show the development of sporadic AD in terms of behavior and histology. which was caused by the decrease in the number of neurons in different areas of the hippocampus, as well as the increase in inflammation in the brain of sporadic AD model mice.

Metformin decreased inflammatory cells and reactive astrocytes as well as the dark neurons in the hippocampus region and the cortex in STZ model of sporadic AD and improved cognitive performance. Transplantation of NPCs separately and together with metformin injection also reduced inflammation in the hippocampus. And on the other hand, following the NPCs transplantation, neurogenesis in CA1+DG and CA3 areas of the hippocampus was strengthened, and the lost neurons were replaced. In this regard, improvement in cognitive memory and learning was evident in these two linked groups. In general, therefore, in the field of cognition and memory according to the new object recognition behavioral test, stem cell transplant alone showed better results than stem cell transplant combined with metformin injection and compared to metformin treatment alone. On the other hand, metformin has been helpful in learning.

It can be concluded that due to the anti-inflammatory effect of metformin and NPCs transplant treatments, inflammation in SAD model mice has been reduced to a favorable extent. However, their simultaneous use of both treatments could not show a synergistic effect. On the other hand, these two treatments alone can affect the survival of neurons. This increased neuronal survival in SAD mice treated with metformin alone was because the drug prevented inflammation by activating the AMPK pathway and facilitating neurogenesis. Also, the transplantation of NPCs in the hippocampus also caused the replacement of neurons that were destroyed by STZ. Increased neuron survival leads to improvement in signal processing and improved cognition in SAD model mice.

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VII. APPENDIX

pharmaceuticals

MDPI

Review

Metformin a Potential Pharmacological Strategy in Late Onset Alzheimer's Disease Treatment

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Abstract: Alzheimer's disease (AD) is one of the most devastating brain disorders. Currently, there are no effective treatments to stop the disease progression and it is becoming a major public health concern. Several risk factors are involved in the progression of AD, modifying neuronal circuits and brain cognition, and eventually leading to neuronal death. Among them, obesity and type 2 diabetes mellitus (T2DM) have attracted increasing attention, since brain insulin resistance can contribute to neurodegeneration. Consequently, AD has been referred to "type 3 diabetes" and antidiabetic medications such as intranasal insulin, glitazones, metformin or liraglutide are being tested as possible alternatives. Metformin, a first line antihyperglycemic medication, is a 5′-adenosine monophosphate (AMP)-activated protein kinase (AMPK) activator hypothesized to act as a geroprotective agent. However, studies on its association with age-related cognitive decline have shown controversial results with positive and negative findings. In spite of this, metformin shows positive benefits such as anti-inflammatory effects, accelerated neurogenesis, strengthened memory, and prolonged life expectancy. Moreover, it has been recently demonstrated that metformin enhances synaptophysin, sirtuin-1, AMPK, and brain-derived neuronal factor (BDNF) immunoreactivity, which are essential markers of plasticity. The present review discusses the numerous studies which have explored (1) the neuropathological hallmarks of AD, (2) association of type 2 diabetes with AD, and (3) the potential therapeutic effects of metformin on AD and preclinical models.

Keywords: Alzheimer's disease; diabetes mellitus; metformin; insulin resistance; beta amyloid; tau protein hyperphosphorylation; AMP activated protein kinase (AMPK)

1. Introduction

Currently, there are 44 million people dealing with dementia, being the second leading cause of death in people aged 70 and over [1–4]. Alzheimer's disease (AD) is the most prevalent form of dementia [1] and it is also one of the most significant causes of morbidity and mortality in the elderly population worldwide [3]. AD prevalence is estimated to reach

115 million by 2050 due to an increased ageing population pattern, unless novel drugs are available to slow or cure this disease [2–4]. Neuropathological modifications of AD, such as tau hyperphosphorylation and Aβ toxicity, led to the main current hypothesis trying to explain neuronal and synapse loss, associated with cognitive and memory impairment [2–6].

Since the neurodegenerative process of AD is closely related to the aging process, it has also been called the late onset AD (LOAD). LOAD lacks a clear origin, and it is mainly found in patients over 65 years old. These patients comprise 95% of AD cases, although about 1% are attributed to autosomal dominant mutations in amyloid metabolism-related genes, e.g., beta amyloid precursor protein (βAPP-chromosome 21), presenilin 1 (PS1-chromosome 14), and presenilin 2 genes (PS2-chromosome 1) [6].

In spite AD or dementia is often related with the geriatric community, AD may also affect younger adults. Early-onset AD (EOAD) is referred to those affecting people who are diagnosed before 65 years old (between 30 and 65). As outlined by McMurtray and colleagues, early-onset dementia patients account for 20% to 34% of AD cases in most trials [7]. Three gene mutations playing a key role in EOAD have been identified: APP, PS1, and PS2. PS1 and PS2 are two proteins that constitute the catalytic core of γ-secretase. These gene mutations—including APP, PS1, and PS2—belong to the familial form of AD and have been shown to increase the development of amyloid-β (Aβ) leading to an increase in the Aβ1–42/Aβ1–40 ratio, hence favoring the formation of senile plaques [1]. A combination of genetic and environmental factors and lifestyle elements play a significant role in LOAD, which does not have a clear etiology and it is considered multifactorial [7].

While LOAD has not a well-established etiology, two of the most relevant genes conferring a significantly risk factor are the E4 apolipoprotein allele (APOEε4) and the triggering receptor expressed on myeloid cells 2 (TREM2) [8–10]. The ε4 allele of the APOE gene codes for the main apolipoprotein of the CNS, whose functions are lipid transport and neuron homeostasis [8–12]. It has been reported that one copy of the ε4 allele in APOE increases LOAD risk by 3~4-fold [10,11]. Its mutation results in a higher lipid binding capacity of APOE4, and it is associated with a less efficient clearance of Aβ and an increase in pathological changes responsible for cognitive decline [10]. On the contrary, carriers of the APOEε2 allele have two times less risk of suffering from LOAD than non-carriers, being considered a protective genetic factor against this disease [12]. There are significant clinical and basic evidence that shows early driving amyloid pathology in the brains of APOEε4 carriers [8–10]. Furthermore, in several brain homeostatic pathways, including lipid transfer, synaptic integrity and plasticity, glucose metabolism and cerebrovascular activity, APOE4 is either pathogenic or a decreasing factor of performance [8–11]. In turn, TREM2 is a very abundant receptor on the surface of microglia and plays an important role in its activation and regulation [13,14]. Certain mutations can condition the affinity of TREM2 for its ligands, decreasing phagocytosis of Aβ peptide by microglia and promoting a systemic inflammatory response. Thus, TREM2 deficiency is involved in the development of LOAD due to an insufficient microglial function. In addition, TREM2 regulates the function of microglia in LOAD and other neurodegenerative diseases, and also participates in inflammatory responses and metabolism, either alone or in close association with other molecules, such as APOE [15].

Type two diabetes mellitus (T2DM) and LOAD have become worldwide pandemics, with recent projections indicating that they will get worse in the coming decades. In this respect, obesity, T2DM and associated comorbidities have been described to be involved in the development of LOAD [14]. Thus, LOAD has been recently described as a "metabolic disease", related with the inefficient utilization of glucose by the brain and associated with insulin resistance and chronic mild inflammation in the brain [15–20]. Likewise, and due to the insulin resistance generated in the brain, LOAD has been also referred to as "type 3 diabetes" [19,20]. Like T2DM, which is characterized by a decreased ability of peripheral tissues to metabolize glucose, in LOAD the decreased ability of brain to metabolize glucose (brain glucose hypometabolism) could contribute to the neurodegenerative process,

together with the classical neuropathological LOAD hallmarks, such as Aβ deposits and hyperphosphorylated tau (p-tau) in neurofibrillary tangles (NFTs) [19,20].

Preclinical research studies and clinical and epidemiological trials reported that T2DM has been related not only to the development, but also to the progression of LOAD. A retrospective study of 20 prospective clinical trials concluded that the prevalence of LOAD in patients with T2DM was 56% greater than in people without diabetes [21]. Brain insulin resistance, decreased insulin signaling, inflammation, hyperglycemia, vascular alterations, hypoglycemic events, and impaired amyloid metabolism are proposed causes for this relationship [9]. Consequently, it has been shown that both T2DM and LOAD possess multifactorial risk profiles and a wide variety of molecular connections. The intersection between the molecular pathways of these two diseases could give birth to the appearance of the cognitive anomalies of LOAD patients with underlying T2DM [22–25].

2. Type 2 Diabetes Mellitus Related with Alzheimer's Disease

T2DM is a widely known chronic metabolic condition characterized by high levels of blood glucose and insulin resistance [24–26]. Epidemiological findings indicate that, relative to normal people, some diabetic patients have an elevated chance of developing dementia. Hence, in the 'Rotterdam Study', Ott and colleagues were the first who reported the potential connection between both pathologies, disclosing that diabetes significantly increased the risk of dementia [27,28]. Thus, it was suggested that LOAD can be viewed as a metabolic disorder because brain of LOAD patients showed several features in common with compromised insulin signaling pathways [21]. Additionally, clinical and epidemiological studies have confirmed this association, demonstrating that the alteration of metabolic parameters, such as hyperglycemia and hyperinsulinemia, are positively correlated with the development of LOAD neuropathology [29]. In this sense, the 'Hisayama Study' reported an association between diabetes plus APOEε4 and Aβ plaques, but not with neurofibrillary tangles formation [30]. In turn, in a study performed in cognitively healthy middle-aged adults enrolled in the 'Wisconsin Registry for Alzheimer's Prevention study', Willette and colleagues reported an association between brain insulin resistance (BIR) and Aβ brain deposition in LOAD patients, given support to the hypothesis that BIR is a risk factor in the early stages of LOAD [31,32]. Therefore, this study concluded that BIR is a modifiable risk factor during the preclinical stage of the pathology, which opened a new therapeutic window for the design of new strategies focused on the prevention of LOAD [31]. Likewise, some reports conclude that, under certain conditions such as metabolic disorders, the body's diabetic status will enhance the occurrence of LOAD by disrupting the transfer of glucose into the brain and decreasing its metabolism [21]. Overall, impaired insulin signaling pathway is associated with metabolic disturbances such as glucose/lipid metabolism, protein modifications, mitochondrial dysfunction, and oxidative stress. In addition, BIR can exacerbate Aβ accumulation, increase tau hyperphosphorylation, devastate glucose transportation and energy metabolism, and impair hippocampal framework pathways [18,19,30]. Besides, it has been demonstrated that insulin has many positive effects on the brain, including synaptic trophic effects and dendritic spine development promotion [33–35]. Although the evidence of a correlation between diabetes and neurodegenerative pathology in LOAD is mixed, some human postmortem findings suggest a link between brain insulin tolerance and increased LOAD pathology, including increased Aβ deposition [35–39]. Moreover, as we have already mentioned above, several clinical studies have discovered that patients with diabetes have considerable levels of Aβ and p-tau in cerebrospinal fluid as well as lower scores of cognition [40–44]. However, evidence from pre-clinical and limited clinical trials indicate that insulin and agents that promote insulin signaling could reduce neuropathology and boost cognition in diabetes and LOAD [41,42].

The most common hypothesis recognizes Aβ as the main cause of LOAD [43–49]. This starts with amyloid beta precursor protein (APP), which breaks down to release Aβ due to the activation of a whole series of enzymes of the amyloidogenic cascade, such as β and γ

secretases. This increases the levels of harmful Aβ 1-42 [46–48], enabling fibril production of extracellularly deposits that destroy neurons and organize classical senile plaques [46]. Hence, the initial amyloidogenic hypothesis suggested that amyloid plaques or insoluble amyloid fibrils were responsible for the loss of synapses since amyloid plaques are found in postmortem brains of LOAD patients and in preclinical models of AD that present cognitive deficits [46–49]. Although mice models generally develop amyloid plaques, they also show synaptic dysfunction and cognitive deficits prior to plaque formation. In this way, this hypothesis has been challenged and it is now accepted that soluble Aβ oligomers, also known as Aβ-derived diffusible ligands (ADDL), would be the powerful neurotoxins of the central nervous system (CNS) that accumulate in the brain in LOAD [46,47]. Based on these observations, Aβ toxicity is mediated not only by insoluble amyloid fibrils but also by ADDL, and synaptic failure is likely to be one of the earliest events in the pathogenesis of AD [46]. Moreover, it has been shown that ADDL correlate better with the disease severity (cognitive decline) than with the accumulation of insoluble Aβ peptides into plaques triggering AD pathophysiology [46,47]. Likewise, previous studies have demonstrated that ADDL are able to impair the function of insulin receptor in brain (detected at dendritic level in synapses), inducing an intracellular localization of this receptor which takes it away from the neuronal surface of dendrites. Therefore, this process is associated with a decrease of glutamatergic neurotransmission [46–49].

Furthermore, Aβ is linked to both neuronal oxidative stress and mitochondrial dysfunction [47]. Unfolded protein response or endoplasmic reticulum stress are also involved in the development of Aβ neuronal cell damage, also being closely correlated with the pathology of tau protein. Through the raise of Aβ pathology, these metabolic agents could increase the occurrence of LOAD in diabetic patients [50].

Apart from the effects of Aβ oligomers at the brain, a very interesting point is the role of plasma Aβ oligomers on peripheral IR. In line with this concept, it has been reported that these oligomers also have an inhibitory effect on the peripheral insulin signaling pathway through different mechanisms mediated by oxidative stress and inflammatory responses, hence demonstrating that Aβ oligomers modify peripheral glucose metabolism through multiple ways [51–54]. Thus, Aβ oligomers have important effects on the systemic metabolism of glucose, where insulin is critical for proper glucose homeostasis through effects on the liver, skeletal muscle, and adipose tissue [41,55]. Therefore, these studies give support to the hypothesis that LOAD is a metabolic disease in which Aβ oligomers production in the brain play a key role in peripheral metabolism alteration.

One of the characteristics of LOAD is the brain hypometabolism that is due to a decrease in glucose uptake. The drop in brain glucose levels is mainly related to a reduced glucose uptake associated with the decreased expression of glucose transporters in neurons, mainly GLUT1 and GLUT3 [56]. Therefore, it has been suggested that increasing glucose transport to neurons, for example with antidiabetic drugs such as metformin, may be a therapeutic approach in AD [56]. Likewise, alteration of different brain pathways associated with T2DM has been reported in LOAD patients. In LOAD, as we have already discussed, it has been demonstrated that an alteration of IR levels can affect the cognitive process due to synaptic impairment, in addition to increasing oxidative stress, thus favoring mitochondrial dysfunction that ultimately leads to neuronal apoptosis [38–44].

Chronic hyperglycemia can be responsible for the appearance of diabetic complications since it can generate glycolipotoxicity. In this way, hyperglycemia generates advanced glycation end products (AGEs) that are also a crucial link between diabetes and LOAD [48–53]. It has been proposed that an increase in AGE levels in the brain may be directly related to cognitive dysfunction in LOAD patients. For this reason, AGEs can contribute to LOAD by promoting the formation of fibrillar tangles and amyloid plaques, which are the main neuropathological characteristics of LOAD, in addition to increasing the cytotoxicity of Aβ [16–20]. AGEs induce the expression of their receptor, RAGE, which is also a putative receptor for Aβ [16–18]. Previous studies have shown that RAGE levels are increased in various types of LOAD brain cells. For example, glial cells of the brain show elevated levels

of RAGE and, furthermore, a colocalization of RAGE with intracellular Aβ and tau has been observed in LOAD patients.

Besides, the key enzymes glycogen synthase kinase 3β (GSK3β) and insulin degrading enzyme (IDE) are altered in bothT2DM and LOAD, being a potential link between the two diseases. GSK3β is regulated by the insulin receptor and its activation in LOAD favors the phosphorylation of tau protein and the formation of neurofibrillary tangles [40,41]. IDE plays a key role in the metabolism and elimination of Aβ and also insulin. IDE degrades both insulin and the Aβ peptide, however, insulin binds to IDE with higher affinity. Therefore, in both diseases, T2DM and LOAD hyperinsulinemia sequesters IDE, presenting a greater affinity for insulin than for Aβ and, for this reason, it ends up facilitating the accumulation of Aβ levels and increasing the risk of LOAD [55].

Supporting this metabolic hypothesis of LOAD, a preclinical study with a neuron-specific human BACE1 knock-in mouse model (PLB4) conducted by Plucińska and colleagues demonstrated that the brain BACE1 overexpression by itself increased the risk of peripheral T2DM [56]. Therefore, this study suggested that LOAD progression can promote T2DM comorbidities in mice, independently of the classical obesogenic process, which could be a potential link between T2DM and LOAD.

As we have already mentioned, tau is the other main biomarker related to the neurodegenerative process in LOAD [1]. Tau is a hydrophobic protein involved in the neuronal stabilization of microtubules and axonal transport. According to the "tau hypothesis", tau protein's dysfunction leads to form NFTs [4,6,57]. Specifically, tau hyperphosphorylation interrupts its connection with microtubules, which disrupt the entire microtubules assembly [1,4,6,58–61].

In this sense, brain insulin has been shown to play a key role in the regulation of tau phosphorylation through the activation of its receptor located in the brain. Indeed, as we have commented above, BIR is associated with the activation of tau kinases, including GSK3β, through the phosphatidylinositol kinase (PI3K)/protein kinase B (AKT) signaling pathway [60]. Thus, BIR leads to an overactivation of GSK3β, which in turn promote tau hyperphosphorylation. Likewise, tau hyperphosphorylation, oligomerization, misfolding, and aggregation are involved in the impairment of synaptic plasticity and contribute to the neurodegenerative process due to its location on dendrites in the postsynaptic terminals [61]. It has been reported that deposits of p-tau in the CA1 and CA3 regions of hippocampus are related with the decrease of density and shape of dendritic spines, as well as neuronal loss [60,61]. Yarchoan and colleagues reported that BIR was related to IRS1-pS616 and IRS1-pS312 expression in LOAD and brain tauopathies, including Pick's disease, corticobasal degeneration, and progressive supranuclear palsy [61]. Thus, in this study, the authors suggest an association between BIR and tau, in which IRS-1 pS616 phosphorylation increases favor an abnormal tau phosphorylation [61].

In this line, Marciniak and colleagues demonstrated the role of tau as a key modulator of BIR and the insulin receptor signaling pathway, as well as the mechanisms whereby tau could modulate insulin receptor function [59]. Hence, tau deletion is involved in the control of peripheral and brain insulin metabolism, the modulation of hippocampal BIR can contribute to cognitive function and hypothalamic BIR regulates metabolic alterations in LOAD patients and in tauopathies [59]. Therefore, chronic BIR is involved in the development of tau pathology by altering the balance between kinases and phosphatases and vice versa. Moreover, it has been demonstrated that tau hyperphosphorylation leads to an increase in uptake and intraneuronal accumulation of insulin as insoluble oligomeric aggregates in LOAD patients and in several tauopathies [58]. Interestingly, this process occurs independently of T2DM, suggesting that BIR is associated with alterations of insulin signaling pathway independently of the presence of clinical T2DM.

Likewise, tau has recently been identified as a key regulator of peripheral insulin signaling, with evidence linking tau to IR in the brain and peripheral tissues, as well as beta cell dysfunction [60,62,63]. Tau is widely expressed in insulin-secreting beta cells in the pancreatic islets. At a young age, mice with a global tau knockout exhibit a rise

in body weight, defects in glucose-stimulated insulin secretion, and reduced glucose tolerance [60,61].

Although it is well known that insulin can modulate the phosphorylation of tau protein, its role in the regulation of the insulin receptor has been only studied in the recent years. In accordance with this idea, mice which do not express insulin receptors at the neuronal level (NIRKO mice) as well as IRS-2$^{-/-}$ mice showed an increased phosphorylation of tau through the inhibition of phosphoinositide 3 kinase (PI3K)/AKT signaling pathway [18,19,62,63]. This link between insulin and tau signaling is mainly based on the modulation of downstream signaling pathways involving different kinases such as GSK-3β, c-Jun N-terminal kinase (JNK) and AMPK and phosphatases including protein phosphatase 1 and 2 (PP1 and PP2A, respectively) [18,19].

3. Metformin as an Antidiabetic Drug Strategy for Alzheimer's Disease Treatment

Metformin is an antidiabetic drug derived from galengine, a natural product of the *Galega officinalis* plant [18]. Metformin is a biguanide that contains two couple guanidine molecules [64–66], with a highly hydrophilic chemical structure (1, 1-dimethylbiguanide hydrochloride) properties [65]. Therapeutically, metformin is the first-line treatment for T2DM and is prescribed by most health guidelines because its low side-effects, it is usually well absorbed, and not associated with weight gain [67]. Metformin decreases liver gluconeogenesis and reduces insulin resistance, leading to lower levels of plasma glucose [65]. Likewise, metformin is able to cross the blood–brain barrier (BBB) and has been involved in increased cognitive performance [68]. Furthermore, metformin could alter gut microbiota composition, which may play a role in AD pathogenesis [69].

3.1. Preclinical Animal Studies with Metformin

For the purpose of designing therapeutics or disease modifying agents for AD treatment, a wide variety of animal models have been developed to replicate the human environment of the disease [70–74]. In particular, the first aim of most AD animal models is to develop the neuropathological features that precede the cognitive dysfunction [75–77].

The transgenic mice are important models for deciphering familial AD pathology pathways. These models do not display all the anomalies found in human AD and do not duplicate the sporadic forms of AD, because they only reproduce the pathological features of AD common mutated genes [77]. However, transgenic innovation gives special opportunity to replicate the cause of familial AD by transfecting a mutant human APP [74,75]. Mice models enabled our understanding of Aβ-production, deposition, and clearance-related molecular pathways and the impact of Aβ on the neuronal network and synapses [73,74]. A wide variety of parenchymal and vascular amyloid deposits similar to those of human AD were developed successfully by the APP mouse model [75]. Transgenic mice models developed by over-expression of mutated human PS1, APP and tau, are the majority of these animal models. The triple-transgenic 3xTg-AD mice contains three mutated genes (human PS1M146V, APPSwe and tauP301L) and develops increasing age-dependent amyloid plaques and NFTs as well as memory deficits [76–78] (Table 1).

On the other hand, many of the signature features of AD are reproduced by the injection of pharmacological or chemical agents into the brain or by the activation of lesions in specific brain regions [76–78]. For instance, the injection of Aβ peptide into the brain of rat or rhesus monkey has been used in several studies. Although these models cause some of the clinical signs, they do not specifically mimic AD pathology. Lesion models include the chemical or physical degradation of particular regions of the brain such as hippocampal, cortical, and striatal regions that are normally either cholinergic or active in cognitive processes [1]. In general, interventional models will be effective for detecting symptomatic or therapeutic interventions as a disease model. These models can include valuable observations such as the streptozotocin (STZ)-induced AD model, scopolamine-mediated amnesia model that led to learning and memory loss and cognition dysfunction [76–80]. For instance, in scopolamine-induced amnesia models, inflammation

is activated by endotoxins and the brain metabolism interacts with other chemical action models [73].

Since LOAD represents more than 95% of AD cases, associated animal models are valuable research resources for studying pathogenesis and designing experimental treatments for sporadic AD [76–84]. STZ is a diabetogenic agent that is widely used to induce diabetes in animals because it damages and induces IR in pancreatic beta cells. Decrease of glucose/energy metabolism in brain, corresponds to the severity of dementia symptoms in AD, and is a well-established brain abnormality of sporadic AD [77–93]. In ICV-STZ animal models, BIR reduced brain glucose metabolism, tau and Aβ accumulation, gliosis, cholinergic deficits, oxidative stress, and learning and memory deficits [77–85]. Brain insulin signaling controls the metabolism of cerebral glucose, and impaired transduction of brain insulin signaling was reported in AD [82]. Tau hyperphosphorylation, increasing of Aβ40/42 and both GSK-3β and BACE1 activities have also been observed in rats treated with STZ, which also exhibited a lack of dendritic and synaptic plasticity [85–87]. In January 2013, Dr Hoyer hypothesized that ICV STZ is the non-transgenic metabolic form of sporadic AD [85]. Hoyer's reasoning began with the observation that while both oxygen and glucose intake in the brain decreases in LOAD, the decrease in brain oxygen consumption is significantly smaller [85–89]. These findings lead to the hypothesis that the main biochemical change in incipient LOAD is related to the regulation of cerebral glucose metabolism, which leads to an alteration of a signal transduction deficiency of the cerebral insulin receptor [89]. According to findings of Saffari and colleagues, an i.c.v. injection of STZ caused a substantial decrease in spatial learning and memory, while metformin administered in therapy phosphatidylserine nanoliposomes formulation improved learning and memory. Thus, metformin increased spatial learning and decrease neuroinflammation in the STZ rat model of LOAD [89]. In addition, the recent results of Pilipenko et al. showed that metformin reversed STZ-induced impairments in spatial learning/memory capacity and sociability, as well as normalization of brain glucose transport, uptake, and metabolism, together with an improved microgliosis and astrogliosis in a LOAD rat model [84].

According to the studies of Ditacchio and colleagues, metformin is an efficient treatment to improve insulin sensitivity, with a higher drop in blood glucose levels in the AβPP AD model [90]. In addition, Farr and colleagues examined the effect of metformin on the expression of APPc99, AβPP, Aβ, G3DPH, and p-tau in SAMP8 mice [91]. They showed that the expression of APPc99 and p-tau decreased after metformin treatment. Thus, metformin treatment in SAMP8 significantly reduced hyperphosphorelated tau and APPc99 proteins, leading to an improve in learning and memory processes. Moreover, Ditacchio and colleagues also showed that Aβpp transgenic female mice that were treated with metformin showed increased cognitive abilities [90] (Table 1). These results were seen in another genetic model of AMPK activation, where some unexplained structural mechanism disrupted AD-related cognitive activity in these animals downstream of liver AMPK activation [91]. Additionally, the authors carried out studies in males and females and the results demonstrated that beneficial effects of metformin were greater in females than in males [91]. This supports the idea that there may be an effect of the gender in the effectivity of this drug.

Finally, Lu and colleagues demonstrated that metformin improved learning and memory performance in APP/PS1 transgenic mice, according to Morris water maze and Y-maze results [93]. Another study demonstrated that metformin improved microglial autophagy in the APP/PS1 mice model, allowing pathological Aβ and tau proteins to be phagocytized, and thus reducing Aβ deposits and restricted the distribution of tau pathology [83]. Similarly, Ou and colleagues also reported that metformin treatment in APP/PS1 exerts multiple beneficial effects in the brain neuropathology [82]. Thus, metformin treatment improved the cognitive process and neurogenesis, exerting neuroprotective effects on the hippocampus. Moreover, metformin, probably through the modulation of the AMPK/mTOR/S6K/BACE1 signaling pathway, also improved amyloidogenic pathway and prevented the neuroinflammatory process [82].

Table 1. Effect of different dose of metformin on treatment of preclinical Alzheimer's disease.

Row	Reference	Animal Model/Gender	Starting Age	Metformin Dose	Duration of Therapy	Main Finding
1	[82]	B6C3-Tg (APPswe, PS1dE9) 85Dbo-fAD/F	26 weeks old	200 mg/kg/d	14 days	Neuroprotection, Enhanced memory, reduced inflammation, regulation of AMPK/mTOR/S6K/Bace1 pathway.
2	[91]	SAMP8 mouse model of random onset-AD/M	12 months old	20–200 mg/kg/d	8 weeks	Increased PKC, improved pGSK-3ser9,reduced pTau404 and APPc99, enhanced learning and memory.
3	[90]	PDAPP (J9) mice-AD/M&F	6–8 weeks	350 mg/kg/d	Until 14–16 months-old	increases insulin sensitivity in male, lifespan extension and delayed degradation of the estrous cycle in female
4	[94]	C57BL/6 mice-PD/M	10-weeks	200 mg/kg/d	10 days.	Stimulate AMPK, mediating the pleiotropy
5	[95]	Wistar rats-AD/M	Five-month old	50, 100–200 mg/kg/d	3 weeks	Decreasing Memory loss, preserved the pAMPK and CREB levels, Improved TAS & SOD levels, increased antioxidant function
6	[96]	Wistar rats-AD/M	Adult	100 mg/kg/d	8 weeks	Enhances neuronal activity and neuropathological modifications, prevent synaptic plasticity impairment
7	[83]	Wistar rats-sAD/M	9 weeks	75–100 mg/kg/d	21 days	Modulation of glucose delivery and uptake, anti-neuroinflammatory function, maintenance of synaptic plasticity
8	[86]	C57BL/6 mice-sAD/M	12–14 weeks	200 mg/kg/d	21 days	Suppress glycemic levels and cognitive dysfunction, increases insulin receptor sensitivity, facilitate neuronal survival
9	[76]	APP/PS1 transgenic mice/F	9 months old	4 mg/mL in drinking water	2 months	Promoted the phagocytosis of Aβ and tau proteins by enhancing microglial autophagy capability

3.2. Metformin in Clinical Studies

It has been shown that metformin stops or slows the onset of dementia in adults with diabetes [97]. In 2019, Shi and colleagues focused on the effect of metformin in elderly adult US veterans with T2DM and neurodegeneration [98]. According to the results of this study, metformin therapy over 2–4 years provides a strong risk reduction in the occurrence of neurodegeneration in patients with T2DM compared with patients without metformin treatment [98].

A pilot study of 80 people with amnestic moderate cognitive disorder was undertaken at Columbia University in New York City from 2008 to 2012. The participants were overweight, but none of them had diabetes. They were given either 2000 mg of metformin separated into two doses or a placebo for one year. The selective reminding test (SRT) for recall and the ADAS-Cog were the primary outcomes [99]. The secondary endpoint was FDG-PET glucose absorption in the posterior cingulate/precuneus, as well as plasma levels of Aβ42, the most toxic form of the Aβ peptide. The metformin group performed slightly higher on the SRT than the placebo group. There were no differences in the ADAS-Cog, glucose uptake, or plasma Aβ42 between classes. Only 10% of patients were able to take the peak dosage of metformin, with the majority receiving 1000 or 1500 mg a day [92,93]. The main conclusion of the study was that metformin improve of efficacy for recall in the SRT.

From 2013 to 2015 a small study at the University of Pennsylvania assessed the impact of metformin on biomarkers of AD in 20 non-diabetic individuals with moderate cognitive

dysfunction or dementia related to the disease. MRI, FDG-PET, and amyloid biomarkers were used to validate the diagnosis of AD [100,101]. Each participant was given metformin at a daily dose of 2000 mg/day for eight weeks, then placebo for eight weeks, or vice versa, in a crossover study. The ADAS-Cog and CANTAB batteries were used to assess cognitive performance in multiple learning and memory domains, executive processing, focus, expression, and motor speed. Cerebral spine fluid (CSF) concentrations of Aβ, total tau, and tau were also evaluated, and blood flow in the brain was determined by arterial spin marking [9]. In the treated population, the Trails B test of executive function showed a statistically significant increase, as well as improvements in learning, memory, and focus. Metformin had little effect on blood supply in the areas where it was tested. The compound was found in the CSF, but the AD biomarkers remained unchanged [101].

In February 2020, Swedish researchers began testing the impact of a year of metformin treatment plus exercise and diet on memory in 80 people with T2DM and moderate cognitive dysfunction. Recruitment, adherence, and retention rates are the primary consequences, while metabolic improvement and memory capacity are secondary measures. The research will last until December 2021 (https://www.alzforum.org/therapeutics/metformin) (accessed on 23 August 2021).

In turn, Samaras and colleagues compared the efficacy of metformin on cognitive decline and dementia risk in diabetic patients. After 6 years of research, authors concluded that the administration of metformin in older people with T2DM was associated with a decreased risk of dementia [102]. In addition, in an interesting study performed in aged African American and white patients, with data taken from Veterans Health Administration (VHA) medical record, Scherrer and colleagues showed that the administration of metformin decreases the risk of dementia by 29% and 40% in African American patients aged 65 to 74 years and 50 to 64 years, respectively. These results are very interesting because they give support to the hypothesis that metformin is able to decrease the risk of dementia in aged patients [103].

On another front, Sluggett and colleagues demonstrated that Finnish patients with T2DM and long-term metformin treatment had lower risk of developing AD. Again, the results of this study give support to the hypothesis that glucose lowering drugs may be important pharmacological alternatives that modify the course of the disease and delays the risk of dementia [104].

On the contrary, other studies such as those of Koo and colleagues showed that metformin treatment was not effective and even worsened the cognitive state in older Korean patients [105]. For this reason, more studies are necessary to clarify metformin effects in diabetic patients with cognitive loss [106,107].

4. Molecular Mechanism Involved in Neuroprotective Effects of Metformin in Alzheimer's Disease

4.1. Metformin Effects on Amyloid and Tau

Previous studies have reported that AMPK is highly expressed in the hippocampus —a brain region that plays key roles in synaptic plasticity, memory, and cognition—and aberrant AMPK activity has been reported in the brains of transgenic mouse models of AD and AD patients [108,109]. Based in the amyloidogenic hypothesis of AD, it has been reported that Aβ oligomers inhibited AMPK and thus could increase the risk of a metabolic dysfunction in hippocampal neurons that may play a key role in early metabolic defects in the LOAD brain [110]. Thus, metformin, which is able to promote AMPK activation, could be an attractive target that can compensate this energy loss in the in the nervous system. In addition, AMPK activation can reduce Aβ by reducing BACE1 expression and thus decrease brain Aβ levels [111,112]. Moreover, AMPK could play an additional favorable role in LOAD by promoting autophagy [70–72]. Indeed, previous studies have reported that activation of autophagy decreased Aβ pathology and improved the cognitive process in preclinical models [113]. Therefore, metformin could improve the brain autophagic function, helping to remove waste proteins and improving the treatment of AD [113].

It is well known that phosphorylation of tau is regulated by several kinases, including AMPK, which is a tau kinase that acts by phosphorylating multiple tau sites [114,115]. However, the process of tau regulation by AMPK is complex since it can be regulated by direct and indirect mechanisms. Wang and colleagues reported that both salicylate, an AMPK agonist, and wortmannin, a GSK-3β inhibitor, reduce tau phosphorylation [61]. Likewise, AMPK can phosphorylate the Ser9 site of GSK-3β, triggering its inhibition, and therefore it may explain its participation in the modulation of this regulatory process in the phosphorylation of tau [116,117]. Apart from the direct regulation of tau phosphorylation, AMPK also activates SIRT1, a deacetylase enzyme which, by improving or enhancing the deacetylation process, can inhibit the hyperphosphorylation of tau [115]. Likewise, another mechanism that regulates both the acetylation and phosphorylation of tau involves the protein phosphatase 2A (PP2A). Interestingly, it was reported that metformin induces tau dephosphorylation by directly activating PP2A [117]. In addition, PP2A activity is increased by AMPK-mediated phosphorylation at Ser298 and Ser336 [98,99].

In general, the role of metformin on AMPK activation and phosphorylation of tau is not fully understood, since involves both direct and indirect mechanisms. Because different hypotheses have been proposed, more research studies are needed. However, it is accepted that metformin improves mitochondrial defects, promotes the autophagy, and regulates insulin sensitization through the modulation of different intracellular pathways and consequently could improve LOAD neuropathology in preclinical AD models [104–107]. Therefore, metformin could be a suitable potential therapeutic treatment of metabolic risk factors target for LOAD.

4.2. Metformin Effects on Mitochondria

A strategy based on "brain energy rescue" in the treatment of LOAD has currently been proposed [118]. The objective of this strategy is based on preserving and/or restoring the energy state of the brain. In this sense, metformin treatment could be a potential brain energy rescue strategy by improving mitochondrial function and improving peripheral and cerebral glucose metabolism. It is well known that mitochondrial metabolic abnormalities are involved in the pathogenesis of LOAD [114,115]. Thereby, it has been proposed that AMPK can regulate mitochondrial synthesis and the main functions of mitochondrial autophagy. Previous studies have shown that mitochondrial damage is an early sign of LOAD that appears before NFTs and is accompanied by phosphorylation of the tau protein [115]. Thus, the activation of AMPK through metformin can favor the process of mitochondrial biogenesis regulating the function of peroxisome proliferator activated receptor γ coactivator-1α and peroxisome coactivator-1α (PGC-1α, a transcriptional coactivator nuclear) [110]. Furthermore, as we have already commented above, metformin could promote the mitochondrial autophagy process through AMPK activation, hence favoring the elimination of damaged/defective mitochondria, increasing ATP production, and reducing the production of reactive oxygen species [116]. Therefore, it can be hypothesized that the activation of AMPK by metformin could generate an increase in cellular autophagy and ATP production and helps to improve the symptoms of LOAD.

4.3. Metformin Effects on Neurogenesis: The AMPK/aPKC/CBP Signaling Pathway

Metformin is involved in two distinct molecular pathways to facilitate the proliferation/regeneration and differentiation of adult neuron progenitor cells (NPCs) [119]. In the first pathway, metformin activates AMPK, which activates the cascade of aPKC-CBP to facilitate neuronal differentiation. Atypical protein kinase C (aPKC) is stimulated upon activation of AMPK, which ultimately phosphorylates CREB-binding protein (CBP) at Ser133 to facilitate neurogenesis and increase spatial memory development in adult mice [119,120]. In the second pathway, metformin significantly upregulates the expression of TAp73 mRNA, which in turn increases the production of essential proteins involving in self-renewal of adult NPCs. P73 is a transcription factor that plays a key role in neural stem cells and its expression increases following their differentiation [120]. For the treatment

of patients with cognitive dysfunction associated with T1DM and T2DM, the ability of metformin to stimulate neurogenesis is potentially promising [117,118,120]. According to the previous studies, it can be shown that long-term usage of oral metformin therapy improves hippocampal neurogenesis and spatial memory, followed by an induction of chronic microglial activation and improved glucose-lowering impact of phosphorus-relation of AMPK/aPKC f/k/IRS1 serine residues in the hippocampus of middle-aged diabetic mice [120]. These findings are consistent with previous research demonstrating the neuroprotective effects of chronic metformin administration on high fat diet-induced deterioration in hippocampal neurogenesis and neurological disorders [117]. Taken together, considering the crucial roles of AMPK in intracellular metabolism in LOAD, metformin could be introduced as suitable and attractive therapeutic target [116].

Studies by Ma and colleagues showed that metformin improves the composition of the gut microbiota of obese mice. This peripheral effect could inhibit the neuroinflammatory process in the hippocampus. Likewise, this drug could prevent the deterioration of newborn neurons in the hippocampus and therefore improve the learning process and memory in obese mice. These results reinforce the hypothesis of the benefit of acting at the microbiota level to improve the cognitive process in LOAD [121].

4.4. Metformin Effects on Learning and Memory

Cognition is one of the most complex features of the brain, and it involves perception, registration, consolidation, storage, and memory over the course of human life [118]. Any memory deficiency, such as amnesia, has a significant impact on an individual's quality of life and is regarded as a major CNS disease attributed to a decline in neuronal population as a result of ageing, neurodegenerative disorders, head injuries, brain defects, genetic anomalies, and other factors [122]. Accumulating evidence suggests that diabetic therapies in model animals or humans with diabetes can improve cognitive functions. Likewise, thiazolidine-based diabetic therapy, i.e., pioglitazone, decreases the risk of dementia in patients with diabetes and increases both glucose metabolism and memory performance in patients with LOAD and diabetes [122,123]. Treatment with metformin has shown to substantially enhance memory deficits. Mostafa and colleague's studied the acute administration of metformin in a scopolamine-amnesic mice model (with impaired learning and memory skills), for about two weeks, and demonstrated the valuable effects of metformin on improving memory [71]. The molecular mechanism involved in the neuroprotective action of metformin was multiple since it showed significant antioxidant and anti-inflammatory activity. However, the authors propose that its protective effect against scopolamine-induced cognitive impairment is probably through the signaling pathway of Akt/GSK3 beta and prevention of phosphorylation of tau protein. Likewise, according to some trials, scopolamine therapy has been shown to decrease pAMPK and CREB levels, and metformin treatment has successfully restored pAMPK and the transcription factor CREB levels in the hippocampus [71,121]. Furthermore, metformin was able to increase the hippocampal levels of antioxidant enzymes, such as superoxide dismutase levels [121]. These results give support to the hypothesis that metformin could be a potential preventive drug against cognitive and memory impairment [94,95,121,124–132] (Table 1). Similarly, metformin was shown to prevent cognitive damage in the chronic L-methionine model of memory impairment, probably by normalizing oxidative damage [124].

Kodali and colleagues reported that after 10 weeks of metformin treatment, C57BL6/J mice with late middle age improved recognition memories in old age [72]. Metformin treatment in the hippocampus modulated microglial cells in an anti-inflammatory M2 phenotype and reduced hypertrophy of astrocytes. Furthermore, it reduced the concentration of proinflammatory cytokines and enhanced autophagy processes through the activation of AMPK and inhibition of mTOR signaling.

Likewise, since the hippocampus is an essential part of the brain for memory and cognition and it is widely affected in AD, the hippocampus' neuronal activation and synaptic transmission are important for improving these functions. According to Chen and

colleagues, metformin improved synapsis, memory, and cognitive deficits with disrupted hippocampal synaptic communication [131]. This process could be explained through the increased presynaptic glutamate release, which would be responsible for the increased elevated miniature excitatory postsynaptic currents (mEPSC) into CA1 pyramid neurons in hippocampus [131]. Likewise, Asadbegi and colleagues demonstrated that metformin treatment was able to improve significantly long-term potentiation in rats after the Aβ-injection. Moreover, rats were under a high-fat diet (HFD), and metformin treatment showed neuroprotective effects against detrimental effects of Aβ and HFD on hippocampal synaptic plasticity [96].

In turn, Li and colleagues studied the effects of intraperitoneal injection of 200 mg kg^{-1} d^{-1} of metformin for 18 weeks in db/db mice, which have multiple AD-like brain changes such as alterations in cognitive functions, increased phospho-tau and Aβ, as well as decreased synaptic proteins. Metformin decreased hippocampal levels of total tau, phospho-tau, and activated c-Jun-N-terminal kinase [133]. Moreover, metformin treatment increased the levels of synaptophysin, a synaptic protein, in the hippocampus of db/db mice [133]. Notwithstanding, metformin did not attenuate spatial learning and memory deficits. However, it was effective in enhancing biochemical changes like those of AD in the hippocampus of these mice.

4.5. Metformin Effects on Synaptic Density and Dendritic Spines

The theory that activating insulin receptors enhances cognition has been confirmed by clinical and preclinical trials. Hence, it is accepted that insulin improves emotional function in active and elderly people, as well as Alzheimer's patients [128]. Insulin signaling can influence the synaptic plasticity by controlling glutamate receptor expression and trafficking, and insulin receptors are enriched at hippocampal synapses, where they are proposed to control synaptic plasticity by interactions with the glutamatergic system [95,128–131]. Furthermore, various studies have shown that synaptic markers and/or dendritic spine dysfunction appear before the development of Aβ plaques and NFTs, thus implying that these events are closely linked to cognitive decline in AD [95,128–132]. In older rhesus monkeys, selective loss of thin spines is closely associated with decreased learning capacity [134–136]. In addition, according to Morrison and Baxter, reducing spine form can have a detrimental impact on prefrontal synaptic plasticity, which is essential for normal functioning in aged people [137]. In this line, the maintenance of thin and mushroom spine populations (another spine type) combined with cumulative increased spine extent in the dorsal-lateral-prefrontal-cortex (DLPFC) distinguish cognitively normal older individuals with AD pathology from patients with AD dementia [134]. This changes may be linked to the mild cognitive impairment (MCI) that can be detected early in AD patients [134–136], confirming that synaptic loss is key to the development of the disease [60] and supplying cellular evidence that dendritic spine remodeling could be a process of cognitive resilience. All these findings support the idea that synaptic function and behavior are directly related to cognition ability [138–141]. As a result, the cellular and molecular events that regulate synapses may be used to treat cognitive dysfunction in AD [136].

Loss of synaptic activity in the AD brain can be correlated with observed cognitive deficiencies [12,138–141]. Metformin has been shown to mediate memory forming through synapse plasticity [142,143]. In addition to synaptic impairment and lack of neuronal integrity in mature neuronal circuitry, in the AD-associated neurodegenerative phase, aberrant adult hippocampal neurogenesis is also involved. Cyclin-dependent kinase 5 (CDK5) is a serine/threonine kinase triggered by p35/p39 neuron-specific activators that plays a key role in synaptic plasticity neuronal and cognitive behavior [144]. The proteolytic cleavage of p35 to p25 contributes to protracted and aberrant activation of CDK5 and results in synaptic depression, which closely mimics early AD pathology [145]. Consequently, a possible promising strategy for the development of AD drugs is CDK5 inhibition. It has been found that metformin inhibited CDK5 hyper-activation and CDK5-dependent tau hyper-phosphorylation in the hippocampus of APP/PS1 mice [144–146]. Liu and colleagues reported that CDK5-activation by hyperglycemia is involved in neuronal apop-

tosis [145]. Furthermore, it was shown that CDK5 phosphorylates the PPARγ receptor on serine residue 273, thus preventing the transcription of antiobesity effects and favoring weight gain. In this sense, Cai and colleagues reported that CDK5 could be the link between AD and T2DM hyperacetylation of H3K9 histone on CDK5 promoter [146].

The transgenic APP/PS1 mice presents loss of spines as demonstrated by reduced spine density from CA1 pyramidal neurons. According to Wang and colleagues' study, chronic metformin administration for 10 days improves synaptic defects, including surface GluA1 expression, decrease spine disappearance, and reduction in basal synaptic transmission in the hippocampus of APP/PS1 mice [144]. Furthermore, in highly primed hippocampal slices from APP/PS1 mice, theta burst stimulation-induced CA3-CA1 long term potentiation (LTP) was compromised, while the LTP deficiency was saved by chronic therapy with metformin for 10 days too [144]. Increased presynaptic glutamate release from terminals innervating CA1 hippocampal pyramidal neurons was observed using paired-pulse ratios (PPR), but the excitability of CA1 pyramidal neurons was not affected. These findings indicate that metformin improves glutamatergic rather than GABAergic signaling in hippocampal CA1, revealing new information about metformin's actions on neurons.

4.6. Metformin Effects on Neuroinflammation

According to Ha and colleagues, metformin possesses anti-inflammatory effects [147]. The neuroinflammatory response requires microglial cells, which are resident phagocytes in the CNS. Microglial cells initiate an innate immune response when they are triggered by danger-associated molecular patterns (DAMPs) such as S100A8, S100A9, Aβ, or pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides (LPS) [147]. According to reduction of multiple inflammatory responses in BV-2 microglial cells by metformin treatment, including the secretion of pro-inflammatory cytokines such as tumor necrosis factor(TNF)-α and interleukin (IL)-6, it could be considered an important autophagy regulator and anti-neuroinflammatory drug [147]. Pursuant to Liu and colleagues, metformin reduced the incidence of clinical stroke in adults with diabetes and attenuated post-stroke brain atrophy volume 24 h after therapy in mice with temporary middle cerebral artery occlusion (tMCAO). In normal mice, metformin therapy not only stimulated neurogenesis through the modulation of the CREB-binding protein (CBP)-protein kinase C (PKC) pathway, but also increased lifespan by alleviating chronic inflammation [148]. Accumulation of Aβ increases the proinflammatory factors IL-1β and IL-6 levels in APP/PS1 mice [76]. It has been reported that metformin decreases the levels of IL-1β and IL-6 in APP/PS1 mice [76]. In addition, different studies have highlighted the anti-inflammatory and antioxidant function of metformin, with several pathways playing a key role in the activation of AMPK [147–152]. In certain cases, metformin suppresses inflammation and decreases or removes inflammatory factors largely by dependent pathways and often independently of AMPK at the cellular level and elsewhere at the systemic level [148,149]. Metformin is also efficient in decreasing the amount of oxidative stress factors by controlling the cell's antioxidant function [64,65]. Interestingly, metformin anti-inflammatory effects could be due to the decrease of the expression of nuclear factor kappa B (NF-κB) [65]. NF-κB is involved in multiple inflammatory pathways, cell death, and tissue degradation [147]. Moreover, it is widely demonstrated that AGEs are one of the most important inflammatory factors in the development process of diabetes. Macrophages actively participate in this inflammatory process, which act by amplifying the expression of pro-inflammatory cytokines (IL-1, IL-6, and TNF-α), in addition to increasing the expression of receptor for RAGE and activating the NF-κβB pathway. Indeed, RAGE/NF-kB signaling plays a role in the inflammatory activity of AGE-stimulated macrophages/microglial cells. By activating AMPK and inhibiting NF-κβ, metformin suppresses the RAGE/NF-kβ pathway, which leads to inhibited effects of AGE and at the brain level it can decrease the activation of microglia favoring the M2 (anti-inflammatory) phenotype over M1 (classic or inflammatory) [149].

On another front, it has been reported that metformin can decrease ROS production through direct inhibition of the chain of the electron transfer complex of complex I (NADH ubiquitin oxidoreductase (NADH) [151–153]. Other described mechanisms involved in the reduction of ROS may be due to the activation of antioxidant enzymes such as catalase, which is the main decomposer of H_2O_2, inducing the endogenous antioxidant system that includes glutathione reductase (GSH), superoxide dismutase (SOD), and catalase (CAT [151,152]. It has also been described that metformin can stabilize the nuclear factor related to erythroid 2 (Nrf2), a sensor of oxidative stress, and induce its gene expression, through AMPK. Induction of the Nrf2 pathway is associated with an increased level of antioxidant system enzymes such as CAT, GSH, and SOD [151–153]. Thus, through the induction of AMPK activation, metformin stimulates the initiation of this pathway and may explain its antioxidant function.

In several studies performed on mice with traumatic spinal cord injury, reactions of local inflammation along with microglia proliferation, activation, and phagocyte infiltration are used [154]. In a demyelinating context induced by lysolecithin, metformin treatment reduced demyelination and inflammation and protected the functional integrity of optic tract, as measured by visual evoked potential recording [155]. Moreover, potential application of metformin in multiple sclerosis has been recently reviewed [154].

All these data support that metformin has an antioxidant and anti-inflammatory function in different circumstances. Therefore we can conclude that, for several neurodegenerative diseases whose inflammatory pathways and oxidative stress play a role in their pathogenesis, metformin may be an effective therapeutic choice [150–152].

4.7. Neuroprotective and Neurorestorative Potential of Metformin

Chung and colleagues studied the genes and proteins whose expressions or functions were either directly or indirectly influenced by the AMPK pathway to understand the role of metformin between multiple signal pathways in neuroprotection [124]. AMPK can work independently via various roles of many basic cell type functions (e.g., mitochondrial biogenesis, cellular synthetic activity, anti-inflammation, anti-oxidative stress, cell growth, and proliferation) and molecular pathways (e.g., incorporation of proper effects through AMPK-PPARγ, AMPK-PGC1 alpha, AMPK-PFK, AMPK-FOXO, and AMPK-mTOR signaling cascades) [124]. Downregulation of AMPK and downstream signaling pathways, lead to AGEs production, to an increase in human neural stem cells (hNSC) death and to mitochondrial dysfunction. Several studies have also shown that AGEs reduce mitochondrial capacity, and Wareski and colleagues demonstrated that AMPK stimulation promotes mitochondrial activity through the activation of PGC1α [153]. In age treated hNSCs, metformin also improves AMPK, PGC1α, NRF-1, and Tfam expressions that may contribute to the observed elevation in mitochondrial functions. In addition, metformin-enhanced neuroprotective gene expression can help to protect hNSCs against toxicity caused by AGE [150].

Interestingly, Fatt and colleagues demonstrated a potential neurorestorative effect of metformin [153]. They reported that treatment with metformin improves NPCs proliferation, self-renewal, and neuronal differentiation [153]. Metformin therapy orchestrated this process mainly through the activation of TAp73 gene expression in adult NPCs and through AMPK activation by triggering the cascade of aPKC-CBP [153].

5. Conclusions

Dementia is linked to a number of co-morbid disorders in the elderly, including diabetes, asthma, dyslipidemia and cardiovascular disease, among others [1,4,6]. Therefore, all these factors could substantially complicate LOAD treatment. For this reason, it has been proposed that a combination therapy with more than one drug may be necessary to slow or delay the evolution of the disease [2–5]. In this regard, in a combinatory therapy with 3 or 4 drugs (anticholinergics, memantine, aducanumab, sodium oligomannate (GV-971), anti-inflammatories) it may be interesting to add a drug such as metformin, which may be key

in improving hypometabolism and increasing glucose uptake in the brain. For this reason, metformin (or other antidiabetic drugs) can provide added value by increasing glucose transport to neurons and increasing ATP levels [65,149–153]. Thus, based on literature, metformin can be used to inhibit dementia progression and can be a novel therapeutic medication for strengthening LOAD-related cognitive dysfunction [65].

In general, metformin is considered a safe and well-tolerated drug. However, the appearance of gastrointestinal adverse effects—such as diarrhea, nausea, and vomiting—has been reported [154]. Less frequent may be the appearance of headache, hypoglycemia, weakness, and rhinitis. However, one must be very careful as metformin has a serious warning for the risk of lactic acidosis [156]. This side effect is rare but serious and has an incidence rate of 1 in 30,000 patients. In this way, metabolic acidosis results in a decrease in the pH in the blood causing nonspecific signs and symptoms, such as respiratory distress, elevated lactate levels and acidosis [156]. Lactic acidosis can, in turn, cause hypotension, hypothermia, and death.

Metformin, through multi-directional pathways, could be a promising candidate for prevention of not only LOAD, but also of other neurodegenerative diseases, due to beneficial effects at the central and peripheral level (Figure 1). Metformin crosses the BBB and acts centrally via exerting a neuroprotective effect. It may also facilitate neurogenesis and enhance spatial memory development. In addition to cognitive and behavioral changes that follow the emergence of LOAD, recent findings indicate that metformin could play a neuroprotective role by correcting the hallmarks of brain damage (metabolic dysfunction, synaptic dystrophy and cellular loss). Preclinical results of metformin treatment on transgenic mice, demonstrate that spatial memory can be improved as well as neuroprotection and neurogenesis in hippocampus. In addition, amyloidogenesis and inflammatory reactions can be affected by metformin to decreasing through regulation of AMPK/mTOR/S6K/Bace1 signaling and block the NF-kβ. Regarding clinical trials, the authors generally suggest that future studies should include biomarkers of AD in CSF or image markers such as PET associated with amyloid ligands, so that the results reinforce the modifying role of metformin in the LOAD. In turn, studies with metformin in older people with diabetes showed that this drug was associated with an improvement in global cognition and reduced the risk of dementia compared to older people with diabetes who were not treated with metformin. Therefore, we must wait for the results of more clinical studies to confirm the role of metformin in a potential combinatorial therapy in the prevention of LOAD.

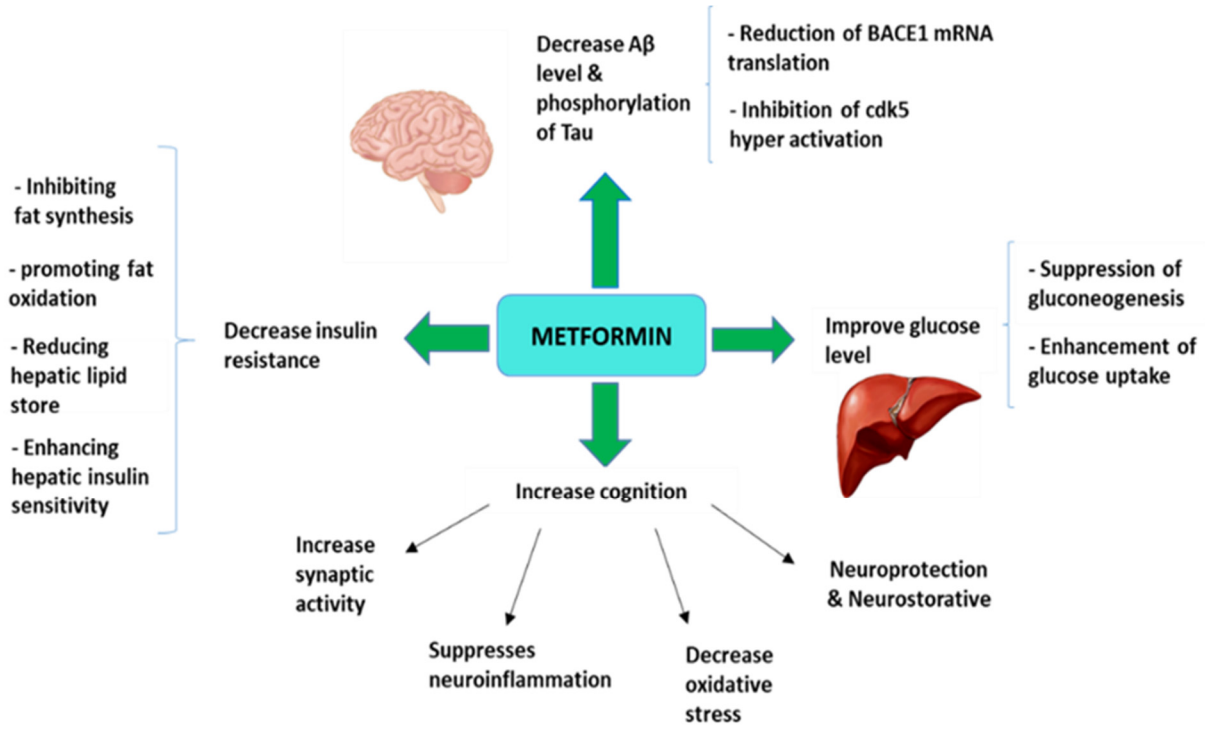

Figure 1. Metformin decreases the insulin resistance by multiple mechanisms and increases insulin sensitivity [18]. Reduction in level of Aβ and phosphorylation of Tau protein by reduction of BACE1 translation [67] and improving the glucose level by suppression of gluconeogenesis are the other beneficial effects of metformin treatment in neurodegenerative disorders such as AD [18]. Metformin also improves cognition by increasing synaptic activity [64,65,131], suppressing inflammation and decreasing oxidative stress [75]. In addition, metformin has neuroprotection and neurorestorative effects to increase memory, learning, and cognition in AD cases [101].

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Metformin restores cognitive dysfunction and histopathological deficits in an animal model of sporadic Alzheimer's disease

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ABSTRACT

Background: Metformin has been introduced as a neuroprotective agent in recent years. Here we evaluate the therapeutic effects of metformin in sporadic mouse model of Alzheimer's disease (SAD).
Methods: AD was induced by streptozotocin (STZ, 0.5 mg/kg) on days 1 and 3. Metformin (MET, 200 mg/kg per day) was used for two weeks. Novel objective recognition (NOR) and Barnes Maze test were used to test the learning and memory. Nissl staining was used as s histological method for counting the dying neurons in different regions of hippocampus. Immunofluorescence staining against glial fibrillary acidic protein (GFAP), ionized calcium binding adaptor molecule 1 (Iba1) and NeuN were used to visualize reactive astrocytes, microglia and neurons, respectively.
Results: In NOR test, the discrimination indices in the STZ group were significantly lower than the control and treatment groups. Goal sector/non-goal sector (GS/NGS) ratio index in Barnes maze was increased in metformin group compared to other groups. The number of dying neurons was increased by SAD and metformin reduced it. GFAP level was increased in CA1, CA3 and cortex of STZ group and reversed following the treatment. Iba1 level was significantly higher in STZ group in CA3 and cortex regions compared to Control and decreased by metformin in CA3 and cortex. Counting $NeuN^{+}$ cells demonstrated significant reduction of neurons in DG+CA1 and CA3 after SAD induction.
Significance: Metformin decreased inflammatory cells and reactive astrocytes as well as the dying neurons in the hippocampus region and the cortex in SAD, and improved the cognitive performance.

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1. Introduction

One of the most prevalent neurological diseases of the century is Alzheimer's disease (AD) which is characterized by progressive cognitive decline [1]. It is neuropathologically characterized by the accumulation of extracellular neuritic plaques and fibrils in the brain, mainly consisting of aggregated amyloid beta (Aβ) peptides and intracellular neurofibrillary tangles (NFT), composed of hyperphosphorylated Tau (p-Tau) [2]. Soluble Aβ peptides through binding to their receptors would be responsible for generating the neurodegenerative process. Likewise, synaptic damage is mediated by soluble Aβ oligomers, which have been shown to better correlate with the disease severity than with the accumulation of insoluble Aβ peptides into plaques triggering AD pathophysiology [2]. Familial form of AD (FAD) 2) is associated with mutations in the amyloid precursor protein (APP) and presenilin-1 and 2 PSEN 1 and PSEN genes. In sporadic AD (SAD) which accounts for more than 95% of all AD patients, the hallmarks seem somewhat different, and the inflammatory processes have been proposed as the main mechanism [3]. Most of the AD cases are sporadic which are less obviously impacted by a single gene mutation. The cause of SAD is unknown and additional factors, other than genetic and age may be involved in the neurodegenerative process pointing out that AD is a multifactorial pathology. There are a number of risk factors identified in SAD development among them obesity, type 2 diabetes mellitus (T2DM) and neuroinflammation have been identified as well-known risk factors [2].

SAD affects people without a family history of the disease. Insulin resistance is one of the most important causes of SAD [2]. As the major hallmark of AD especially in sporadic AD, the innate immune cells, microglia as well as the astrocytes mediate the neuro-inflammatory response. Synaptic dysfunction, neural death and neurogenesis suppression can be done by pro-inflammatory molecules such as tumor necrosis factor (TNF), interleukin-6 (IL-6), IL-1β, IL-18, and small-molecule messengers such as nitric oxide (NO) in response to insulin resistance and the presence of Aβ toxicity in the brain. According to previous epidemiological reports, diabetes has been linked to dementia [4]. Interventions that target astrocyte and microglial priming in the preclinical phase of the disease and control their response in the brain once the AD process has been started, show anti-inflammatory strategies as new approach for AD treatment [4]. Obesity, T2DM and associated comorbidities, all have been linked to the development of late onset AD (LOAD) [5]. LOAD has recently been labeled as a "metabolic disease" (type 3 diabetes) [6] associated with inefficient glucose utilization by the brain, insulin resistance, and chronic mild inflammation in the brain [7,8].

This association could be related to inflammation, oxidative stress, vascular involvement, increased level of brain amyloid peptides, and hyperinsulinemia [9,10]. Streptozotocin (STZ) can be utilized to induce the activation of pathophysiological processes which mimics the pathophysiology of SAD [11]. STZ is produced by *Streptomycetes achromogenes* and because of its capability to impair the pancreatic β cells and induce insulin resistance, in the systemic use, it initiates diabetes. STZ inhibits the insulin receptor function in the brain and disrupts the glucose and energy metabolism [12].

Metformin is a biguanide that contains a couple of guanidine molecules [13,14] and its chemical structure is highly hydrophilic (1, 1-dimethylbiguanide hydrochloride). This antidiabetic drug reduces liver gluconeogenesis and insulin resistance, resulting in lower plasma glucose levels [15]. It can cross the blood–brain barrier (BBB) and has been previously linked to improved cognitive performance [16]. Inhibition of hyperinsulinemia results in limited formation of amyloid plaques and the advanced glycation of end-products in the brain and decreases the inflammation and oxidative stress [17]. According to studies on transgenic mice APPswe, PS1dE9 and PDAPP (J9); and AD models induced by chemicals such as mice sporadic models of AD induced by STZ and etc., metformin prevents hippocampal insults and spatial memory decline, reduces inflammation and regulates the AMPK/mTOR/S6K/Bace1 pathway. In addition, metformin increased insulin receptor sensitivity and facilitates neuronal survival [18]. According to Shi et al. the effect of metformin on neurodegenerative diseases (ND) such as dementia, AD, Parkinson's disease (PD) and Huntington's disease (HD) has been evaluated. Based on the findings, metformin showed a significant reduction in the incidence of ND in patients with T2DM [19]. Additionally, metformin was suggested to restore the abnormal blood-brain barrier transport of amyloid-β (Aβ), improve memory, and neurogenesis by activating protein kinase C/CREB binding protein (PKC-CBP) and AMPK pathways [20,21]. In a recently published study, it was shown that administration of metformin was associated with memory and learning improvement in SAMP8 mouse models of AD with accelerated aging [22]. However, to our knowledge the restorative effect of metformin on histopathological and memory deficits in animals with developed SAD model needs further clarification.

We designed the current study to evaluate the effects of metformin on an established sporadic Alzheimer's disease (SAD) in mice. We performed behavioral evaluation of the cognition performance, as well as the neuroinflammation and gliosis levels assessments. The potential of metformin to preserve neurons from further degeneration was assessed through dying neurons counting. Total neuron counts were assessed by NeuN staining. Histopathological evaluations were assessed in both hippocampus and cerebral cortex.

2. Material & methods

2.1. Animals and the interventions

In this study, 5–6-month-old male mice (C57BL/6) were used. Mice had access to food and water ad libitum and were housed in an environment with 12/12 h light/dark cycle and temperature maintained at 22 °C ± 2 °C. Attempts were made to keep the number of animals utilized to a minimum and to alleviate their suffering. All protocols and procedures followed the ethical guidelines stablished by the European Communities Council Directive 2010/63/EU. The stereotaxic surgery protocol and animal endpoint was approved at Tarbiat Modares Ethics Committee (approval number: D52/6725).

Animals were initially divided to 2 main groups: 1) eleven mice without any treatment as Control; 2) eighteen mice treated with STZ as SAD model. These mice were treated with intracerebroventricular (ICV) injections of STZ in aCSF (0.5 mg/kg) at the first and

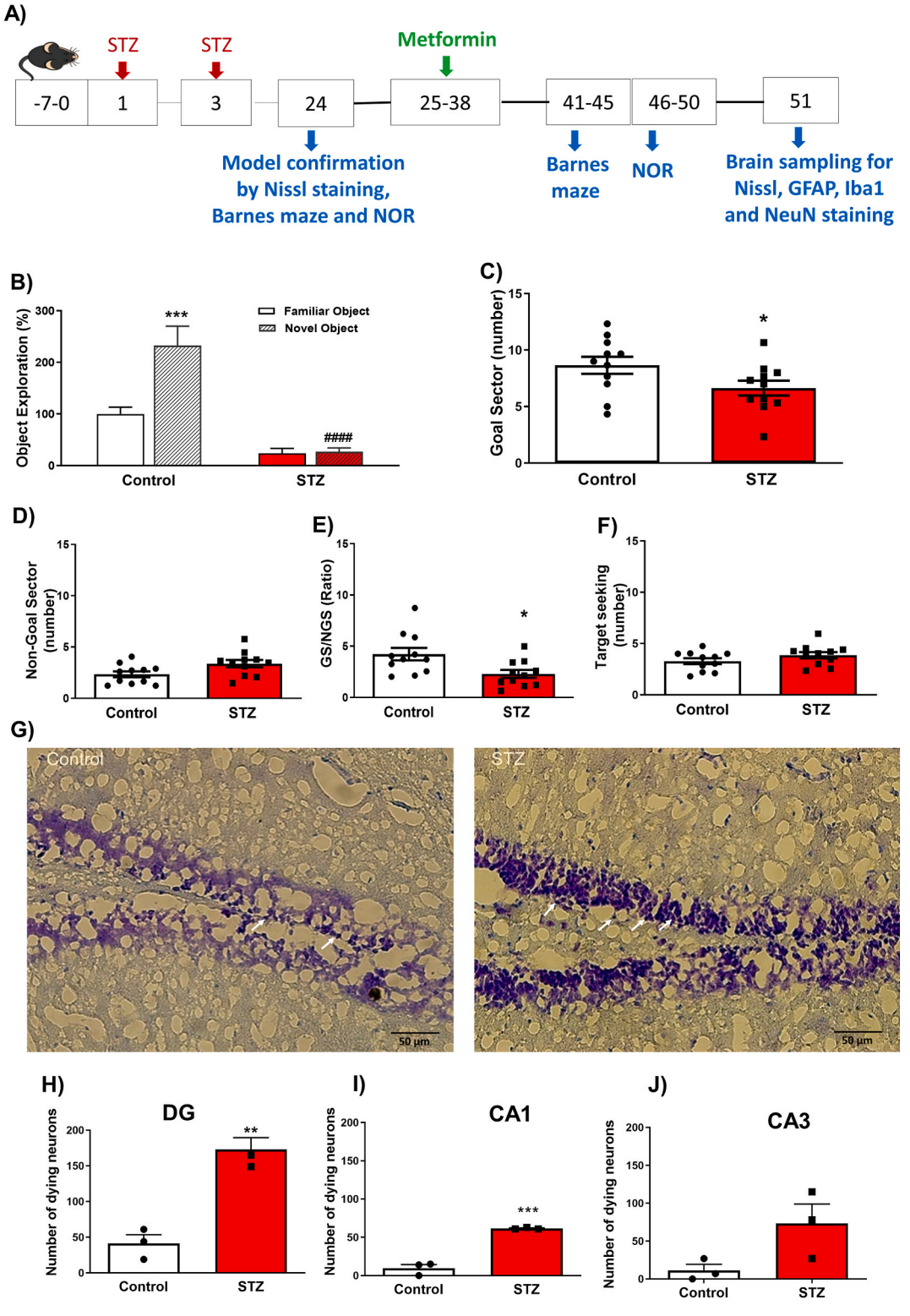

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Fig. 1. Streptozotocin (STZ) injection into mice ventricles caused cognitive impairment and neural damage as assessed 3 weeks post STZ injection. A) Schematic representation of experimental timeline. B) Novel Object Recognition (NOR) test demonstrated a significant memory loss in SAD mice compared with control group. Object exploration percentage was analyzed using two-way ANOVA. C–F) Barnes Maze test results. C: Goal Sector exploration number data showed significant differences between control and STZ model group. D: Non-Goal Sector exploration number and E: GS/NGS ratio data demonstrated significant differences between the Control and STZ model group. F: Target seeking analysis showed no significant differentiation. G-J) Nissl staining sections obtained micrographs as representatives for Control and STZ groups and the quantitative analysis data. G: Nissl-stained micrographs images of dentate gyrus represents samples of dying neurons in two experimental groups. H–J: the average number of dying neurons in DG, CA1 & CA3, and the mean differences between the groups. *P < 0.05, **P < 0.01 and ***P < 0.001 vs Control, and $^{\#\#\#\#}$P < 0.0001 vs novel object in Control.

third day of experiment [23]. First, animals were anesthetized using administration of ketamine/xylazine and placed in a stereotaxic device. The injections were done bilaterally at 0.9 mm lateral to the midline, 0.02 anteroposterior (AP) from bregma and 2.4 mm depth from dura, using two stereotaxic surgeries for each animal at days1 and 3. STZ powder (0.5 mg) was dissolved in aCSF (Ingredients of artificial cerebrospinal fluid: 147 mM NaCl, 2.9 mM KCL, 1.6 mM MgCl2, 1.7 mM CaCl2 & 2.2 mM dextrose) and injected in the volume of 2 μl in each side (total volume: 4 μl). After 21 days, mice which showed SAD phenotype, underwent the behavioral tests, then divided to 2 subgroups; 11 mice were kept as SAD group with no further treatment, and 7 SAD mice received metformin as treatment (STZ + MET group). Metformin 200 mg/kg dissolved in PBS, was injected every day (100 μl at morning and 100 μl at afternoon (every 12 h)) for 2 weeks (days 22–35) (Fig. 1). Metformin dose was selected base of previous studies which reported its neuroprotective/neuro-restorative effects [24]. Animals underwent Barnes maze test and novel object recognition (NOR) test to assess their learning, memory and cognitive performance prior to sacrifice for histopathological evaluations as mentioned in the timeline presented in Fig. 1A.

2.2. Cognitive assessment using barnes maze test

The Barnes maze was used to measure the spatial memory and learning skills in animal groups on days 38–42. Barnes maze test was performed on a circular table with a diameter of 90 cm and a height of 90 cm from the floor. Twenty holes, each with a diameter of 5 cm, were regularly spaced around the table's circumference. The target hole (main hole) was the only one that led to an escape chamber where the animal could hide. To enhance the animal's motivation to hunt for the target hole, illumination was increased and at the middle of the table, was kept at 1350 lux. The animal was introduced into the start box placed in the maze center for the habituation phase, at the beginning of each trial. The start box was removed 5 s later, and mouse was given 300 s to explore the area. The procedure included four training days (every day 4 trials, each 5 min) and one test day. In all stages, the Barnes maze area and the start and escape boxes were cleaned with 96% ethanol prior to introducing the animal to remove the interfering smell. Movement strategy to find the escape box (target hole) after moving out the start box at the center of Barnes maze has been classified as direct, serial and random. When radially moved toward the escape box, the movement was considered as direct strategy. If animal moved from one hole to the next one and continued until finding the target hole, the strategy was named serial. The strategy was named random when animals moved randomly, without any plan, till finding the escape box [25]. On the test day, the escape box was removed, and the animal was placed in the center of Barnes maze and observed for 5 min. The video tracking system Ethovision XT 11 (Noldus Company, Netherland) was used to collect and evaluate the behavior [26,27].

2.3. Cognitive assessment using novel objective recognition

NOR test was used to assess the cognitive impairment in mice. There were three steps in the task procedure: habituation, familiarization and testing. During the habituation phase, mice investigated a square open-field area with 39 × 39 × 20 cm inner dimensions without an item for three days, 10 min per session. Each mouse was placed in the area with two identical items (A + A) in the middle of the field for 10 min on the fourth day (familiarization phase). For the test phase, mice were reintroduced to the area 24 h later with two different objects; one that was identical to those presented the day before (A) and the other that was novel (B) for 10 min. In all stages, the area and objects were cleaned with 96% ethanol prior to animal introduction to remove smell signals. Exploration was described as an animal's snout pointing toward an object, smelling, or touching it. The percentage of discriminating index (DI) was used to determine the difference in exploration time between familiar (A) and unfamiliar (B) objects (equation I). As a result, each object's exploration time was divided by the total exploration time, and expressed as a percentage [28]. The Ethovision XT 11 (Noldus Company, Netherland) software was used for NOR test evaluation.

Equation I:

$$DI\% = \frac{new\ object\ exploration\ time}{total\ exploration\ time} \times 100$$

2.4. Nissl staining

In this study 0.1% Cresyl violet acetate (IHC world, USA) was used to stain the coronal hippocampus 10 μm-sections. In the first step, the brain samples were passaged in ethanol gradient 96%, 80% and 70% each for 1 min. After washing in tap water for 2–3 min, samples were stained in Cresyl fast violet for 3 min. After washing in water, samples entered to ethanol 70% and 80% each for 15 s and

ethanol 90%, 96%, 100% each for 2 min. Finally, samples were placed in Xylol for 5 min. Three mice per group and 3 sections per mouse were stained and photographed under an Olympus BX-51 microscope and DP72 camera. The number of dying neurons in DG, CA1 and CA3 regions was measured. Dying neurons were those with dark cytoplasmic Nissl staining, and not-prominent nucleoli [29, 30]. Neurons with abnormal morphologies of shrunken and hyperbasophilic appearance were distinguished as dying neurons.

2.5. Immunofluorescence staining

For immunofluorescence staining, mice were sacrificed under deep anesthesia and then perfused with 4% paraformaldehyde (PFA) diluted in 0.1 M phosphate buffer (PB). The brains were extracted and post-fixed for an additional night; then cryoprotected in a 30% sucrose-PFA-PB solution at 4 °C. The samples were kept frozen at −80 °C. A cryostat apparatus was used to make 10-μm thick coronal sections. Fixed sections collected on the positive charged slides were washed for 3 times each for 5 min with PBS 1% in room temperature. In next step, sections were incubated with Triton 0.3% for 20 min. Then samples were incubated with blocking solution (NGS 10% + 10 μl Triton100 x) for 1 h. Afterward, the slides were washed with PBS 3 times, each 5 min, and incubated with polyclonal rabbit anti-GFAP (1/500), anti- (1/1000) and mouse anti-NeuN (1/1000) primary antibodies at 4 °C, overnight. Sections were washed 3 times for 5 min each, in PBS, before been incubated for 1 h in dark at room temperature with red goat anti-rabbit and anti-mouse IgG secondary antibody (1:500; Invitrogen, Eugene, OR, USA) [28,31,32]. The images were obtained by fluorescence microscope, Olympus BX-51 and DP72 camera, with magnifications of 100 or 200. Three sections with ~10-μm thickness were selected, then photographed, and quantified using Fiji Software (NIH, USA) for each animal and averaged. This average value was entered to group mean calculation.

2.6. Statistical analysis

GraphPad Prism V8.0 was used for statistical analysis and the graphs preparation. All data was presented as means ± SEM and the minimum significance level of mean differences was set at $p<0.05$. The normal distribution was assessed using Shapiro-Wilk test. Student's *t*-test was used to compare control and STZ groups in model conformation experiments. One-way ANOVA with Tukey's post-hoc were used to compare behavioral and IHC data of Control, treated and non-treated groups. Two-way ANOVA was used to compare animal performance in NOR and Barnes maze during trial days.

3. Results

3.1. AD phenotype was developed following STZ treatment

Evaluation using NOR test showed that STZ caused significantly lower performance in exploring the novel object. The significant difference was observed between Control and STZ groups ($p < 0.0001$) (Fig. 1B).

The Barnes maze results showed that STZ-treated animals stayed in the goal sector less than Control group (Fig. 1C). The results also show that STZ animals appeared more frequently in non-target holes compared to Controls and spent more time in non-goal sectors

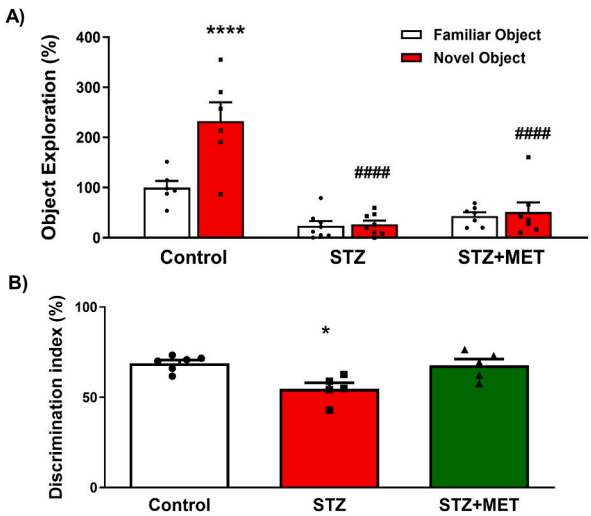

Fig. 2. Novel Object Recognition (NOR) test in control, STZ and STZ + MET groups demonstrated a significant memory loss in SAD mice compared to control group and a relative improvement in metformin-treated group. A) Object exploration percentage was analyzed using two-way ANOVA, significant differences were demonstrated between Novel and Familiar object in control group. B) Discrimination index percentage showed the positive effect of metformin on cognitive performance. *$p < 0.05$ and ****$p < 0.0001$ vs. control, ####l < 0.0001 vs. novel object in Control group.

(Fig. 1D). The GS/NGS ratio was significantly lower in STZ group (Fig. 1E), while the ratio of touching the entire holes/20 was not significantly different between the groups (Fig. 1F).

Fig. 1G demonstrated Nissl-stained dentate gyrus of control and STZ groups. The average number of dying neurons in dentate gyrus

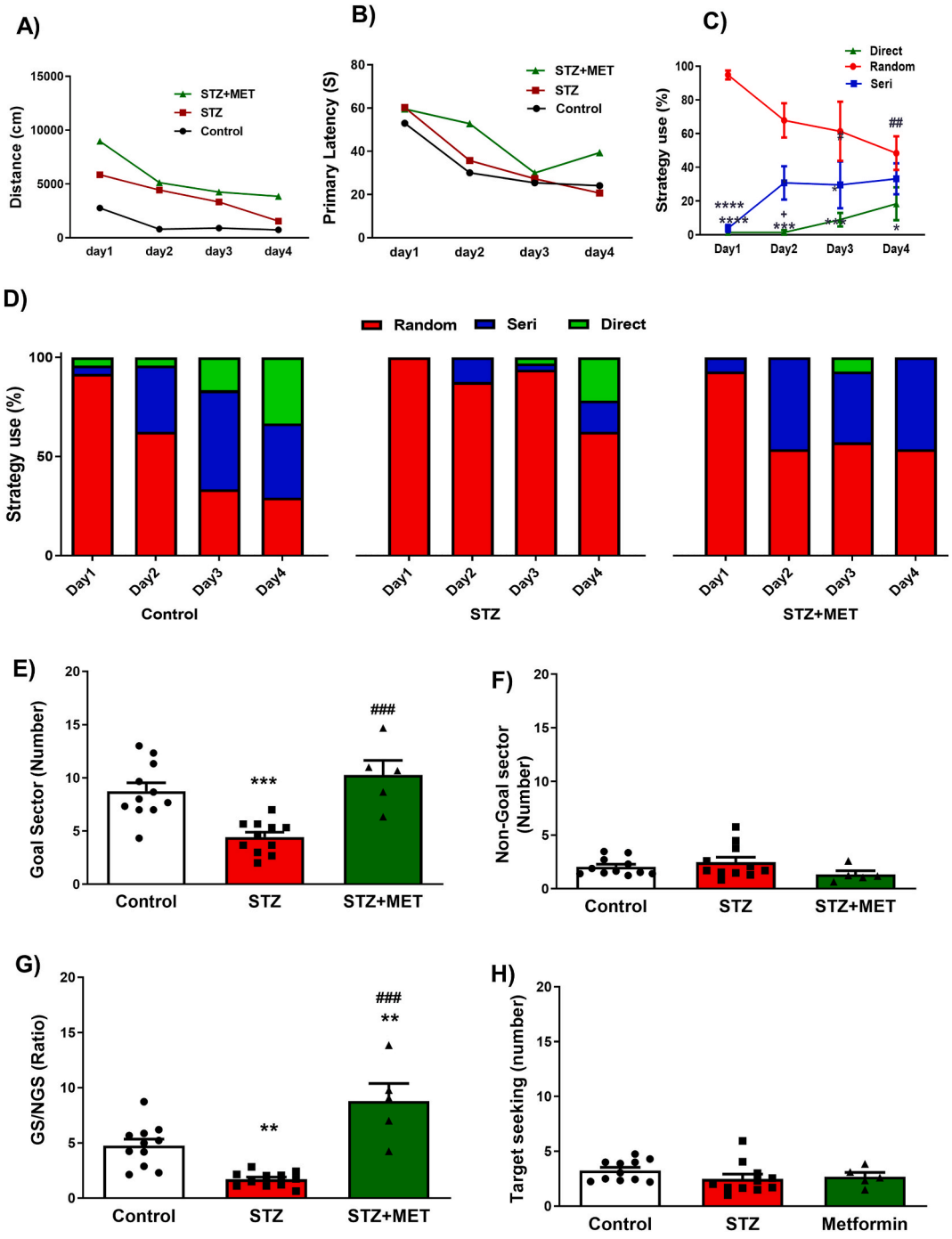

Fig. 3. Barnes Maze test performance in trial and the test day. A) Distance measures in 4 trial days demonstrated no significant difference between 3 groups. B) Primary latency calculated as duration to enter the escape box showed no significant difference between 3 groups. C-D) movement strategy to find the escape box in trial days. E) Goal sector exploring number at the test day showed remarkable difference between Control, STZ and STZ + MET. F) Non-Goal Sector exploration number showed no significant difference between 3 groups. G) GS/NGS ratio demonstrated significantly difference between Control and STZ and control and treatment group (STZ + MET). H) Target seeking number showed no difference between the groups. $^{**}P < 0.01$, $^{***}P < 0.001$ vs Control group, $^{\#\#\#}p < 0.0001$ vs STZ group.

(DG) and CA1 regions was significantly increased in STZ group (Fig. 1H and I; $p < 0.01$ and $p < 0.001$, respectively). Number of dying neurons observed in CA3 was not statistically different (Fig. 1J).

3.2. Metformin improved cognitive performance in SAD mice

Results obtained from NOR test showed that the total time spent to explore the familiar and novel objects was significantly higher in the Control group (Fig. 2A). Animals treated with metformin (STZ + MET) in novel object exploration percentage demonstrated a significantly elevated index ($p < 0.0001$). The percentage of discrimination index was significantly higher in Control group and was the lowest one in STZ group (Fig. 2B). No significant difference was observed between Control and treated group.

Barnes maze data on trial days (days 1–4) showed that the distance travelled to find the target hole was reduced in all animal groups during the trial days (Fig. 3A), although the total distance was lower for Control. Primary latency in the trial days of Barnes maze test was decreased (Fig. 3B).

The movement strategy of animals was analyzed as direct, serial and random types. At the initial trials animals in different groups mostly used the random strategy while it was gradually reduced in the next trials. Control animals showed the highest amount of reduction in random strategy and replaced it mainly by serial strategy and in lower extent by the direct strategy. STZ showed lower amount of reduction in random strategy. STZ + MET group showed a prominent decline in using random strategy by replacing it mainly with the serial strategy. Direct strategy to find the escape box mainly observed in Control by time (Fig. 3C–D). Compared to

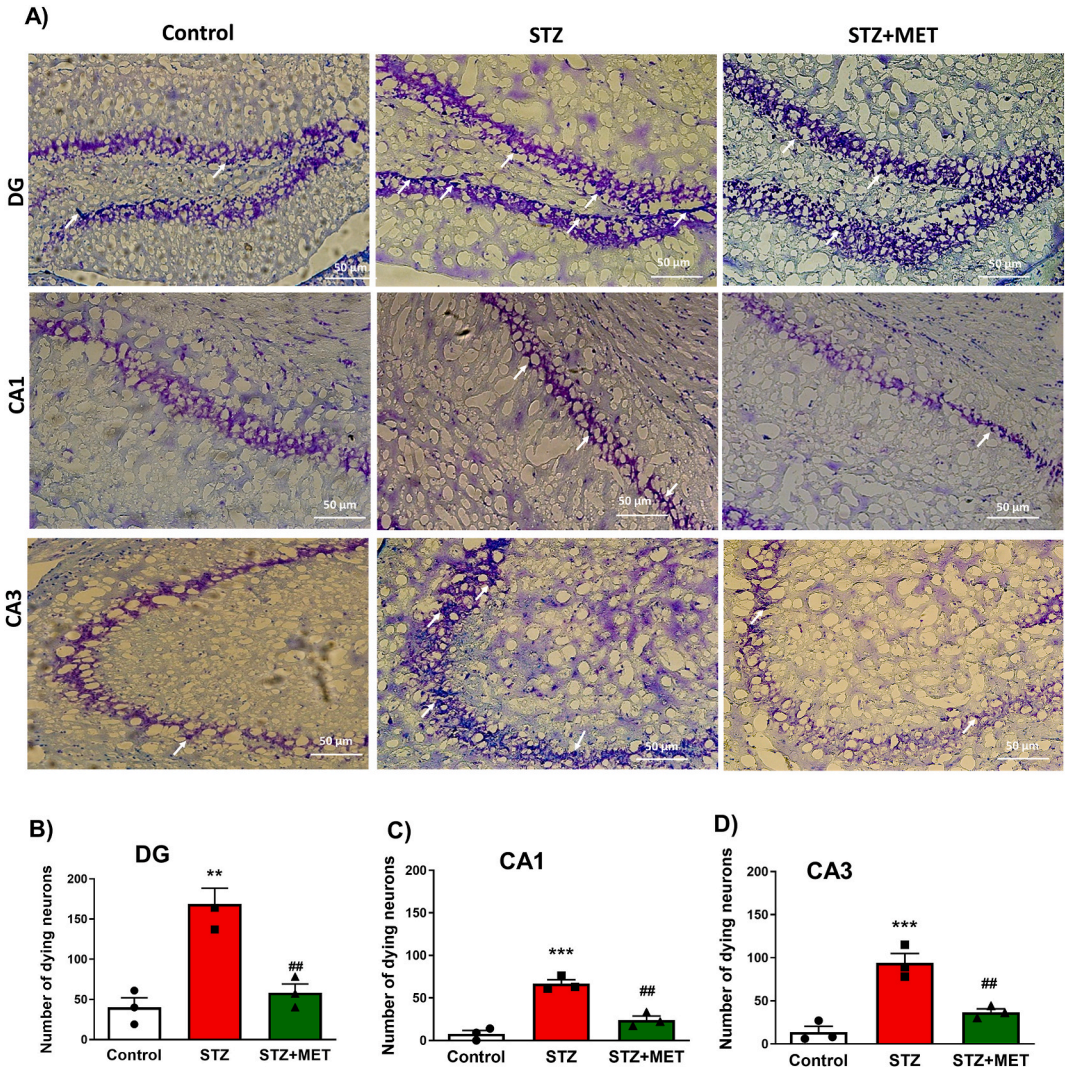

Fig. 4. Nissl Staining demonstration of average number of dying neurons in the hippocampus in three experimental groups (Control, STZ and STZ + MET). A) Representative Nissl-stained images of DG, CA1 and CA3. B) The average number of dying neurons in DG of Control, STZ and STZ + MET mice. C) The average number of dying neurons in CA1 of Control, STZ and STZ + MET mice. D) The average number of dying neurons in CA3 in different groups. $^{**}P < 0.01$, $^{***}P < 0.001$ vs Control group, $^{\#\#}p < 0.01$ vs STZ group.

STZ, STZ + MET group mice were more successful in substituting random to serial strategy as it was done in Control.

On the test day, (day 5), poking the goal sector (number of pokes of correct holes (including main, right & left holes)/3 within 5 min was at the highest level in the treatment group (STZ + MET) and showed significant difference with STZ group ($p < 0.001$). GS number in Control group was 9.0 vs. STZ group that was 4.5 ($p < 0.001$, Fig. 3E). Non-goal Sector (the number of poking of incorrect holes (the other 17 holes)/17), was the highest in STZ animals and no significant difference was observed between the experimental groups (Fig. 3F). GS/NGS ratio in test day in Barnes Maze was significantly the highest one in the treatment group (STZ + MET) as compared with Control and STZ groups ($p < 0.01$ and $p < 0.0001$, respectively). The lowest one presented in STZ group with significant difference between control vs. STZ ($p < 0.01$, Fig. 3G). Target seeking parameter (the number of poking of both correct and incorrect holes/total holes (20)), in the test day was not significantly different between the groups (Fig. 3H) [27].

3.3. Metformin reduced dying neurons in SAD mice

Number of dying neurons was evaluated within the DG, CA1 and CA3 using Nissl staining (Fig. 4A). According to the quantified data presented in Fig. 4 (B-D), the average number of dying neurons in DG was significantly increased following the SAD induction compared to Control group ($P < 0.01$). Metformin treatment reduced the number of dark cells (dying neurons) to the control level with

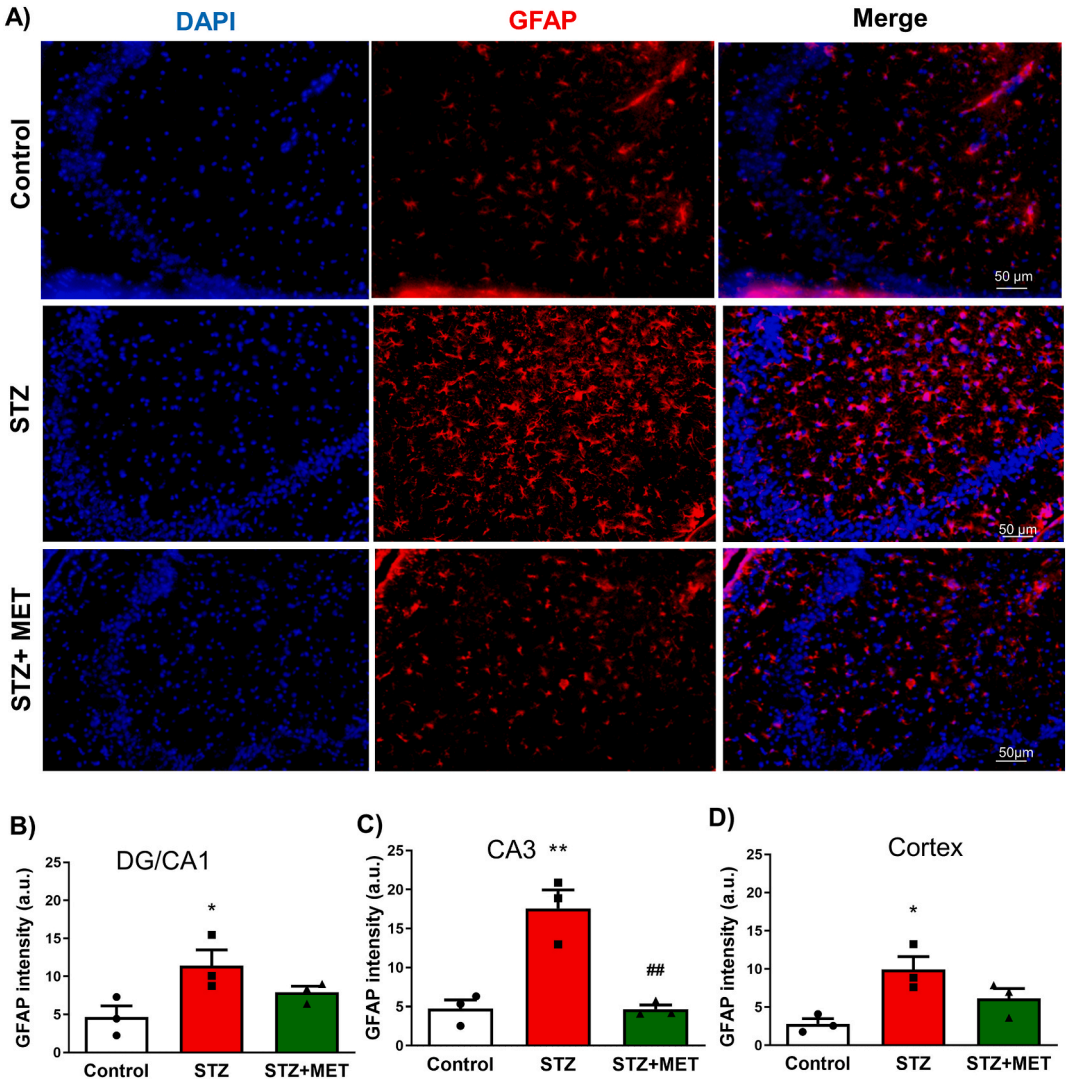

Fig. 5. A) Representative micrograph for GFAP staining in CA3. B) Quantification of GFAP immunofluorescence of DG/CA1 regions of hippocampus showed significantly difference between control and STZ groups. C) Quantification of GFAP intensity of CA3 region of hippocampus in three experimental groups demonstrated significant difference between control and SAD groups and also between SAD model and treatment groups. D) Quantification s of GFAP intensity of Cortex in three groups of study showed significantly difference in control group Vs. STZ group. *P < 0.05, **P < 0.01 vs Control group, $^{\#\#}p < 0.01$ vs STZ group.

significant difference Vs. STZ group, ($P < 0.01$, Fig. 4B). In CA1, number of dying neurons was significantly higher in STZ group as compared to Control ($P < 0.001$) and the number of dying neurons significantly decreased in treated group (STZ + MET) Vs. STZ group ($P < 0.01$, Fig. 4C). In CA3, the population of dying neurons was significantly increased in STZ group compare with control group ($P < 0.001$), metformin treatment significantly reduced the dying neuron in CA3 Vs. STZ group ($P < 0.01$) (Fig. 4D).

3.4. Metformin ameliorated gliosis in SAD mice

To measure the extent of reactive astrocyte, following SAD induction and the treatment, the intensity of GFAP staining was measured in different areas of brain section including DG/CA1, CA3 and the adjacent brain cortex in same sections. Based on immunofluorescence studies, GFAP intensity in DG/CA1, CA3 and cortex regions were significantly increased in STZ group compared to Control group, in addition treatment with metformin was effective to reduce the intensity of GFAP in three selected area of brain compared to STZ group (Fig. 5A–D). In DG/CA1 hippocampal area significant difference was observed between control and STZ groups ($p < 0.05$, Fig. 5B). Mean fluorescence GFAP intensity was highest in CA3 of STZ group in compare with Control and treated groups (P

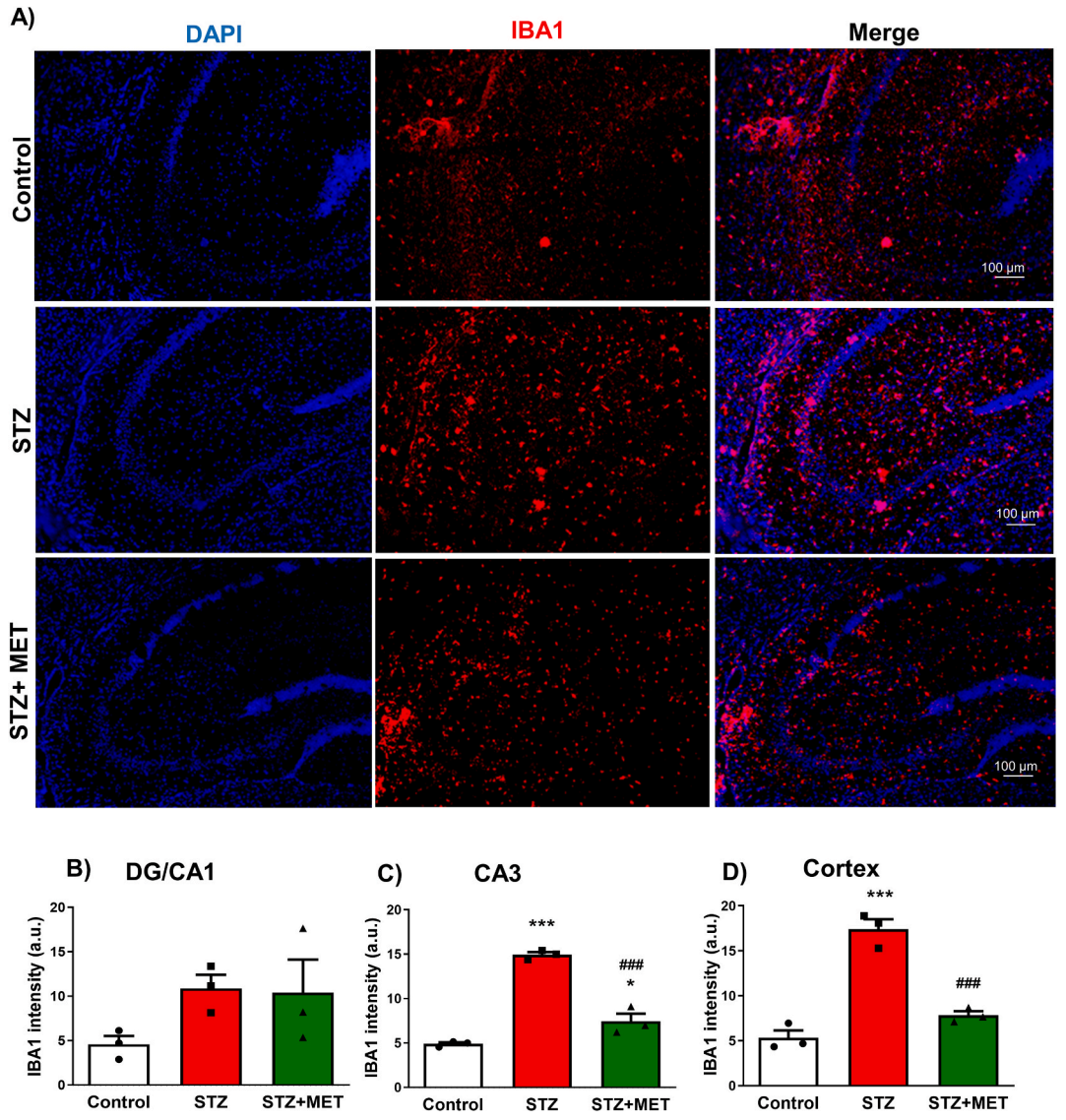

Fig. 6. A) Representative micrographs for IBA1staining in CA3. B) Quantification of IBA1 immunofluorescence of DG/CA1 regions of hippocampus showed no significantly differences between experimental groups. C) Quantification of IBA1 intensity of CA3 region of hippocampus in three experimental groups demonstrated significant differences between control and two other experimental groups such as STZ and treatment group and also between significant difference can be seen between STZ and STZ + MET groups. D) Quantification s of IBA1 intensity of Cortex in three groups of study demonstrated significantly differences in control Vs. STZ groups and in STZ Vs. treatment groups. ***$P < 0.001$ vs Control, $^{###}p < 0.001$ vs STZ.

< 0.01) Vs. control group and ($P < 0.01$) Vs. STZ + MET group (Fig. 5C). In cortex evaluation of Mean fluorescence GFAP intensity demonstrated significant difference between STZ Vs. control group ($P < 0.05$) (Fig. 5D).

To evaluate the neuroinflammation extent, we measured the intensity of IBA1 staining in the selected areas. Fig. 6A–D shows the intensity of IBA1 in DG/CA1, CA3 and the adjacent cortex. In DG/CA1 region, the IBA1 intensity was higher in STZ group compared to Control in view of mean but no significant difference observed among three experimental groups (Fig. 6B). Compared to Control, Intensity of IBA1 in CA3 was significantly increased by STZ induction ($p < 0.0001$) and reversed in STZ + MET group ($P < 0.05$). Based on the measurement of IBA1 intensity and the evaluation of reactivated microglia in the CA3, metformin was able to reduce and improve the inflammation in CA3 and significant difference observed in STZ + MET Vs. STZ group ($P < 0.01$) (Fig. 6C). The intensity of IBA1 in cortex was significantly increased in STZ group as compared to Control ($P < 0.0001$). According to measurement of Mean fluorescence IBA1 intensity, metformin treatment can be affective for reduction the neuroinflammation in cortex. Between STZ and STZ + MET groups significant difference was observed ($P < 0.001$, Fig. 6D).

NeuN as a mature neuronal marker stains the nuclei and was used to evaluate changes in the number of neurons in different areas of brain such as DG/CA1, CA3 of hippocampus and adjacent cortex (cortex area in same sections studied for hippocampal changes). Fig. 7A shows the sample micrographs obtained from DG/CA1. Immunofluorescence findings and $NeuN^+$ cells counting in DG/CA1

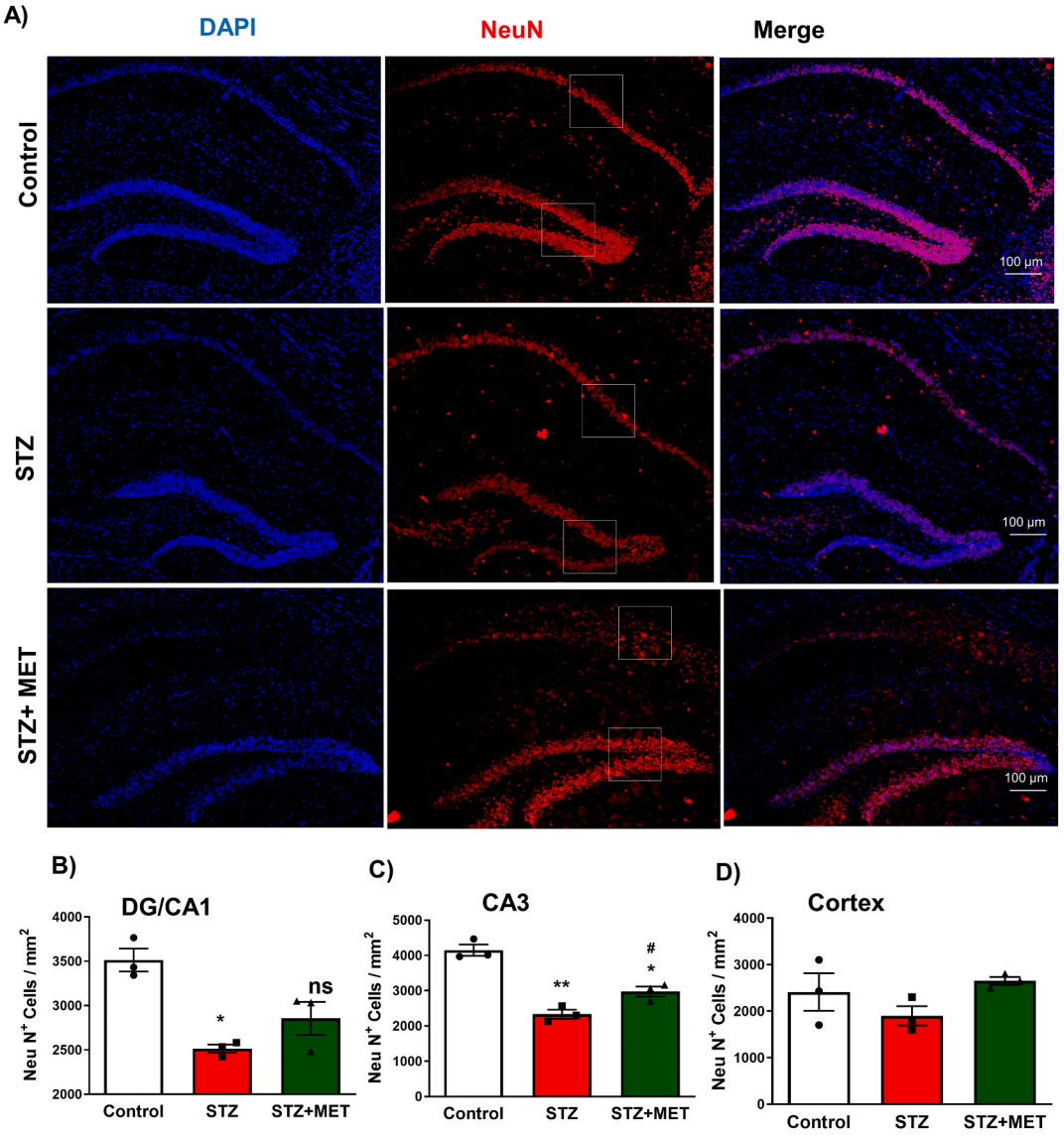

Fig. 7. A) Representative micrographs for $NeuN^+$ cells in DG/CA1. B) the number of NeuN positive cells in DG/CA1. This graph showed significant difference between control and STZ groups. C) the number of NeuN positive cells in CA3 that demonstrated significant differences between three experimental groups. D) the number of NeuN positive cells in cortex. There is no significant difference between three experimental groups. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs Control, $^{\#}p < 0.051$ vs STZ.

showed significant reduction following the SAD induction compared to Control group ($P < 0.01$) and declined from 3500/mm^2 to 2500/mm^2. Compared to STZ group, the number of NeuN$^+$ cells were increased in treatment group (STZ + MET), showing that metformin was able to ameliorate neuronal loss, however NeuN counts in STZ + MET group were still lower than the Control group ($P < 0.05$, Fig. 7B). Neuronal loss was affected by metformin in CA3 area and significantly restored, compared to the STZ group ($p < 0.05$). The number of NeuN$^+$ cells in CA3 was significantly decreased in both STZ and STZ+MET groups Vs. Control ($P < 0.001$ and $P < 0.01$, respectively, Fig. 7C). Counting of NeuN$^+$ cells in the adjacent cortices demonstrated no significant differences between the experimental groups (Fig. 7D).

4. Discussion

In this study, STZ affected healthy mice and induced sporadic AD-like pathology after 21 days. Treatment with metformin improved the animal behavioral performance including learning, and memory, and played a restoring effect on histopathological deficits induced by STZ administration.

Like diabetes, AD hires multiple pathophysiological mechanisms that impair insulin sensitivity, cognition and glucose metabolism [21]. Anti-diabetic agent, metformin hydrochloride, which acts through activation of AMPK, has showed positive effects on the survival of the neurons, neurogenesis and the formation of spatial memory [33,34]. Accordingly, it was quite rational to observe anti-AD effect following metformin administration in mice that had already developed AD-like pathology.

In behavioral evaluations, including NOR and Barnes maze tests, two I.C.V. injections of STZ led to induction of SAD –like pathology in adult healthy mice (SAD mice). According to results of NOR, memory impairment in SAD mice was shown in and the percentage of discrimination index was significantly decreased in SAD group as well as novel object exploration percentage that decreased considerably in SAD group compared with Control. According to the NOR results, the time spent for exploring the novel object was decreased significantly in SAD mice. However, the percentage of exploration for novel object in Control mice was about twice the SAD group. In Barnes maze test, the ratio of goal sector to non-goal sector time in the SAD mice was significantly decreased that may imply for impairment of spatial memory and learning in the STZ group. In histology, SAD mice showed increased number of dying neurons in DG and CA1 which confirmed the results of behavioral tests. These finding showed successful development of SAD–like pathology in our experimental setting. In the next step, we tried to mention the effect of metformin on the disease parameter in a condition that the pathology is already established. Therefore, our outcomes have the potential to be extrapolated to the possible effect of metformin in patients which are already diagnosed with AD.

Based on NOR, metformin treatment in a good extent reversed the cognitive deficits. In Barnes maze test, the time spent in GS area was significantly increased by metformin. Same findings were observed in other parameters like ratio of GS/NGS. These findings indicate that metformin administration improves spatial memory in mice. Metformin treatment was reported to protect against STZ-induced impairments in spatial learning and memory [34]. In the trial days, Pilipenko et al. found that rats which received metformin as a protective drug spent 43% more time in the target quadrant and crossed the platform zone twice more in Morris water maze test. Metformin enhanced learning and memory impairment after 14 days administration [35]. Mostafa and colleagues found that administration of 100 mg/kg metformin for two weeks would protect spatial learning and memory in a rat model of scopolamine induced learning/memory impairment [1]. In another study, A previous report investigated that metformin could improve learning and memory dysfunction while applied during the AD developing phase in transgenic mice [1]. Another report administered that 100 mg/kg metformin for two weeks in a rat model of scopolamine-induced learning/memory impairment, and reported spatial learning and memory preservation [36].

In this study, all groups (control, STZ and STZ + MET) had similar searching patterns at the beginning of the training. Random search strategies were used in more than half of the trials and the rest were divided between the direct and serial strategies. However, there was a significant difference between the groups in using the direct and serial strategies. According to Harrison FE et al. reports, it is expected that control mice will be able to find the escape box quickly by using serial and direct search strategies [34]. Learning and memory impairment, as well as cognitive deficits in SAD mice, prevent to find the escape box location as well as remember the signs that used as an escape box guide. These mice are mostly using random search strategy to find the escape box location. However, in metformin-treated mice, because cognitive memory and learning were somewhat improved, most of these mice used the serial search strategy to find the escape box. Preferred strategy for metformin-treated mice was serial after the first trial days. The Lack of direct strategy in metformin-treated mice compared to control mice indicates that metformin was able to improve spatial learning and memory somewhat over a 14-day period of treatment, but this improvement may not be as elaborate as in Control mice. Here, after developing SAD we started the metformin administration for two additional weeks. Therefore, in addition to protection, metformin possessed restoring effect in the context of an established SAD model which imply for the possibility of administrating this FDA approved drug in patients with progressed AD.

We also performed histological studies to know the neuronal cell number in hippocampus areas. The results of the Nissl staining performed in the present study indicated a decline in the number of neurons and alterations in their morphology in hippocampus. According to the results of counting the number of dying neurons of DG, CA1 and CA3 regions of hippocampus in Nissl-stained sections, treatment with metformin was able to reduce the rate of dying neurons a factor that may contribute to the memory restoration following metformin treatment.

Reactive gliosis and neuroinflammation are known as the neural tissue consequences of neurodegenerative disorders and can be monitored through the increased activity of astrocytes and microglial cells. Hippocampal astrogliosis was remarked by increased GFAP staining in CA1 and DG regions. The results of this study demonstrated that GFAP level was significantly increased in STZ group and decreased after administration of metformin. The results come to an agreement with Pilipenko et al. report [37]. In their study GFAP

level was lower in animals treated with 100 mg metformin during the disease progression. Intensity of reactive astrocytes (inflammation) in CA1 was reduced following administration of metformin 100 mg/kg, while at the dose of 75 mg/kg, decreased astrogliosis observed only in CA1 and DG. Oliveira et al. reported significant decreased level of GFAP in diabetic mice that received metformin 200 mg/kg [38]. They also found that astrocyte activity was reversed to the control level. It was suggested that metformin up regulates AMPK expression in glial cells and results in decreased Aβ deposition [37].

Iba-1 protein is a marker of microglia and macrophages. Olivia et al. found higher activation level of microglia in diabetic mice and metformin decreased this activity [37]. In current study, we found that Iba-1 level was significantly higher in CA3 and cortex in STZ group and metformin was effective in reducing the level of this protein reactivity, indicating that inflammation response in STZ group was inhibited by metformin.

Quantifying the NeuN in three different regions of hippocampus and cortex demonstrated that neuronal loss was accrued in SAD mice. Significant decrease in the neuronal count was shown in hippocampus. Treatment with metformin to some extent prevented the neuronal death in different parts of the hippocampus and cortex or restored it. In CA3 of treatment group, significant increase observed in the number of neurons compared to STZ group.

To reduce the number of animals used in this study, same samples were used for both immunofluorescence and Nissl staining. Using frozen sections reduced the quality of images in Nissl staining in some extents. As another limiting point, the high density of neurons in DG, may have been caused underestimation of dying neurons count, especially in STZ group. This underestimation may not interfere with our interpretation on the effects of metformin.

5. Conclusion

Our results demonstrated the potential neuroprotective effects of metformin in SAD mice as mentioned by restoring the cognitive performance and NeuN positive cells. Additionally, metformin decreased the neuroinflammation in the hippocampal region of the brain as well as the gliosis and dying neurons. While the previous reports on the effects of metformin on preclinical AD animal models mainly reported the neuroprotection during the disease settlement, our data showed that metformin had both protective and restorative effects on established AD. Relevant clinical trials seems reasonable to check its possible administration in AD management.

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Production notes

Author contribution statement

Saghar Rabieipoor: Conceived and designed the experiments; Performed the experiments; Wrote the paper.
Meysam Zare: Performed the experiments; Analyzed and interpreted the data.
Miren Ettcheto: Analyzed and interpreted the data; Wrote the paper.
Antoni Camins; Mohammad Javan: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Antoni Camins reports financial support was provided by Center for Networked Biomedical Research on Neurodegenerative Diseases. Antoni Camins reports financial support was provided by European Regional Development Fund.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e17873.

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