The effects of chronic metformin treatment on behaviors and pathology of a transgenic Alzheimer's model mouse

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The effects of chronic metformin treatment on behaviors and pathology of a transgenic Alzheimer's model mouse

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ABSTRACT

The effects of chronic metformin treatment on behaviors and pathology of a transgenic Alzheimer's model mouse

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Purpose: Alzheimer's disease (AD) is the most common neurodegenerative disease with multidimensional cognitive impairment. Metformin, the first-line medication for type 2 diabetes mellitus (T2DM), ameliorates metabolic dysfunctions, such as hyperglycemia and insulin resistance, by activating AMP-activated protein kinase (AMPK), a key energy regulator. As numbers of research have shown that T2DM is highly associated with AD progression, metformin has been considered a promising AD treatment. However, although many studies have been conducted to assess its effects on AD pathology, the results are still conflicting. Especially, some studies have shown that metformin exacerbated cognitive decline and AD pathology. If these findings come out as a fact, this might hinder metformin usage in the patients with AD and drug repositioning research of metformin from T2DM to AD. Therefore, this study evaluated the safety and potential of metformin as AD therapy by assessing the effects of metformin on behaviors and AD pathology.

Method: Male (n = 26) and female (n = 12) 3xTg-AD mice were used as

transgenic AD model mice. 2 mg/ml of metformin was orally treated to mice since 3-month-old. A touchscreen operant system was conducted as a cognition-behavior assessment to accurately evaluate the effects of chronic metformin treatment on diverse domains of cognitive functions impaired in AD of male mice with food restriction throughout the age since 4-month-old. The effects of chronic metformin treatment on AD pathologies, such as amyloid-beta (Aβ) and tau pathogenesis, and AMPK regulation in the hippocampus of female mice without food restriction were measured by western blot analysis and immunohistochemistry.

Results: Chronic metformin treatment impaired learning and memory consolidation of 3xTg-AD mice. It worsened attention retrieval, object-location associative learning and memory, and long-term memory without altering the appetite and whole-body energy metabolism regulation. Metformin worked as an AMPKα-subunit regulator in 3xTg-AD mice by upregulating AMPKα1-subunit expression under long-term administration. Chronic metformin treatment also impaired AD pathology in 3xTg-AD mice. It exacerbated Aβ and tau pathogenesis by increasing Aβ oligomers levels, Aβ plaques number and aggregation, phosphorylated tau (p-tau) Ser356 and p-tauThr231 levels, and GSK3β expression.

Conclusion: Chronic metformin treatment had detrimental effects on behaviors and AD pathology by upregulating AMPKα-subunit isoform expression in 3xTg-AD mice. Taken together, the long-term prescription of metformin should be reconsidered for familiar AD gene-containing people. Furthermore, metformin might not be a promising candidate for AD treatment.

Keywords: Alzheimer's disease, metformin, cognitive functions, touchscreen operant system, Aβ, p-tau, AMPK

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I. INTRODUCTION

1. Alzheimer's disease (AD)

Alzheimer's disease (AD) is one of the most prevalent neurodegenerative diseases, accounting for 60-80% of dementia.[1] Its main pathological hallmarks are extracellular amyloid-beta (Aβ) plaques and intracellular neurofibrillary tangles (NFTs) with hyperphosphorylated tau.[2] Increased inflammation responses are also one of AD pathology which can exacerbate Aβ and tau pathogenesis. These pathogeneses interfere with proper neuron-to-neuron communication through synapse reduction and dysfunction.[3] Ultimately, impairments of multidimensional cognitive functions, such as attention, episodic memory, and executive memory, occur in AD.[4]

There are two types of AD: early-onset Alzheimer's disease (EOAD) and

late-onset Alzheimer's disease (LOAD).[5] EOAD, also known as familiar AD, mostly appears between the 30s and mid-60s and only consists of less than 10% of total AD incidence. LOAD, also called sporadic AD, mainly occurs older than 65 and represents about 90% of total AD incidence. Different factors cause EOAD and LOAD. EOAD occurs by gene mutation, while LOAD is affected by the *APOE* ε4 allele and environmental factors. There are differences between EOAD and LOAD in cognitive functions, psychological symptoms, and other features.[6] As AD progression in EOAD is more aggressive than in LOAD, memory decline is faster in EOAD patients.[7] EOAD patients show poorer cognitive functions, such as executive functions and visuo-constructional abilities, than LOAD patients. However, semantic memory was more impaired in LOAD. Behavioral and psychological symptoms, such as delusion and hallucination, occur less frequently in EOAD than in LOAD.[6] AD can also be subdivided into two different types: typical AD and atypical AD.[7] Typical AD is common in LOAD. In contrast, atypical AD is prevalent in EOAD. Therefore, hippocampal atrophy, a symptom of typical AD, appears more often in LOAD, whereas temporoparietal atrophy and frontoparietal atrophy, symptoms of atypical AD, are more prevalent in EOAD.

A. AD risk factors

Amyloid precursor protein (APP), *PSEN1 (presenilin 1)*, and *PSEN2 (presenilin 2)* are inherited genetic mutations of familiar AD.[8] PSEN1 and PSEN2 are the major components of the γ-secretase complex and mediate the cleavage of APP to produce Aβ. Diverse mutations of APP, PSEN1, and PSEN2 lead to an excessive increase of Aβ. Allelic variation of *APOE* is the most important genetic risk factor of sporadic and late-onset familial AD.[9,10] *APOE* ε2, the least common allele, decreases the risk of AD. *APOE* ε3, the most

common allele, does not affect the risk of AD. *APOE* ε4 allele is highly involved in AD.[11] As the number of *APOE* ε4 alleles increases, it increases the risk of AD and reduces the onset age of AD. One *APOE* ε4 allele increases AD risk by 2-3 fold. Two *APOE* ε4 alleles increase AD risk by 15-17 fold and accelerate AD development up to 10 years earlier than the *APOE* ε4 allele non-carrier.[7] *APOE* is found in Aβ plaque and modulates the deposition and clearance of Aβ.[12] Therefore, *APOE* ε4 allele levels exacerbate AD pathologies, such as Aβ accumulation and cognitive decline.[13] Compared to *APOE* ε3, *APOE* ε4 decreases Aβ clearance and increases Aβ deposition.

Gender is also a risk factor for AD as the incidence of AD is different between the sexes.[14] In females, the incidence of AD was higher, and the cognitive decline was more severe than in males.[15] The prevalence of AD is higher in females due to the concentration differences of sex steroid hormones. For example, estrogens and androgens regulate key processes in AD pathogenesis, especially the accumulation of Aβ.[16] Not only genetically non-modifiable factors, but several modifiable factors, such as aging, diabetes mellitus, obesity, level of education, and environmental exposures, can increase the incidence of AD.

B. Amyloid-beta (Aβ)

(A) Aβ synthesis

Aβ has a crucial role in AD pathology. It is derived from APP through cleavage. There are two types of cleavage: α-cleavage and β-cleavage.[17] α-cleavage, a non-amyloidogenic pathway, prevents the synthesis of Aβ. APP is divided into sAPPα and C-terminal APP fragment α (CTFα) through α-secretase. CTFα is cleaved by γ-secretase and divided into p3 and APP intracellular domain

(AICD). β-cleavage, an amyloidogenic pathway, synthesizes Aβ and impairs neuronal survival. sAPPβ and CTFβ are released from APP cleaved by β-secretase. Aβ and AICD are generated from CTFβ by γ-secretase. Therefore, Aβ with 36 to 43 amino acids is synthesized. Further cleavage via γ-secretase produces $A\beta_{40}$ and $A\beta_{42}$, the main final forms of Aβ in AD. $A\beta_{40}$ (80-90%) is more abundant than $A\beta_{42}$ (5-10%) in the brain.[18]

(B) Aβ aggregation

Aβ, especially Aβ42, is prone to aggregate into oligomers, fibrils, and plaques owing to its hydrophobic and fibrillogenic sequence.[19,20] Aβ monomers are secreted to the extracellular space and self-aggregates into diverse forms. Soluble Aβ oligomer is one of the assembles of Aβ monomers with 2 to 6 peptides. This form of Aβ is the most neurotoxic as it triggers synaptic dysfunction and cognitive decline in AD.[21] It spreads throughout the brain and binds to diverse receptors, such as p75 neurotrophin receptors, metabotropic glutamate receptors, and N-methyl-D-aspartic acid receptors (NMDARs).[22] The binding of Aβ oligomers to receptors induces oxidative stress, mitochondrial dysfunction, inflammatory responses, and tau hyperphosphorylation. Insoluble Aβ fibrils are the assembles of Aβ oligomers and the components of Aβ plaques.

(C) Aβ plaques

There are four types of plaques: diffuse plaque, primitive plaque, classic dense core plaque, and compact plaque.[23] Diffuse, primitive, dense-cored, and compact plaques can progress in sequence following progressive Aβ fibrillation processes, and each plaque has different components. Diffuse plaques are the

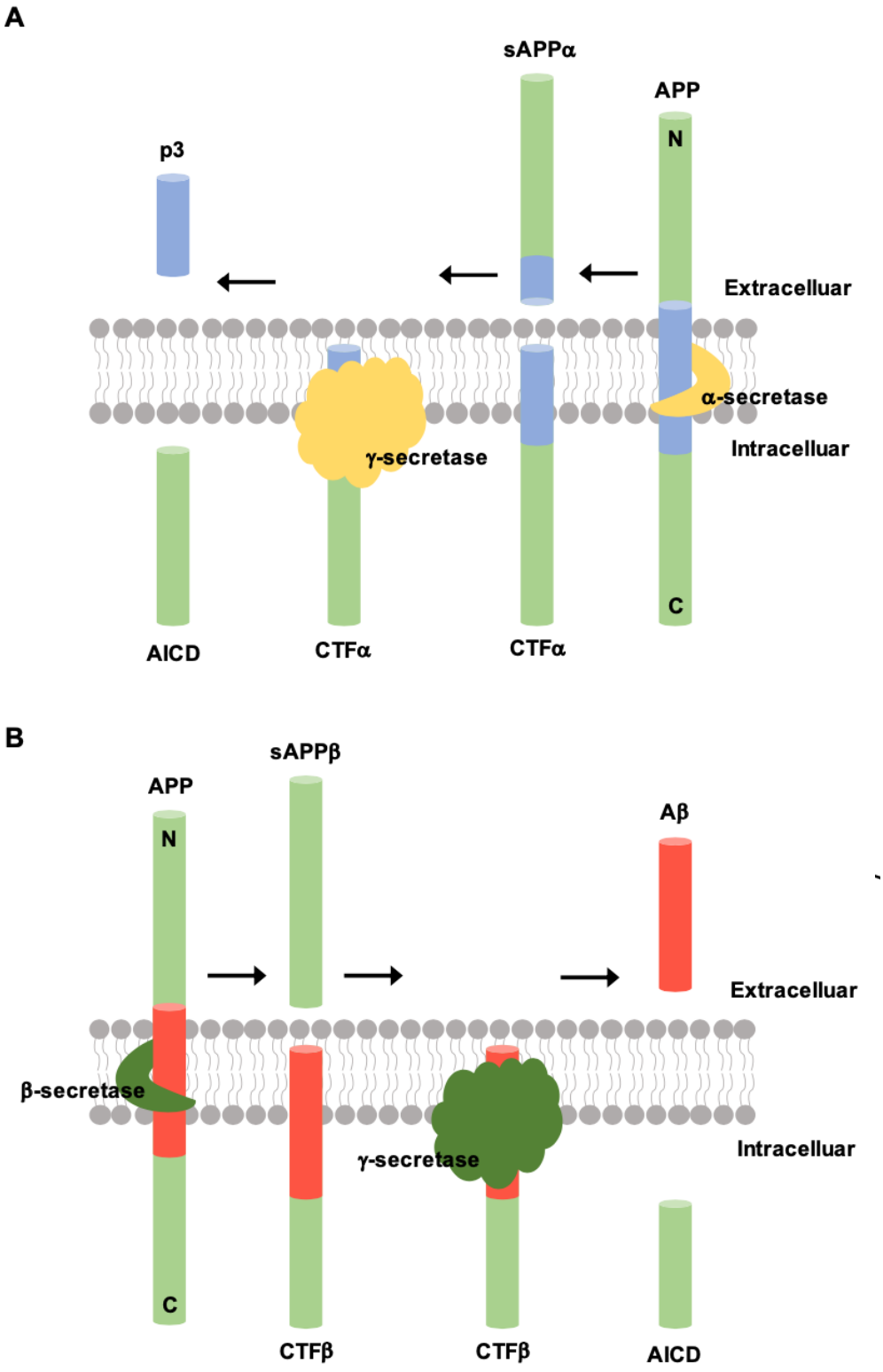
most prevalent plaques type in AD and are irregularly shaped with diffuse boundaries lightly stained with Aβ peptides antibodies.[24]

In diffuse plaques, non-fibrillar and non-aggregated Aβ, such as Aβ oligomers and early cleavage of APP including $A\beta_{42/43}$, are found.[25] Diffuse plaques do not mostly possess fibrillar Aβ and dystrophic neurites containing PHF. They are not highly associated with inflammatory cells, such as activated microglia and reactive astrocytes. Moreover, neuronal cell bodies have a close relationship with diffuse plaques.[26] One or more neurons are found in the plaques. The number of neurons is positively correlated to the plaque diameter, the mass of Aβ, and the distance to which Aβ has diffused within the parenchyma.[23] Neurons seeded with Aβ deposits rapidly attached to $A\beta_{40}$ or $A\beta_{42}$ and survived as long as neurons with the control substrate.[27] Therefore, diffuse plaques are not toxic enough to cause synaptic abnormalities or neuronal degeneration.[28] Diffuse plaques are deposited during the early phase of AD and observed in the neocortex, cerebellum, presubiculum, basal ganglia, and brain stem.[29]

Primitive plaques have more symmetrical shapes and are stained darker than diffuse plaques.[30] In primitive plaques, Aβ aggregates into fibrillar form, β-pleated sheet configuration of amyloid, and clusters of dystrophic neurites without distinct amyloid core observed. Inflammatory cells are more associated with primitive plaques than diffuse plaques. Primitive plaques are subdivided into two types: globular and curly plaques.[31] Globular dystrophic neurites containing C-terminal APP are found in globular plaques. Elongated dystrophic neurites containing PHF and N-terminal APP are found in curly plaques. Both globular and curly plaques are observed in AD brains, whereas normal brains only have curly plaques. However, neurons are mostly absent in primitive

plaques. Primitive plaques are deposited in the neocortex and CA1 and DG regions of the hippocampus.

Dense-cored plaques have a distinct central core surrounded by a ring structure with dystrophic neurites.[24] The dense amyloid core consists of condensed fibrillar $A\beta_{42/43,}$ which associates with $A\beta_{40}$ and one or more neurons,[32] while the ring structure has non-Aβ domains of APP and neurites with neurofilaments and PHF.[33] In dense-cored plaques, inflammatory cells are clustered, and astrocytic processes occur. Dense-cored plaques are insoluble as they contain the nuclear and cytoplasmic components resistant to proteolysis by lysosomes released during neuronal cell lysis.[30] Therefore, they are toxic and may cause synaptic dysfunction and loss. Unlike previous studies, a recent study showed that dense-cored plaques might have a protective role by isolating toxic Aβ.[34] Microglia phagocytosis by the TAM (Tyro3, Axl, and Mer) system inhibits the growth of plaques. Microglia detect and engulf Aβ plaques and increase dense-cored plaques. Dense-cored plaques are found in the neocortex and hippocampus. Compact plaques are the most aggregated form among the plaques and have an amyloid core without a ring and inflammatory cells.[24] They are deposited in the neocortex and hippocampus.[23] Still, not much information has been found about compact plaques.



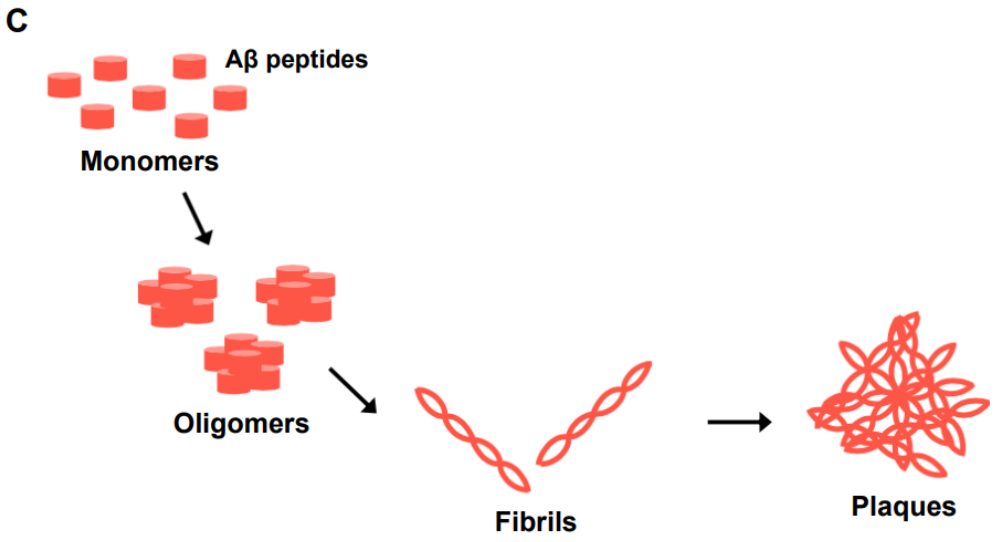


Figure 1. Aβ pathogenesis in AD. (A) Non-amyloidogenic α-cleavage pathway, (B) amyloidogenic β-cleavage pathway, and (C) Aβ peptide aggregation pathway in AD pathology. p3, a non-toxic APP fragment, is synthesized by α-secretase and γ-secretase in the α-cleavage pathway. Aβ, neurotoxic APP fragment, is synthesized by β-secretase and γ-secretase in the β-cleavage pathway and secreted into the extracellular space. Aβ monomers aggregate and become oligomers, fibrils, and plaques in order. APP, amyloid precursor protein; CTF, C-terminal fragment; Aβ, amyloid-beta; AICD, APP intracellular domain.

C. Tau

Tau is one of the microtubule-associated proteins (MAPs) that stabilize neuronal microtubules for cell processes development, cell polarity establishment, and intracellular transport and are most abundant in axons.[35] There are four major domains in tau: an amino-terminal projection domain, a proline-rich domain, microtubule-binding (MTB) repeats, and a carboxy-terminal domain of short tail sequence.[36] Through alternative splicing of exons

2, 3, and 10, six different tau isoforms are synthesized: 0N3R tau (tau-352), 0N4R tau (tau-383), 1N3R tau (tau-381), 1N4R tau (tau-412), 2N3R tau (tau-410), and 2N4R tau (tau-441).[37] As exon 10 has an additional MTB repeat, 3 or 4 MTB repeats are presented in 3R or 4R tau isoforms, respectively.

(A) Tau phosphorylation

Tau has 85 putative phosphorylation sites, 45 with serine, 35 with threonine, and 5 with tyrosine in the longest human tau isoform composed of 441 amino acids.[38] The mixtures of 3R and 4R tau isoforms are found and hyperphosphorylated in AD. The abnormal tau phosphorylation is abundant at Ser-Pro or Thr-Pro motifs.[35] Therefore, hyperphosphorylation of Ser202, Ser 262, Ser356, Ser396/404, Ser422, Thr205, and Thr231 sites are found in AD.[39] Phosphorylation of Ser199, Ser202/Thr205, and Ser262 is associated with early tau pathogenesis as they are discovered in pre-tangles in the neuronal processes. However, Ser396/404 phosphorylation is prominent in later tau pathogenesis as found in NFTs. Ser202 and Thr205 phosphorylation generate precise patterns that lead to aggregation and disruption in the turn-like structure of tau in AD. Thr231 phosphorylation triggers prolyl-isomerization which dissociates tau from microtubules and facilitates tau aggregation. Ser262 and Ser356 sites are located at KXGS motifs, and their phosphorylation causes tau detachment from the microtubule. The phosphorylation of Ser396/404 generates more fibrillogenic tau, decreasing tau binding to microtubules and increasing tau-to-tau interaction. Eventually, this event generates tau oligomers, tau filaments, and NFTs in order. Ser422 phosphorylation might decrease Aβ-mediated tau solubility and increase the generation of paired helical filament (PHF)-like filaments. It also prevents tau from caspase-3 cleavage and induces the conformation change of tau and tau polymerization.

Hyperphosphorylation of tau causes microtubule breakdown. Post-translational modification triggers the aggregation of hyperphosphorylated tau, known as PHFs, in cell bodies and dendrites. The aggregates destroy intracellular transport and neurons and ultimately trigger cognitive impairments in AD.[40]

(B) Tau kinase

Tau kinases have crucial roles in microtubule stability, axonal transport, and protein aggregation by phosphorylating different sites of tau.[41] They are divided into three main groups: proline-directed protein kinases (PDPK), non-PDPK, and tyrosine protein kinases (TPK). Glycogen synthase kinase 3 (GSK3), cyclin-dependent protein kinase-5 (Cdk5), and MAP kinases, such as p38, extracellular signal-regulated kinase 1 and 2 (Erk1/2), and c-Jun N-terminal kinases (JNK) are PDPK. Casein kinase 1 and 2 (CK1/2), microtubule affinity-regulating kinases (MARK), protein kinase A (PKA), and protein kinase C (PKC) are non-PDPK. Src family kinase (SFK) members, lymphocyte-specific protein tyrosine kinase (Lck), and spleen tyrosine kinase (Syk) are TPK.

GSK3, CK1/2, and PKA are the essential tau kinases, as they phosphorylate large numbers of sites which highly related to AD pathology (e.g., Ser202, Ser262, Ser356, Ser422, Thr205, and Thr231) and colocalize with NFT.[40] GSK3β and Cdk5 affect tau-microtubule interaction by reducing the microtubule affinity of tau. GSK3β activity is increased in AD brains and triggers tauopathy, especially promoting phosphorylation at the Ser396 site.[42] Its overexpression induces tau hyperphosphorylation, which causes apoptotic cell death and neurodegeneration.[43] GSK3β also exacerbates tau pathology through the response of Aβ.[44] Cdk5/p25 complex, the active form of Cdk5, is hyperactivated

in AD brains and increases tau hyperphosphorylation, NFT accumulation, and neurodegeneration.[45] Tau phosphorylation by Cdk5 makes tau a better substrate for GSK3β and promotes excessive tau phosphorylation.[46]

CK1 and CK2 regulate microtubule dynamics through tau phosphorylation and are involved in apoptosis and survival pathways.[47,48] MARK phosphorylates KXGS motifs sites (Ser262 and Ser356) which cause microtubule disruption by tau dissociation from microtubules[49] and tau aggregates in AD.[50] It also regulates microtubule stability, differentiation processes, neuritic growth, cell division, and organelle trafficking.[51] PKA is a pro-apoptotic kinase and makes phosphorylated tau a better substrate for GSK3β.[52]

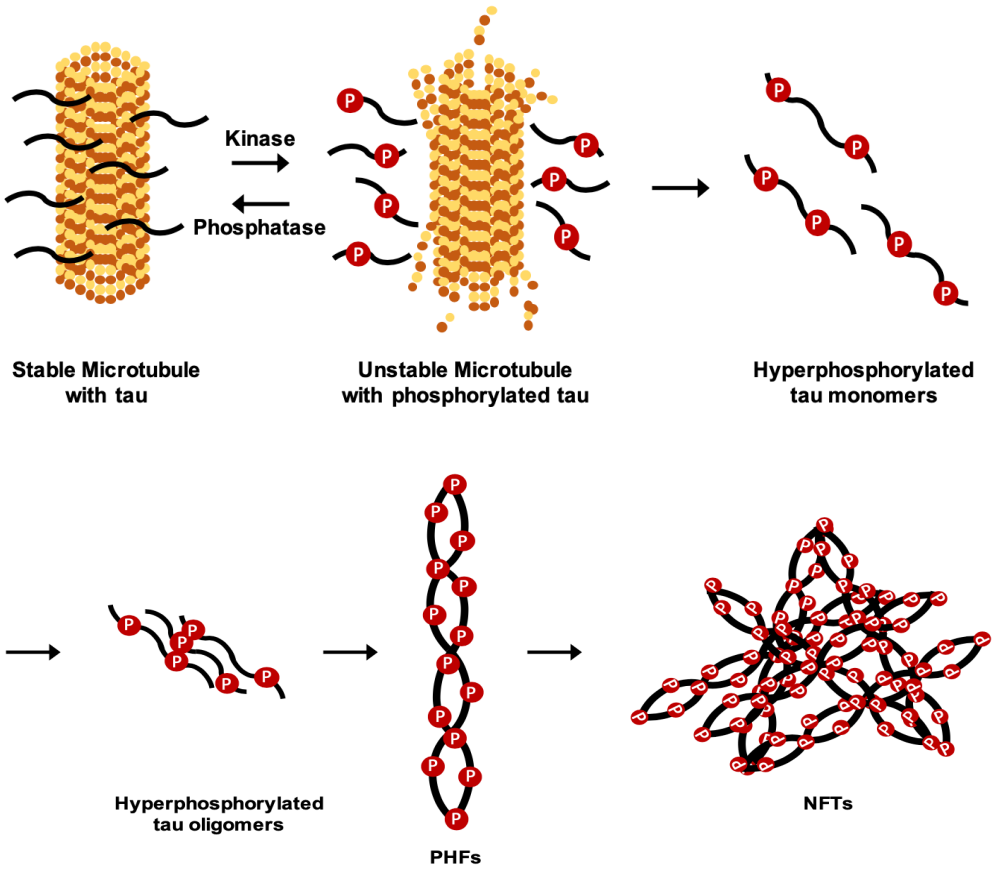


Figure 2. Tau pathogenesis in AD. Tau originally regulates the stabilization of the microtubule. Through tau phosphorylation, the microtubule is depolymerized, and its structure is collapsed. Hyperphosphorylated tau monomers aggregate and become oligomers, PHFs, and NFTs, in order. PHFs, paired helical filaments; NFTs, neurofibrillary tangles.

D. Inflammation

The presence of Aβ plaques and NFTs activates inflammatory cells, such as microglia, astrocytes, macrophages, and lymphocytes.[53] The activation of inflammatory cells alters the levels of pro- and anti-inflammatory mediators, such as cytokines, chemokines, neurotransmitters, and reactive oxygen species (ROS). Thus, through excessive inflammatory responses, the progression of AD is accelerated.[54]

(A) Microglia

Microglia, one of the neuroglia in the brain and spinal cord, work as resident phagocytes to support and protect overall brain functions. Thus, they are the first and foremost form of active immune defense in the central nervous system (CNS).[55] In addition, microglia have critical roles in the cellular responses to pathological lesions, such as Aβ and neuritic plaques.[56]

Activated microglia under Aβ presence have neuroprotective effects, such as Aβ clearance through phagocytic (fibrillar Aβ) and scavenger (soluble Aβ) functions.[57] TREM2 regulates microglial chemotaxis, phagocytosis, survival, and proliferation. It binds to *APOE* and *clusterin*, AD risk genes, and regulates fibrillar Aβ clearance.[58] The mutations of TREM2 alter the interaction with its lipoprotein ligands (e.g., APOE, LDL, and clusterin) and Aβ removal. Toll-like receptor-2 (TLR2) and its coreceptor CD14 are also involved in fibrillar Aβ uptake via microglia.[59] Microglial fractalkine receptor (CX3CR1) inhibits microglial inflammatory responses. The deficiency of Cx3CR1 activates microglia and increases Aβ clearance in AD mouse models.[60] Scavenger receptor-A and lysosomal cathepsin B are critical in microglial soluble Aβ

clearance.[61] Macrophage scavenger receptor type I (SCARA I) is also involved in clearing soluble Aβ in myeloid cells both *in vitro* and *in vivo*.[62] However, a recent study showed a novel mechanism of soluble Aβ degradation through microglia. Microglia partially degrade synthetic or physiological soluble Aβ via the secretion of the insulin-degrading enzyme, not by uptaking soluble Aβ.[63] However, decreased expressions of Aβ-binding receptors and Aβ-degrading enzymes suppress the phagocytosis of microglia in AD and deposit Aβ.[64]

Prolonged activation of microglia can exacerbate AD pathology. Activated microglia attach to Aβ fibril-coated surfaces via their scavenger receptors and release ROS.[65] Activation of microglia increases the expression of MHC II and triggers neuroinflammation by increasing the secretion of cytokines and chemokines, such as IL-1, IL-6, and TNF-α.[66] The clusters of activated microglia are attracted by Aβ and are located at the sites of aggregated Aβ deposition and dense-cored plaques.[67] Inflammatory responses, triggered by microglia, have critical roles in the early stages of Aβ plaques formation and Aβ fibrils synthesis and accompany neurotoxic effects.[68]

Tau is also regulated by microglia. Tau is taken up by microglia *in vivo* and colocalized with activated microglia in AD brain.[69] This finding suggests that microglia have potential roles in tau internalization and extracellular tau clearance. However, others found that tau pathology is exacerbated by microglia activation. Microglia activation upon CX3CR1 deficiency increases tau phosphorylation and aggregation and impairs cognitive functions, even though it had beneficial effects on Aβ clearance.[70] Reactive microglia are also correlated with the spread of tau in the brain.[70] This pathogenesis is regulated by TLR4, IL-1 receptors, and p38 MAPK.[71] Therefore, microglia-induced neuroinflammation can accelerate neurodegeneration via tauopathy.

(B) Astrocyte

Astrocytes, star-shaped neuroglia in the brain and spinal cord, have diverse functions, such as supplying nutrients to nervous tissue, maintaining extracellular ion balance, and healing the brain and spinal cord following traumatic injuries. As astrocytes are the metabolic regulator in CNS, metabolic dysfunction in astrocytes increases AD incidence.[72] Disrupted brain homeostasis triggers increased Aβ production and decreased Aβ clearance. Even though astrocytes can degrade Aβ in the normal brain,[73] their tendency to release proinflammatory molecules may accelerate AD progression.[53] Astrocyte clusters are located at Aβ deposit sites which release chemotactic molecules and induce astrocyte recruitment.[53] Aβ can activate astrocytes or disrupt astrocyte functions and trigger neuroinflammation by increasing cytokine secretion and chemokines, such as IL-1, IL-6, and S100.[74] The chemokines, cytokines, and ROS released from activated astrocytes gather microglia and block Aβ clearance.[75] Through this, neuronal damage and AD pathology are exacerbated. Astrocytes contribute to the synthesis of Aβ plaques as astrocytes accumulate neuron-derived Aβ, and this accumulated Aβ is released from lysed astrocytes.[76] Reactive astrocytes facilitate APP processing and Aβ synthesis and secretion.[77] Like microglia, astrocytes can also be activated by senile plaques.[78] They also interact with Aβ plaques and cause neural network disruption and neurotransmitter imbalance, resulting in cognitive impairments.[79]

Tau pathology is also exacerbated by metabolic dysfunction in astrocytes.[80] Disrupted glutamate homeostasis is associated with AD development and cognitive impairment.[81] Excitatory amino acid transporter 2 (EAAT2; GLT1), an astrocytic glutamate transporter, regulates glutamate uptake in the synaptic cleft. In AD patients, GLT1 expression was decreased, and neurons were

hyperactivated.[82] Decreased activity of GLT1 is closely linked to tauopathy as well as Aβ pathology and cognitive decline. Zumkehr et al. (2015) have found that upregulation of GLT1 could ameliorate tau accumulation and cognitive decline in the AD mouse model.[83] Moreover, Kilian et al. (2017) have shown that deletion of GLT1 amplified neuronal excitatory activity and exacerbated tau-induced neuronal toxicity and behavioral deficits.[84] Tau nitration, found in AD brain, suppresses tau binding ability to microtubule and increases its aggregation.[85] Nitration at Tyr18, one of the tau nitration sites, of tau (Tau-nY18) might be associated with astrocyte activation, as Tau-nY18 was colocalized with activated astrocyte, which interacted with Aβ plaques or NFTs.[86] Astrocytes also increased tau phosphorylation and neurotoxicity in response to Aβ-induced increase of caspase-3 activation.[87]

Reduction in astroglial volume, surface area, and morphological complexity is found in AD transgenic mice models.[79] Morphological atrophy of astrocytes indicates disrupted astroglial homeostasis and reduced astroglial coverage of neurons and synapses.[88] Therefore, astrodegeneration is linked to impaired synaptic connectivity, imbalanced neurotransmitter homeostasis, and neuronal death through increased excitotoxicity.

Ca^{2+} concentration is increased in astrocytes in response to receptor agonists (e.g., glutamate and GABA) or release of Ca^{2+} or inositol-1,4,5-triphosphate (IP_3).[89] Ca^{2+} signaling in astrocytes is important to neuronal information processing as astrocytes releases gliotransmitter (e.g., glutamate, GABA, and ATP) to interact with neurons. It also modulates energy supply to the brain via i) releasing messengers [e.g., 20-hydroxyeicosatetraenoic acid (20-HETE), epoxyeicosatrienoic acids (EETs), and prostaglandin] to vascular smooth muscles, ii) increasing expression of GLUTs into the cell membrane, and iii)

regulating oxygen supply to produce ATP. As astrocytes can regulate neuronal spiking, synaptic plasticity, and brain blood flow, abnormal Ca^{2+} signaling in astrocytes will lead to neural dysfunction and disrupted brain metabolism in AD.[90]

(C) Neuron

Neurons are traditionally known as passive bystanders of neuro-inflammation. However, neurons also induce neuroinflammation by secreting cytokines and chemokines, such as IL-1, IL-6, and TNF-α.[66] Chemokines released from neurons act as messengers between neurons and neuroglia. Moreover, mRNA of classical pathway complement protein expression is increased in the neurons of AD. Through these processes, more severe neuroinflammation is triggered, which increases neuronal damage and exacerbates AD pathology.

(D) Complement system

The complement system is a part of the immune system. It enhances the proteolytic cascades to lyse pathogens or damaged cells from the organism by promoting inflammation. Most inflammatory reactions in AD proceed via the classic complement pathway.[91] Complement protein overexpressed in AD, and activated complement fragments, such as C4d and C3d, are observed in tangles and plaques. Unlike traditional notions, the robust activation of complement protein in AD may occur without the presence of antibodies. Aggregated Aβ and senile plaques are the activators of complement protein. Pentraxins, amyloid P- and C-reactive proteins, are involved in acute immunological responses and activate the complement system in AD. Autolytic attack in dystrophic neurites

of AD brain caused by complement proteins precipitates much of the neurite loss.[91]

E. Proteolytic degradation dysfunction

AD shows abnormalities in not only protein synthesis but also protein degradation. The imbalance between the production and clearance of AD-related proteins (e.g., Aβ and tau) causes toxic form accumulation of each protein in the brain.[92] Endosomal-lysosomal network (ELN), autophagy, and ubiquitin-proteasome system (UPS) are involved in neuronal functions and cognition.[93-95]

(A) Endosomal-lysosomal network (ELN)

Endosomes are membrane-bound cytosolic vesicles that sort intracellular compartments in eukaryotic cells. Lysosomes are acidic organelles that facilitate the digestion of proteins, organelles, and extracellular debris through the action of acid hydrolases. Endosomes are part of the endocytic membrane transport pathway originating from the trans-Golgi network. Molecules or ligands transported to endosomes from the trans-Golgi network, which internalize from the plasma membrane, can be merged to lysosomes for degradation or be recycled back to the cell membrane or Golgi apparatus in the endocytic cycle. Aβ and CTFβ are synthesized through β-cleavage mainly in the endosome and generally degraded in the lysosome.[96] However, Aβ and CTFβ accumulated abnormally in endosomal-lysosomal network (ELN) compartments of AD.[92] CTFβ overactivates Rab5, one of the small GTPase that regulates the maturation of endosomes, and causes endosome morphological abnormalities (e.g., accelerated endocytosis and impaired transport of enlarged endosomes), which occur during the early stage of AD.[97] The assembly of CTFβ and oxidized

substrates, such as Aβ, progressively impairs lysosome function and causes autophagy failure.[98]

Dysfunctions of endosome and lysosome acidification in AD are caused by gene mutations. *APOE* ε4, the risk factor of AD, is associated with endosome function. It accelerates endosome dysfunction and hinders exosome release.[99] These trigger lysosomal expansion and membrane permeabilization. Overexpression of *APOE* ε4 increases the amount of Aβ42 in the lysosome and releases lysosomal enzymes via lysosomal membrane disruption. Ultimately, neuronal degeneration is led.[100] *PSEN1* is crucial for lysosome acidification by regulating vacuolar ATPase distribution.[101] Lysosome pH dysregulation initiates functional derangements in proteostasis, synaptic plasticity, neurotransmitter exocytic release, and synaptic vesicle fusion and recycling.[102] As lysosome has a crucial role in the autophagic pathway, the mutations of *APOE* ε4 and *PSEN1* are also associated with autophagic dysfunction in AD.

(B) Autophagy

Autophagy is a self-digesting mechanism responsible for removing long-lived proteins, damaged organelles, and malformed proteins during biosynthesis by the lysosome. Autophagy is subdivided into macroautophagy, microautophagy, and chaperone-mediated autophagy. Among them, macroautophagy is the most relevant to AD.[103] Autophagy can regulate proteostasis, homeostatic cell signaling, phagocytosis, innate immunity, synaptic function, and neuronal survival in AD.[104] Under nutrient and metabolic stress, macroautophagy, the major autophagic pathway, is neuroprotective, as it is induced to recycle non-essential substrates for energy or to degrade aggregated proteins and damaged organelles.[105] However, when autophagy is induced

continuously by high rates of oxidized or damaged constituents, such as Aβ, lysosomes are overburdened, and a substantial accumulation of undigested substrates is found in the neuron.[106] Therefore, autophagic dysfunction in AD is mainly driven by lysosomal proteolytic failure, resulting in the intraneuronal accumulation of autophagic vacuoles (AVs) in the brain.

Autophagy has an essential role in Aβ regulation: Aβ degradation, production, and secretion.[103] Autophagy can degrade APP and its cleavage products, such as Aβ and CTFs.[107] Aβ is generated from AVs during abnormal macroautophagy in AD.[108] Aβ secretion was reduced in autophagy-deficient mice. In contrast, the restoration of autophagy enhanced Aβ secretion to normal levels.[109] Intracellular accumulation of Aβ was increased through the inhibition of Aβ secretion in autophagy-related gene-7 (ATG-7) deficient mice.[110] Even though tau is mainly regulated by UPS, autophagy can also affect tau pathology.[103] Dysfunction of autophagy-lysosome system promoted tau oligomers and insoluble aggregates formation.[111] In P301S mice, tauopathy model mice, autophagy induction decreased insoluble and phosphorylated tau and increased neuronal cell survival which correlated to tau alteration.[112] However, inhibition of autophagy through mTOR activation increased tau phosphorylation in the same model.[113]

Abnormal axonal transport causes disrupted neuronal homeostasis and axon degeneration and occurs in both the early and late phases of AD. As newly synthesized autophagosomes move along with microtubule tracks, impaired axonal transport triggers the accumulation of AVs in neurons. Disrupted axonal transport of autophagy-related compartments results in neuritic dystrophy associated with AD.[114] Additionally, phosphorylated tau and abnormal protein

aggregates can affect axonal transport and degradation by blocking the fusion of the autophagosome with lysosomes.

(C) Ubiquitin-proteasome system (UPS)

Ubiquitin-proteasome system (UPS) is a major intracellular protein degradation system. Ubiquitins are transferred and tagged to specific proteins by multiple enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3). Proteins with chains of four or more ubiquitins are recognized and degraded by the 26S proteasome. In the AD brain, ubiquitin is accumulated in both plaques and tangles, and proteasome activity is decreased by the oxidized proteins accumulation and proteasome subunit composition changes.[115] Aβ is considered an endogenous inhibitor of the proteasome.[116] $A\beta_{40}$ can directly bind to the inside of the proteasome and inhibits the chymotrypsin-like activity of the 20S proteasome. $A\beta_{42}$ also inhibits proteasome function. Not only Aβ monomers but also $A\beta_{40}$ and $A\beta_{42}$ oligomers affect proteasome activity. [117] They decrease the trypsin-, chymotrypsin-, and peptidylglutamyl-like activity of the proteasome. Conversely, as proteasome degrades Aβ, the age-dependent proteasome dysfunction might result in the accumulation of Aβ in AD brains.[118] Not only Aβ but also tau aggregates decrease proteasome activity. Moreover, as proteasome regulates the turnover and degradation of tau, the dysfunction of UPS initiates tau accumulation.[116] Aβ and tau do not solely work with UPS. Aβ initially impairs proteasome function and triggers tau accumulation.[119] Therefore, the proteasome is a molecular link between Aβ and tau pathology.

Several proteins, such as ubiquitin B (UbB), carboxy terminus of heat shock protein 70-interacting protein (CHIP), and ubiquitin carboxy-terminal hydrolase

L1 (UCH-L1), can regulate the UPS to degrade tau.[120] These proteins are altered in AD and result in the malfunction of UPS and accumulation of tau. UBB^{+1} is a Ub mutant protein that originated from the misreading of the *UbB* gene accumulated in NFTs, neuropil threads, and dystrophic neurites of AD brains.[121] UBB^{+1} inhibits proteasome activity and triggers neurodegeneration. CHIP, the E3 ligase, controls aggresome solubility. It ubiquitinates explicitly phosphorylated tau and promotes its proteasomal degradation.[122] However, the overexpression of CHIP increases tau accumulation by blocking proteasome activity.[123] UCH-L1 has crucial roles in abnormal protein clearance and de-ubiquitination control. It is downregulated in AD and associated with memory functions.[124]

F. Synaptic abnormalities

Synaptic abnormality is also one of the main features of AD. It occurs since the early stage of AD.[125] Both Aβ and tau are involved in the synaptic loss.[126] Synaptic loss is closely linked to neuronal loss, dendritic loss, and tangle formation. Therefore, cognitive functions in AD patients are highly correlated to synaptic loss.[125] As the number of synapse and synaptic markers was reduced in plaques surrounded by soluble oligomeric Aβ, soluble forms of Aβ have more detrimental effects on synaptic functions than aggregated forms.[127] Aβ oligomers impair excitatory synaptic transmission, inhibit long-term potentiation (LTP), reduce dendritic spines, and exacerbate spatial memory.[19] Activation of synaptic NMDARs and a significant increase of $[Ca^{2+}]$ are required for LTP, which induces the growth of dendritic spines and is crucial to the acquisition and stabilization of spatial memory. However, high levels of APP and Aβ impair LTP[128] and induce long-term depression (LTD).[129] They facilitate the internalization of synaptic NMDARs and AMPARs and activate perisynaptic

NMDARs, mGluRs, and a7-nAchRs. Through these, postsynaptic [Ca^{2+}] levels decrease to enhance LTD-related mechanisms, such as mGluR, p38 MAPK, and GSK3, and synaptic loss.[130] Therefore, dysregulation of Aβ in AD can trigger cognitive decline by inducing abnormal neuronal activity and compensatory responses. Aβ also causes network instability and promotes synchrony by abnormal synaptic activity and GABAergic dysfunction.[130] Hypometabolism and atrophy via Aβ deposition are associated with abnormal neuronal and synaptic activity in the AD brain and block normal memory encoding.[131]

Tau is hyperphosphorylated and misfolded in both presynaptic and postsynaptic sites. It disrupts normal synapse functions in AD. Pathologically modified tau causes presynaptic dysfunction and lowers neurotransmission by binding to synaptic vesicles.[132] As spine density was reduced whether tau was aggregated or not (pro- or anti-aggregated), soluble tau has synaptotoxicity.[133] Soluble forms of tau could be oligomeric and misfolded and have post-translational modifications, such as abnormal phosphorylation and truncation.[134] The accumulation of pro-aggregated tau with modifications could trigger cognitive impairment, including synaptic and neuronal loss.[135] Tau also modulates neuronal signaling by regulating synaptic mitochondria. The movement of mitochondria within the axon is impaired by pathological changes in tau.[136] Abnormal tau inhibits axonal trafficking and may reduce crucial organelles and cargo (e.g., mitochondria) in synapses and vesicle release.[137] Tau directly alters mitochondria functions. Overexpression of pathological tau decreases ATP levels and increases susceptivity to oxidative stress of mitochondria.[138] The activity and composition of mitochondrial enzymes are disrupted by tauopathy.[139] Tau can also disrupt mitochondrial dynamics (the balance between fusion and fission) and cause mitochondrial elongation and neurotoxicity.[140] Tau has a vital role in Aβ-induced synaptotoxicity,[141] as

synaptic loss triggered by Aβ was induced with the existence of specific tau phosphorylation.[142]

G. Cognitive impairment

The diverse factors that were previously described cause synaptic malfunction and ultimately cognitive impairments in AD. AD is divided into five stages: preclinical AD, mild cognitive impairment (MCI), mild AD, moderate AD, and severe AD.[143] The progression of AD correlates to not only the alteration of AD biomarkers, such as Aβ and tau but also the degree of severity of cognitive impairment and the difficulty of everyday activities.[144] Multiple domains of cognitive functions, such as episodic memory,[145] executive functions, attention,[146] verbal ability,[147] visuospatial ability,[148] perceptual speed, and long-term memory,[149] are impaired in AD. Cognitive, functional, and behavioral deficits appear differently as the stage of AD proceeds (Table 1).[150,151] The brief progression of cognitive impairment during AD development is 'episodic memory (recall memory) → attention and executive functions in inhibitory controls → language and visuospatial memory → cognitive flexibility and response inhibition.'

For diagnosis of AD and staging its progression, five cognitive functioning and mental status test are commonly used.[152] Mini-Mental State Examination (MMSE) and Alzheimer's Disease Assessment Scale-Cognitive Subscale (ADAS-Cog) are sensitive to AD and highly correlated. Activities of Daily Living (ADL) scale and Global Deterioration Scale (GDS) are also used to determine the individual's degree of impairment with regard to functional status. The Montreal Cognitive Assessment (MoCA) has become increasingly used recently due to its high specificity for detecting cognitive impairment and

sensitivity to detecting early cognitive changes associated with AD development. The fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) is a diagnosis method of various neurocognitive disorders, such as AD and Lewy body disease.[153] It subdivides cognitive functions into six domains: complex attention, executive functions, learning and memory, language, perceptual-motor function, and social cognition. For AD diagnosis, at least two domains must be impaired, and one should be learning and memory. Each domain contains different cognition subdomains (Table 2) simultaneously disrupted in AD.[154]

Table 1. Key deficits of AD by stage

Stage of AD	Domain			
	Duration	**Cognitions**	**Functions**	**Behaviors**
Preclinical	15 years	Slightly impaired learning and memory Slightly impaired executive memory	Normal	Apathy Irritability Dysphoria
MCI	7 years	Memory impairment (Isolated deficit)	Occasional loss of complex social or occupational skills	Apathy Anxiety Irritability Withdrawal (mild)
Mild AD	1-2 years	Impaired learning and memory - Recall and learning Impaired executive functions: - Judgement and problem solving - Calculation impairment Impaired language: - Word finding difficulty	Difficulty in: - Routine chores - Complex meal preparation - Financial matters	Apathy Anxiety Delusions Depression Withdrawal (moderate)
Moderate AD	2-12 years	Moderate memory loss	Loss of IADL:	Agitation

		Impaired perceptual-motor function: - Visuospatial deficits - Disorientation - Confusion	- Getting lost - Difficulty dressing - Poor eating habits - Poor hygiene habits	Anxiety Delusions Depression Insomnia Wandering
Severe AD	1-10 years	Severe memory loss Agnosia Apraxia	Loss of basic ADL: - Dressing - Grooming and bathing - Eating - Continence - Mobility	Agitation in: - Verbal - Physical domain Anger Anxiety Delusions Depression Insomnia Uneasiness to care

Abbreviations: AD, Alzheimer's disease; ADL; activities of daily living; IADL, instrumental activities of daily living; MCI, mild cognitive impairment.

Table 2. Neurocognitive domains

Neurocognitive domain	Subdomain
Complex attention	Sustained attention
	Divided attention
	Selective attention
	Processing speed
Executive functions	Planning
	Decision-making
	Working memory
	Responding to feedback
	Inhibition
	Flexibility
Learning and memory	Free recall
	Cued recall
	Recognition memory
	Semantic and autobiographical
	long-term memory
	Implicit learning
Language	Object naming
	Word finding
	Fluency
	Grammar and syntax
	Receptive language
Perceptual-motor function	Visual perception
	Visuo-constructional reasoning
	Perceptual-motor coordination
Social cognition	Recognition of emotions
	Theory of mind
	Insight

H. Metabolic dysfunction

Metabolic dysfunctions have been shown in AD brains since early AD progression.[155] Insulin metabolism, which involves insulin-related signaling, including insulin receptors (IRs) and insulin-like growth factor (IGF), and insulin itself is dysregulated in AD.[156] Reduced peripheral insulin sensitivity and chronic hyperinsulinemia downregulate IRs of the blood-brain barrier and reduce insulin concentration in the brain.[157] Therefore, insulin resistance and disrupted production or usage of insulin by lowering or dislocating the insulin receptors and decreasing the receptor affinity for insulin have been found in AD.[158]

Insulin is essential for AD pathology as insulin interacts with Aβ and tau. Insulin protects the brain against Aβ synaptotoxicity and increases Aβ clearance with insulin-degrading enzyme (IDE).[159] Abnormal insulin/IDE signaling increases Aβ release and accumulation and decreases Aβ clearance and CTFβ degradation in AD. Decreased expressions of IGF-1 and IGF-2 found in AD increase Aβ accumulation and decrease Aβ clearance.[160] Therefore, insulin malfunction, including IDE and IGF signaling, accelerates Aβ accumulation by increasing Aβ production and decreasing Aβ removal in AD brains. Disrupted insulin/IDE signaling stimulates tau expression and phosphorylation by upregulating tau kinases, such as GSK3β and CDK5, and inhibiting tau phosphatase, PP2A.[157] Hyperphosphorylated tau is accumulated and aggregated into insoluble NFTs via insulin depletion.[161] As insulin and its receptors have an important role in cognition by regulating synapse formation and maintenance,[162] impaired insulin and insulin-related signaling result in cognitive impairment.[163]

Diverse glucose transporter (GLUT) isoforms are distributed in the brain,

especially GLUT1 and GLUT3. GLUT1 is expressed mainly in glia and endothelial cells, and GLUT3 in neurons. GLUT4 is selectively sensitive to insulin and transports glucose into the brain only by insulin stimulation. These transporters are essential to glucose uptake modulation and AD development.[164] The levels of GLUT1 and GLUT3 are reduced in AD.[165] Decreased GLUT1 expression exacerbates AD pathology by triggering vasculoneuronal dysfunction and degeneration.[166] Abnormal insulin metabolism blocks normal glucose metabolism, such as GLUT4 function and glucose uptake and utilization, and regulates neural circuits involved in learning and memory.[167] Therefore, metabolic dysfunctions, such as perturbed glucose metabolism, insulin resistance, and mitochondrial dysfunction, are shown in AD.[168] Because of these dysregulations, AD is regarded as brain diabetes and called "Type 3 diabetes mellitus," and metabolic enhancement has emerged as the new treatment strategy of AD.[169]

I. Relationship between type 2 diabetes mellitus (T2DM) and AD

Type 2 diabetes mellitus (T2DM), noninsulin-dependent diabetes mellitus, is one of the most common metabolic diseases. Insulin resistance, hypertension, and hyperglycemia are the main features of T2DM.[170] T2DM shares pathogenesis, such as increased oxidative stress, upregulated inflammation, and cognitive impairment with AD.[171] T2DM and AD also have common pathological mechanisms highly associated with glucose and insulin metabolism, such as reduced GLUT1 or GLUT3 and impaired insulin receptors. Diverse human studies have shown that T2DM increases the risk of AD.[172,173] AD also increases the incidence of T2DM. Impaired fasting glucose and islet amyloid, pathological features of T2DM, were more prevalent in AD without altering the densities of Aβ plaques and NFTs, AD pathological features, between healthy

controls and T2DM patients.[174] Owing to the overlapping metabolic symptoms between T2DM and AD which exacerbate AD-related symptoms, such as cognitive decline and Aβ accumulation, T2DM treatments have also been regarded as the prospective AD drugs.[175]

J. AD treatment and clinical trial

Among current FDA-approved AD treatments, donepezil, galantamine, and rivastigmine are cholinesterase inhibitors to preserve the levels of acetylcholine as the deficiency of acetylcholine, the chief neurotransmitter of the parasympathetic nervous system, causes AD.[176] Memantine, another FDA-approved AD treatment, works as an NMDAR antagonist to prevent the binding of Aβ oligomers to NMDAR, which induces Ca^{2+} dysregulation, neuronal death, and synaptic dysfunction. The combination of donepezil and memantine is also clinically used as AD treatment. Recently, the FDA accelerated aducanumab's approval for AD therapy even though its effects on cognitive improvement are still unclear.[177] Aducanumab, passive immunotherapy targeting Aβ, is the first disease-modifying therapy strategy that lowers the levels of Aβ plaques in the brain.

In 2021, 28 agents out of 126 were in phase 3 clinical trial of AD treatment.[178] Even though diverse approaches, such as neuropsychiatric symptoms and symptomatic cognitive enhancers, are used to cure AD, disease-modifying therapies are still the most dominant. There are diverse disease-modifying strategies to remedy AD, including i) Aβ, ii) synaptic plasticity and neuroprotection, iii) inflammation, infection, and immunity, iv) metabolism and bioenergetic, and v) tau. Even though clinical trials of anti-Aβ agents, such as lanabecestat and umibecestat, were discontinued because of a lack of efficacy,

AD treatment candidates are still targeting Aβ the most.[179] In addition, developing new agents and repurposing FDA-approved drugs for other diseases such as T2DM are ongoing. For example, metformin, the most commonly prescribed T2DM treatment, is in phase 3 clinical trial of AD treatment.[178]

2. Metformin

A. Functions of metformin

Metformin is a first-line T2DM biguanide medication with glucose-lowering and insulin-sensitizing effects.[180] It lowers glucose production by inhibiting gluconeogenesis mainly and glycogenolysis partially in hepatocytes.[181] Gluconeogenic substrates, such as alanine and lactate, and enzymes are reduced by metformin to block gluconeogenesis and stimulate glycolysis.[182] Glucagon, a peptide hormone, is also inhibited by metformin, suppressing gluconeogenesis and glycogenolysis.[183] Glucagon-like peptide (GLP-1), which increases insulin secretion and reduces glucagon secretion, is enhanced by metformin via interaction with the incretin axis.[184] Metformin increases glucose uptake in the skeletal muscle, adipocytes, and the intestine.[185] Enhanced insulin signaling can mediate glucose uptake. Upregulated insulin receptor and insulin receptor substrate 2 (IRS-2) activities increase the translocation of GLUT1 into the plasma membrane.[186] In skeletal muscle, metformin increases the tyrosine kinase activity of insulin receptors and translocation of GLUT4.[187] Glucose absorption is slightly delayed by metformin through the gastrointestinal tract.[188] Pancreatic β-cell responses are impaired by increased plasma glucose levels and fatty acid production. As the function of the pancreatic β-cells, where insulin is synthesized, is improved by metformin treatment, insulin resistance can be ameliorated.[189] Metformin also upregulates insulin functions, such as insulin binding by

increasing insulin receptors.[190] Metformin regulates not only glucose homeostasis but also lipid homeostasis. It suppresses lipid accumulation by increasing fatty acid oxidation.[191] The levels of lipoprotein, essential to lipid transportation, are altered: very low- and low-density lipoprotein cholesterol levels are decreased, while high-density lipoprotein cholesterol levels are increased.[192] Through these anti-hyperglycemic effects, plasma glucose levels are reduced, and insulin sensitivity is recovered.

B. Mechanism of action of metformin

The main mechanism of action of metformin is through the activation of AMP-activated protein kinase (AMPK), a whole-body level energy homeostasis regulator.[193] Metformin primarily inhibits complex I of the mitochondrial respiratory chain and mimics energy deprivation conditions.[194] It reduces the synthesis of ATP and increases the ratio of AMP/ATP or ADP/ATP.[195] This altered condition can directly inhibit gluconeogenesis through insulin.[180] Increased AMP regulates energy metabolism via diverse downstream pathways. First, it activates AMPK via phosphorylation. Second, it allosterically inhibits glucagon-induced cAMP and PKA signaling by suppressing the activity of adenylate cyclase. Decreased PKA reduces the activity of diverse enzymes involved in gluconeogenic flux [e.g., phosphofructose kinase (PFK) and fructose-1, 6-bis-phosphatase 1 (FBPase 1)]. Third, FBPase 1 can also be allosterically inhibited by AMP directly. Activated AMPK is involved in lipid and glucose metabolism and insulin signaling. cAMP-PKA signaling and FBPase 1 are involved in glucose metabolism, especially gluconeogenesis. Metformin lowers glucose and lipid concentrations and improves insulin sensitivity through these pathways.

As metformin is polar, its uptake and secretion rely on membrane transporters.[196] The main transporters of metformin are solute carrier family 22 members (SLC22A), multi-drug and toxin protein (MATE) 1 and 2, and human equilibrative nucleoside transporter-4 (hENT4), also called plasma membrane monoamine transporter (PMAT). SLC22A transporters have four different types which are involved in metformin pharmacokinetics. SLC22A, OCT1, is the major transporter for metformin uptake expressed in the liver and kidney. SLC22A2, OCT2, mediates metformin secretion in the kidney and 80% of total metformin clearance. SLC22A3, OCT3, is expressed in multiple tissues, including the liver, kidney, skeletal muscle, and brain. Therefore, it might have a crucial role in metformin uptake into muscle. SLC22A4, OCTN1, is involved in the gastrointestinal absorption of metformin. MATE1 is expressed in the kidney and liver and mediates urinary excretion highly associated with lactic acidosis when MATE1 is dysfunctional. MATE2 also regulates the deposition of metformin in the kidney. hENT4 is involved in the intestinal absorption of metformin. The dysfunctions or variants of each transporter can cause metformin accumulation and related side effects.

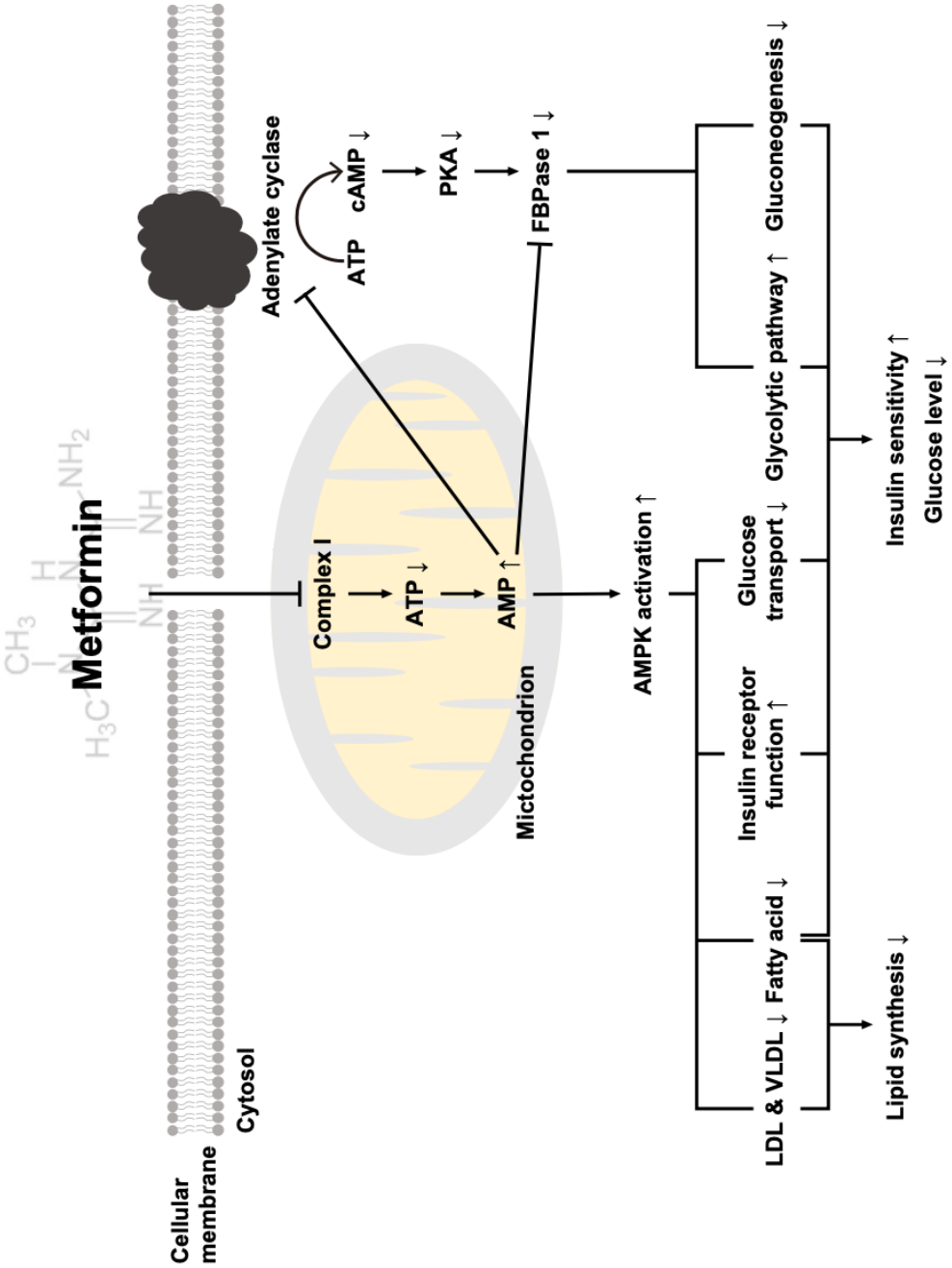


Figure 3. Mechanism of action of metformin. Metformin reduces the levels of ATP by inhibiting the mitochondrial complex I respiratory chain and increases the levels of AMP. As body senses this status as energy deficit and directly

inhibits gluconeogenesis. Increased AMP activates AMPK, reduces cAMP-PKA signaling by inhibiting adenylate cyclase, and inhibits FBPase 1. Activated AMPK decreases fatty acid synthesis and enhances insulin receptor functions and glucose transport. Decreased cAMP-PKA signaling and FBPase 1 activity decreases gluconeogenesis and upregulates the glycolytic pathway. Eventually, metformin can regulate glucose and lipid metabolism and ameliorate hyperglycemia and insulin resistance in T2DM. FBPase 1; fructose-1, 6-bisphosphatase 1.

C. Side effects of metformin

Metformin is commonly used as an anti-diabetic drug, as it is usually safe.[197] However, it does have some side effects. Weight loss in T2DM patients who took metformin is regarded as a favorable side effect.[198] One potential mechanism which causes weight loss is the appetite regulatory pathway in the brain.[199] Metformin might cause appetite suppression and body mass reduction via increasing growth differentiation factor-15 (GDF-15).[200] Most side effects of metformin are gastrointestinal symptoms: nausea, diarrhea, flatulence, and abdominal discomfort.[201] These symptoms occur dose-dependently to metformin and are caused by metformin accumulation in the intestinal tissue.[202] Metformin accumulation can also increase blood lactic levels and induce lactic acidosis.[203] Chronic kidney disease is also developed in metformin-treated patients due to the decreased clearance of metformin and lactic acidosis.[204] In addition, vitamin B12 deficiency has been found in patients with long-term metformin therapy.[205] Therefore, the health condition of patients should be monitored carefully.

D. Metformin on AD pathology

Metformin has diverse neuroprotective aspects: protein dephosphorylation, anti-oxidation, and reducing neuroinflammation.[206] It also has anti-aging effects highly associated with brain functions.[207] Metformin is considered a promising AD treatment, as it can enhance diverse pathways impaired in AD.

(A) Animal studies

Diverse studies have been conducted to assess the effects of metformin on AD pathology. However, the therapeutic potential of metformin as an AD drug is unclear since the studies produced conflicting results. A variety of studies have shown that metformin ameliorates Aβ pathogenesis. Aβ plaque load is reduced by decreasing inflammation responses and expression of BACE1 *in vivo*.[208] BACE1 expressions were decreased by inhibiting the synthesis of the MID1 complex, which regulates the transcription of *BACE1* both *in vitro* and *in vivo*.[209] However, other studies have shown negative results: metformin aggravates Aβ pathogenesis. Metformin increased the synthesis of $A\beta_{42}$ by upregulating BACE1 and CTFβ, the components of β-cleavage, and downregulating sAPPα, the component of α-cleavage.[210] Metformin facilitated autophagosome accumulation and promoted amyloidogenic APP processing by increasing β- and γ-secretase activity *in vitro* and *in vivo*.[211] Metformin upregulated APP and PS1 expression by increasing mitochondria dysfunction, ROS generation, and oxidative stress *in vitro*.[212]

Metformin can also regulate tau pathogenesis. Several studies have found that metformin alters diverse tau kinases and attenuates tau hyperphosphorylation. Metformin inhibited Cdk5 and tau hyperphosphorylation at Ser262 and Ser404

in vivo.[213] It inactivated JNK and decreased total tau levels and tau hyperphosphorylation at Ser396 *in vivo*.[214] Tau hyperphosphorylation at Ser404 was decreased by metformin, as it ameliorated insulin resistance and restored the activities of tau kinases, GSK3β and ERK, *in vitro*.[215] Another pathway that ameliorates tau pathogenesis through metformin treatment is autophagic clearance. Both *in vivo* and *in vitro*, metformin facilitated autophagy activity and decreased tau hyperphosphorylation at Ser396, Ser404, and Ser202/Thr208.[216] However, another study has shown that metformin exacerbates tau pathogenesis. Metformin increased tau hyperphosphorylation at Ser262 and Ser356 and decreased tau and microtubule affinity *in vitro*.[217]

Even though metformin is an AMPK activator, some studies have shown that metformin alters tau pathogenesis independent of AMPK activity. Metformin decreased tau hyperphosphorylation at Ser202, Ser262, and Ser356, the PP2A-sensitive sites, by increasing PP2A activity even before the onset of AMPK activation *in vitro*.[218] However, metformin increased tau hyperphosphorylation by increasing lipogenesis and inflammation responses without altering AMPK activation levels *in vivo*.[219]

Neural and cognitive functions are also controlled by metformin through diverse pathways. Metformin enhanced long-term potentiation (LTP) and spatial memory by restoring spine density and surface GluA1 trafficking *in vivo*.[213] Metformin improved neurogenesis and spatial memory by increasing the atypical PKC-CBP pathway *in vitro* and *in vivo*.[220] Spatial learning and memory and sociability were upregulated by metformin through normalizing brain glucose metabolism, synaptic plasticity, and acetylcholinesterase density *in vivo*.[221] Reduced inflammatory responses by metformin enhanced cognitive functions and neurogenesis *in vivo*.[208] However, metformin aggravated neural

and cognitive functions. Metformin impaired cognitive flexibility and visual acuity by decreasing superoxide dismutase in aged mice[222] and reduced numbers of neurons and synaptic density *in vivo*.[219]

Even though both Aβ and tau pathogenesis are shown in the AD brains and trigger cognitive impairment, the studies mentioned above mostly found the effects of metformin on only one form of AD pathology (Aβ, tau, or cognitive functions). Only a few studies have shown the effects of metformin on both AD pathology and cognitive functions. For example, Farr et al. (2019) showed that metformin attenuated AD pathology, including Aβ and tau pathogenesis and cognitive function in the sporadic AD mouse model.[223] In detail, metformin reduced Aβ pathogenesis by decreasing $A\beta_{40}$ levels and CTFβ. It also enhanced tau pathogenesis by increasing GSK3β phosphorylation and decreasing p-tauSer404. Cognitive functions such as acquisition and memory retention were improved by metformin treatment.[223]

Even though Aβ and tau interact with each other,[224] the effects of metformin on Aβ and tau interaction were not fully elucidated. In a recent study, metformin enhanced microglial autophagy and reduced tau aggregates around Aβ plaques and Aβ deposition in tau injected APP/PS1 mice.[225]

Metformin might simultaneously have positive and negative impacts within the same disease model mice. In P301S mice, metformin decreased tau hyperphosphorylation at Ser202/Thr208, Ser262, Ser396/404, and Thr181 through increased AMPK-dependent PP2A activity.[226] However, at the same time, it increased insoluble tau and β-sheet tau aggregates and triggered hindlimb atrophy and hyperactive behavior.[226] Another study showed that metformin restored impaired spatial memory in aged C57BL/6J mice with a high-fat diet.

At the same time, the mRNA levels of neurotrophic factors and AMPK were decreased without altering protein expression.[227]

Moreover, the effects of metformin could be altered by sex, age, and dosage. Spatial memory was impaired by metformin in male AD model mice but enhanced in the female of the same model.[228] In C57BL/6J, metformin administration impaired spatial memory in young and aged mice without altering spatial memory in middle-aged mice.[227] Visual acuity and superoxide dismutase (SOD) activity were reduced by metformin only in aged mice.[222] When metformin was administered with the dose of average human prescription (100 mg/kg/day) to memory deficit-induced model rats, it enhanced learning and memory by reducing inflammatory responses and oxidative stress. However, metformin administration at a higher dose had no deleterious effect on cognitive functions (300 mg/kg/day).[229]

(B) Human studies

Diverse studies have been conducted to elucidate the relationship between metformin and the risk of AD. Metformin treatment decreased AD or dementia incidence in patients with T2DM.[230-232] Metformin noticeably reduced the risk of AD in T2DM patients when metformin was treated for patients for more than four years.[233] Metformin lowered the prevalence of AD than sulfonylurea or thiazolidinediones.[234,235] The use of metformin reduced cognitive decline in T2DM or cognition-impaired (MCI or mild dementia) patients.[236-238] Metformin slowed the decline of cognition, such as global cognition and executive function, in T2DM patients.[239]

However, negative results also revealed the relationship between metformin and AD development. Metformin increased the incidence of AD and other neurodegenerative diseases (e.g., Parkinson's disease) in T2DM patients.[240] Long-term metformin use increased the risk of AD, while sulfonylureas, thiazolidinediones, and insulin did not affect AD incidence.[241] Metformin exacerbated cognitive impairment.[242] However, cognitive impairment could be recovered through vitamin B12 and calcium supplements.[242]

3. AMP-activated protein kinase (AMPK)

A. Structure of AMPK

AMP-activated protein kinase (AMPK), which is highly conserved across all eukaryotic species, exists as heterotrimeric Serine/Threonine complexes consisting of a catalytic α-subunit and regulatory β- and γ-subunits.[243] There are two isoforms of α- and β-subunits (α1 and α2; β1 and β2) and three isoforms of γ-subunits (γ1, γ2, and γ3) in the AMPK of mammals.[244] The α-subunit has an N-lobe, C-lobe, auto-inhibitory domain (AID), linker peptide, and an α-subunit carboxy-terminal domain (α-CTD). The N-lobe and C-lobe are kinase domains, and upstream kinases of AMPK are bound to the C-lobe. AID reduces the activity of the kinase domains when the α-subunit contains both the kinase domain and AID. A linker peptide, which connects AID and α-CTD, wraps around the γ-subunit to hold it tightly.[245] The β-subunit has a carbohydrate-binding module (CBM) and β-subunit C-terminal domain (β-CTD). Mammalian AMPK is associated with glycogen particles through CMB, where glycogen binds. β-CTD interacts with the α-CTD and γ-subunit to form the core of the complex. The γ-subunit has four tandem repeats of a sequence motif, CBS repeat. Each CBS repeat is numbered 1-4 according to the number of conserved

aspartate residue involved in ligand binding to the repeat. The Bateman domain consists of two repeats with ligand-binding sites in the cleft between the repeats. The Bateman domain with CBS1 and CBS2 can bind to AMP and ATP. Another Bateman domain with CBS3 and CBS4 can bind to AMP, ADP, and ATP. As binding of AMP or ADP to each site causes allosteric activation or modulates the phosphorylation state in the mammalian γ1-subunit,[245] this can explain how AMPK phosphorylation could be maintained. When the levels of AMP and ADP increase during depletion of cellular energy, AMPK is activated by the phosphorylation of conserved threonine of the activation loop of the kinase domain in the α-subunit (Thr174 in α1; Thr172 in α2) to generate ATP. The binding of AMP to the γ-subunit promotes the phosphorylation of the α-subunit and increases the activity of AMPK.

B. Distribution of AMPK

AMPK α-, β-, and γ-subunits are distributed differently in tissues which can alter the function of AMPK. Among them, AMPK α1- and β1-subunits are widely expressed. However, the AMPK α2-subunit is highly expressed in skeletal and cardiac muscle and liver,[246] and the AMPK β2-subunit is highly expressed in skeletal muscle.[247] The AMPK α1-subunit is mainly cytoplasmic, and the AMPK α2-subunit is found in the nucleus and can regulate transcription.[248] As the AMPK α2-subunit is more dependent on AMP than the α1-subunit, different responses to ATP depletion result from the differences in regulation of AMPK α1- and α2-subunits by AMP. The distribution of AMPK isoforms is also different in the brain.[249] The AMPK α1-subunit has low expression in the neuropil, whereas the AMPK α2-subunit is highly expressed in neurons and activated astrocytes. The expression of AMPK β1- and β2-subunits is also altered by location. The AMPK γ1-subunit is found in neurons but not in

astrocytes. The mRNA expression of AMPK α2 and β2-subunits in the brain was increased between embryonic days 10 to 14. However, AMPK α1-, β1-, and γ1-subunit mRNA levels have remained consistent throughout the age. Different expression patterns and cellular localization between the α1- and α2-subunits determine the different physiological roles of AMPK.

C. Regulation of AMPK

(A) Phosphorylation: Activation

Upstream kinases regulate the activation of AMPK: liver kinase B1 (LKB1) and calcium/calmodulin-dependent kinase kinase-β (CaMKKβ).[250,251] LKB1 activates AMPK by sensing the AMP:ATP ratio elevation, AMP-dependent pathway. CaMKKβ senses the increase of Ca^{2+} and activates AMPK, a Ca^{2+}-dependent pathway. TGF-β-activated kinase-1 (Tak1) is also considered an AMPK upstream kinase as it modulates the phosphorylation of the Thr172 site of the AMPK α-subunit *in vitro*.[252] Sirtuin1 (SIRT1), one of the most well-known sirtuins, responds to overfeeding, starvation, energy expenditure, and exercise as AMPK does.[253] SIRT1 can activate AMPK via deacetylation of LKB1, which facilitates AMPK phosphorylation.[254] AMPK also activates SIRT1 by increasing the ratio of NAD^+/NADH or the expression and activity of NAMPT.[255] This AMPK/SIRT1 cycle regulates the energy and redox status of the cells. AMPK can also indirectly modulate the transcription of target genes, as activated SIRT1 via AMPK mediates the deacetylation of histones and transcription factors.[255]

(B) Phosphorylation: Inactivation

Different phosphorylation sites inhibit AMPK activity. Insulin inhibits AMPK via protein kinase B (PKB; AKT) activation.[256] AKT suppresses AMPK activity by phosphorylating Ser485 (Ser487 in humans) of the AMPK α1-subunit without phosphorylating Ser491 in the α2-subunit.[257] This phosphorylation blocks upstream kinases, such as LKB1 and CaMKKβ, which activate AMPK by phosphorylating Thr172 of the AMPK α-subunit. GSK phosphorylates Thr479 of the α1-subunit, which is required for the AKT-mediated AMPK inhibition.[258] Therefore, AKT and GSK are associated with insulin-mediated AMPK inhibition. PKA phosphorylates Ser173 of the α1-subunit for AMPK inhibition and blocks upstream kinases.[259] Leptin, the satiety hormone, inhibits AMPK via p70S6K-induced Ser491 phosphorylation of the AMPK α2-subunit.[260] Diacylglycerol can inhibit AMPK, as it activates PKC and PKD1, one of the PKC isoforms, which phosphorylate Ser487 of the α1-isoform[261] and Ser491 of the α2-isoform, respectively.[262]

(C) Ubiquitination and SUMOylation

Ubiquitination degrades and inhibits AMPK. Cell death-inducing DNA fragmentation factor 45-like effector A (CIDEA) ubiquitinates the AMPK β-subunit in brown adipose tissue.[263] MAGE-A3/6 and TRIM28 ubiquitin ligase complex ubiquitinates the AMPK α1-subunit in cancer cells.[264] WW domain-containing E3 ubiquitin-protein ligase 1 degrades the AMPK α2-subunit in skeletal muscle.[265] SUMOylation is a post-translational modification including apoptosis and translational regulation using a small ubiquitin-like modifier (SUMO). PIAS4 (protein inhibitor of activated STAT 4), the SUMO E3 ligase, catalyzes the SUMOylation and inhibits the AMPK α1-subunit.[266] In contrast,

PIASy activates AMPK by SUMOylation of the AMPK β2-subunit and blocks CIDEA-induced ubiquitination at the C-terminal of the β2-subunit.[267]

(D) Oxidation

ROS can regulate AMPK activity.[268] Oxidative stress and energy stress regulate AMPK differently through direct oxidation at different cysteine residues of AMPK. Hydrogen peroxide (H_2O_2) activates AMPK by oxidation and S-glutathionylation at Cys299/Cys304 of the α1-subunit, the redox-sensitive residues.[269] However, H_2O_2 with glucose deprivation oxidizes Cys130/Cys174 of the α-subunit and inhibits AMPK by facilitating its aggregation and disrupting the interaction with upstream kinases.[270] Therefore, the source of ROS might determine the effects of oxidation on AMPK activity. Nutrient abundance and antioxidant capacity of cells are crucial to AMPK oxidation, as energy stress-induced oxidation and AMPK inhibition were reversed by the expression of the antioxidant enzyme, thioredoxin-1 (Trx1).[270] Another study has found that the redox changes alter AMPK activity, not by its direct action on AMPK, but as a secondary consequence of redox effects on other pathways, such as mitochondrial ATP production.[271]

(E) Protein to protein interaction and subcellular localization

Protein to protein interaction and its subcellular distribution can also regulate AMPK activity. Folliculin (FLCN), a tumor suppressor associated with Birt–Hogg–Dube syndrome, and its binding partners, folliculin interacting protein-1 (FNIP-1) and -2 (FNIP-2), can interact with and inhibit AMPK.[272] The sestrin family of proteins interacts with AMPK and leads to the activation of the AMPK-TSC2 signaling axis to inhibit mTORC1.[273] Scaffold protein axin promotes

LKB1-AMPK complex formation through AMP-dependent conformational change and enables efficient phosphorylation and activation of AMPK by LKB1 under the energy stress condition.[274]

The N-myristoylated AMPK β-subunit works as a scaffolding protein that recruits the AMPK α- and γ-subunits to intracellular membranes, such as mitochondrial membranes, in response to energy stress.[275] The nuclear localization signal (NLS) presented in the α2-subunit but not in the α1-subunit is crucial for the nucleocytoplasmic translocation of AMPK in response to leptin.[276] Through these regulations, the AMPK complex can have different combinations of each subunit isoform which alters the distribution and function of AMPK.

D. Functions of AMPK

AMPK senses energy deprivation and regulates whole-body level energy homeostasis.[277] It is associated with glucose, lipid, and protein metabolism, mitochondrial biogenesis, and autophagy.[278] AMPK generates ATP by activating catabolic pathways, such as glycolysis, glucose uptake, and fatty acid oxidation, and inhibiting anabolic pathways, such as gluconeogenesis and triglyceride synthesis.

(A) Glucose metabolism

AMPK primarily controls glucose metabolism for energy balance.[278] AMPK upregulates glucose uptake and downregulates glucose output by increasing the translocation of GLUT4 to the plasma membrane of skeletal muscle.[279] GLUT1 is also regulated by AMPK activation for glucose uptake. AMPK phosphorylates

6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB) and increases PFK1 activity to increase glycolytic flux (glycolysis). Another pathway to increase glycolysis in muscle is upregulating hexokinase activity. Glycogen synthesis in muscle is inhibited by blocking glycogen synthase through AMPK. Gluconeogenesis in the liver is inhibited as the transcription of gluconeogenic enzymes, such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), are decreased via suppression of the key transcriptional activators, cAMP response element-binding protein (CREB), and forkhead box protein O (FOXO).

(B) Lipid metabolism

To increase glucose utilization, AMPK modulates lipid metabolism.[278] AMPK directly blocks acetyl-CoA carboxylase (ACC), a major lipid regulator, by phosphorylation. Inhibited ACC1 downregulates malonyl-CoA levels and enhances the activity of carnitine palmitoyltransferase 1 (CPT-1), which increases the fatty acid uptake to mitochondria for its oxidation.[280] Downregulated ACC2 suppresses fatty acid synthesis.[281] Fatty acid uptake is also enhanced by stimulating the fatty acid transporter/CD36 (FAT/CD36) translocation to the plasma membrane via AMPK activation. SREBP2 and its important target, HMG-CoA reductase, are also decreased by AMPK activation and cause the reduction of cholesterol synthesis. Inhibited glycerol-3-phosphate acyltransferase (GPAT) via AMPK activation suppresses triglyceride synthesis. Activated AMPK inhibits lipogenesis by suppressing SREBP1c proteolytic processing, nuclear translocation, and transcription of target lipogenic enzymes. Inhibited adipose tissue triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) by AMPK blocks lipolysis. Through these pathways, AMPK reduces lipid storage in muscle, liver, and adipose tissue.

(C) Protein metabolism

AMPK also inhibits protein synthesis by inhibiting the mammalian target of rapamycin (mTOR) in muscle[282] and biosynthetic pathways, such as cell cycles in proliferating cells.[283] In addition, rRNA synthesis is also suppressed by AMPK activation.[278] For example, AMPK inactivates transcription initiation factor IA (TIF-IA), the Pol I-associated transcription factor, through phosphorylation. Inactivated TIF-IA prevents the assembly of functional transcription initiation complexes where the rRNA is produced.

(D) Mitochondria regulation

AMPK is a master regulator of mitochondrial homeostasis. Pharmacologically-activated AMPK increased vital mitochondrial enzymes in skeletal muscle.[284] The expression of proliferator-activated receptor-γ coactivator-1α (PGC-1α), a central regulator of mitochondrial biosynthesis, is increased through physiologically-activated AMPK.[285] Chronic AMPK activation by energy deprivation also regulates mitochondrial biogenesis via PGC-1α and nuclear respiratory factors (NRFs) pathways.[286] AMPK is also involved in mitophagy. Activated ULK1 via AMPK triggers autophagy in mitochondria and removes dysfunctional mitochondria.[287] Additionally, mitochondrial fission and fragmentation are regulated by mitochondrial fission factor (MFF) phosphorylation caused by AMPK activation.[288] Therefore, AMPK can synthesize new mitochondria and remove damaged mitochondria.

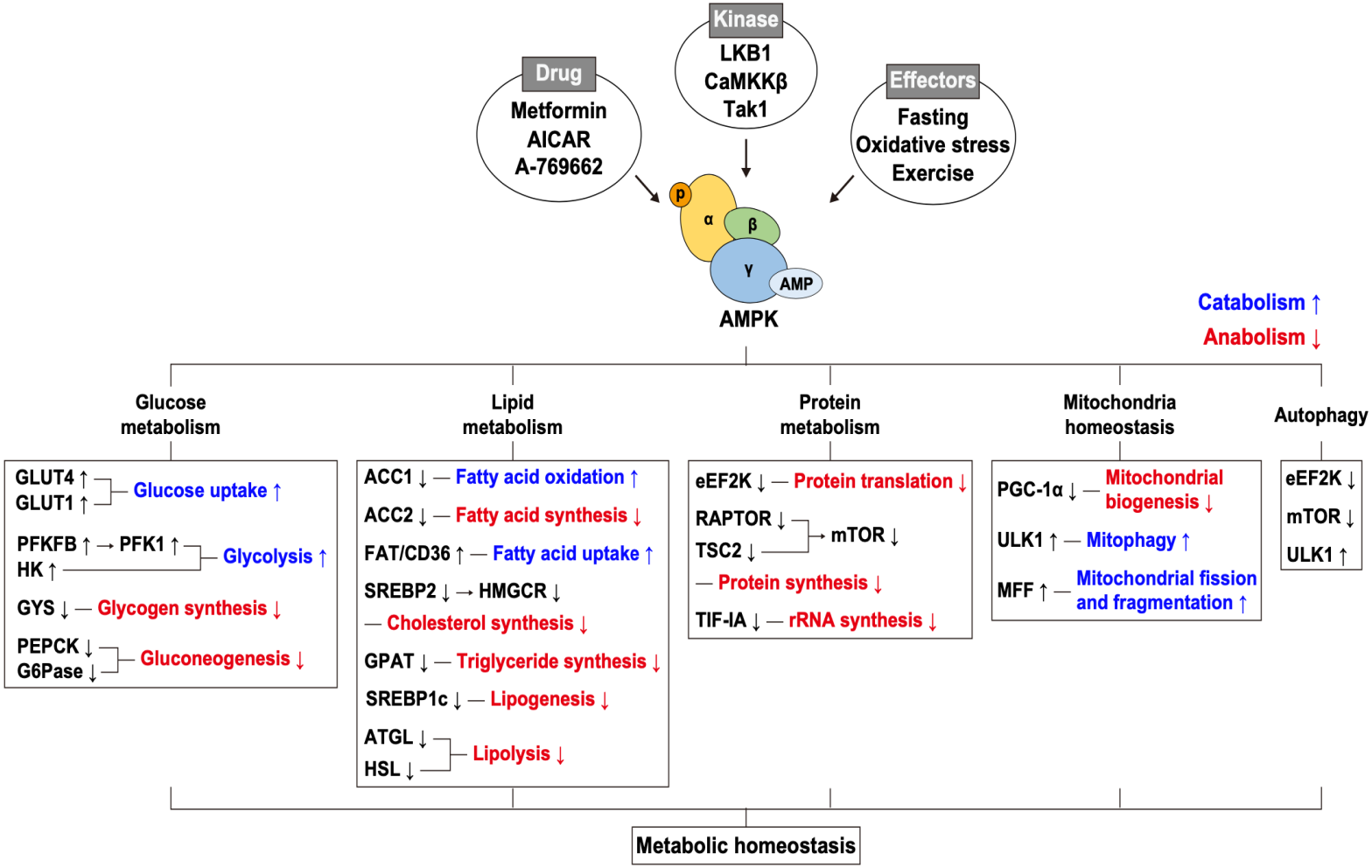
(E) Autophagy

AMPK can modulate the autophagic pathway as it inhibits mTOR and activates ULK1.[289] When autophagy is induced via the AMPK-dependent pathway, it is mostly beneficial. AMPK upstream kinases (LKB1 and CaMKKβ) act as negative regulators of mTOR by activating AMPK directly. Tak1, a potent AMPK activator, also induces autophagy via tumor necrosis factor-related apoptosis-inducing ligand.[290] Another route for autophagy induction via AMPK is phosphorylation and activation of eukaryotic elongation factor-2 kinase (eEF-2 kinase).[291] eEF-2 kinase can also explain how AMPK activation inhibits protein synthesis, regulating translation elongation.[292] However, even though AICAR is a well-known AMPK activator, it is unlikely to inhibit autophagy. The inhibition of autophagy via AICAR might be caused by inhibiting phosphatidylinositol 3-kinase (PI3K) class III, an essential kinase for autophagy.[293] Additionally, AICAR activates AKT, a potential activator of mTOR complex 1, independent of AMPK.[294] AMPK can regulate lipophagy and autophagic degradation of lipid droplets via macrophages. Reduced expression of fatty acid translocase cluster of differentiation (CD36) facilitates the uptake of long-chain fatty acids activated by AMPK and decreases lipid droplets via the phosphorylation of ULK1/Beclin1.[295] However, another study showed that AMPK increases lipophagy. Exercise restored lipid droplet deposition by activating the AMPK/SIRT1 pathway and increased lipophagy in the liver.[296]

(F) Metabolic disease

AMPK is a primary target for regulating metabolic pathways involved in T2DM, non-alcoholic fatty liver disease (NAFLD), and cardiovascular disease (CVD).[297] The metabolism mentioned above and insulin sensitivity are regulated

via AMPK.[244] AMPK inhibits insulin secretion by regulating pancreatic β-cells. AMPK also ameliorates insulin resistance by impeding the negative feedback of the IRS-1 loop by inhibiting mTOR and S6K1, the substrate of mTORC1. AMPK inhibits inflammatory responses by regulating diverse essential proteins which control inflammatory pathways are regulated by AMPK.[297] eNOS, CHOP, and JAK1 are inhibited, whereas p38, MKP1, and PIAS1 are activated. AMPK decreases NF-κB activity by phosphorylating FOXO1, increasing the activity of SIRT1, the deacetylase of NF-κB, and suppressing PGC-1α.[298]


Kinase
LKB1
CaMKKβ
Tak1
Drug
Metformin
AICAR
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Effectors
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AMP
AMPK
Catabolism ↑
Anabolism ↓
Glucose metabolism
Lipid metabolism
Protein metabolism
Mitochondria homeostasis
Autophagy
GLUT4 ↑
GLUT1 ↑
Glucose uptake ↑
PFKFB ↑ → PFK1 ↑
HK ↑
Glycolysis ↑
GYS ↓ — Glycogen synthesis ↓
PEPCK ↓
G6Pase ↓
Gluconeogenesis ↓
ACC1 ↓ — Fatty acid oxidation ↑
ACC2 ↓ — Fatty acid synthesis ↓
FAT/CD36 ↑ — Fatty acid uptake ↑
SREBP2 ↓ → HMGCR ↓
— Cholesterol synthesis ↓
GPAT ↓ — Triglyceride synthesis ↓
SREBP1c ↓ — Lipogenesis ↓
ATGL ↓
HSL ↓
Lipolysis ↓
eEF2K ↓ — Protein translation ↓
RAPTOR ↓
TSC2 ↓
mTOR ↓
— Protein synthesis ↓
TIF-IA ↓ — rRNA synthesis ↓
PGC-1α ↓ — Mitochondrial biogenesis ↓
ULK1 ↑ — Mitophagy ↑
MFF ↑ — Mitochondrial fission and fragmentation ↑
eEF2K ↓
mTOR ↓
ULK1 ↑
Metabolic homeostasis

Figure 4. Role of AMP-activated protein kinase (AMPK) in metabolic regulation. AMPK modulates energy homeostasis by upregulating catabolic pathways (blue) and downregulating anabolic pathways (red). It regulates diverse metabolic pathways: glucose, lipid, protein, mitochondria, and autophagy. When AMPK is activated via diverse activators, such as kinases, effectors, and drugs, its downstream targets (black in squares) are altered to upregulate metabolism. ACC, acetyl CoA carboxylase; ATGL, Adipose triglyceride lipase; eEF2K, Eukaryotic elongation factor 2 kinase; FAT/CD36, Fatty acid translocase; G6Pase, Glucose 6-phosphatase; GLUT, glucose transporter; GPAT, Glycerol-3-phosphate acyltransferase; GYS, Glycogen synthase; HK, Hexokinase; HMGCR, HMG-CoA reductase; HSL, Hormone-sensitive lipase; MFF, Mitochondrial fission factor; mTOR, mammalian target of rapamycin; PEPCK, Phosphoenolpyruvate carboxykinase; PFK1, Phosphofructokinase 1; PFKFB, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; RAPTOR, Regulatory-associated protein of mTOR; SREBP, Sterol regulatory element-binding protein; TIF-IA, Transcription initiation factor IA; TSC2, Tuberous Sclerosis Complex 2, ULK1, UNC-51-like kinase 1.

E. AMPK in brain

AMPK activation in the brain is mainly modulated by CaMKKβ abundant in neurons.[299] AMPK is essential for proper brain function and is involved in neurogenerative disease development as it regulates brain glucose metabolism and mitochondrial functions.[277] A number of studies have been conducted to elucidate the role of AMPK against metabolic stress in CNS (e.g., ischemia). However, the effects of AMPK on the damaged brain are still not well-defined. Some studies showed that AMPK activation has neuroprotective effects,[300-302]

while others suggested AMPK activation is detrimental to neuronal survival.[303,304] The differences in experimental conditions, such as cell types, culture conditions, and glucose concentration of the studies, might trigger the discordance of the results. For example, a 3 mM glucose-containing neuronal culture might correspond more to the physiological condition *in vivo* than 25 mM as brain glucose concentration fluctuates from 0.82 to 2.4 mM.[305]

The AMPK α2-subunit is expressed higher than the AMPK α1-subunit in mouse CNS.[249] Each isoform might have a different function in the brain as its expression and activity are different due to the progression of the disease. A study of the involvement of AMPKα isoforms in ischemia suggested that the activation of AMPKα2 plays a detrimental role in ischemia and the inhibition of AMPK is neuroprotective in stroke.[304] Therefore, AMPKα2 might have a crucial role in regulating the brain, as it has neuroprotective aspects under the ischemia condition.

The degree of AMPK activation also modulates the direction of AMPK effects on brain function.[306] Moderate AMPK activation by caloric restriction to 60% or 25 nM AICAR treatment had beneficial roles in brain function, such as neurogenesis and cognitive functions enhancement. However, excessive AMPK activation by diet restriction to 40% or 1-2 mM AICAR treatment was detrimental to brain function, such as decreased cognitive functions and catecholamines and increased neuronal apoptosis and mortality. Therefore, glucose concentration and the degree of AMPK activation should be considered to generate consistent results in AMPK studies.

F. AMPK in AD

AMPK is highly associated with AD pathology by modulating Aβ generation and tau phosphorylation.[307] AMPK regulates Aβ production via diverse pathways. First, AMPK modulates cholesterol and sphingomyelin levels and alters APP distribution and processing in lipid rafts.[308] Second, AMPK regulates APP β-cleavage by controlling BACE1 expression. The effect of AMPK on BACE1 expression is still controversial. Some studies showed that AMPK exacerbates AD pathology by upregulating BACE1 expression.[210] Others showed that AMPK ameliorates AD pathology by downregulating BACE1.[308] Third, AMPK inhibits mTOR and activates autophagic pathways for Aβ clearance.[309] Lastly, AMPK increases the expression of SIRT1, a target gene of AMPK activation, and reduces the production of Aβ and the number of Aβ plaques.[310]

AMPK modulates tau phosphorylation. AMPK works as tau kinase via NMDAR, CaMKKβ, and p-tau itself (e.g., Ser262 and Ser396) in response to $A\beta_{42}$.[311] Moreover, AMPK directly phosphorylates tau at Ser396/404 and Thr231 *in vitro*.[312] However, AMPK can decrease tau phosphorylation by increasing GSK3β phosphorylation.[313] Increased SIRT1 expression through AMPK activation decreases tau acetylation.[314] Tau acetylation triggers tauopathy by increasing tau phosphorylation and tangle formation and decreasing tau degradation and tau ubiquitination.[315]

AMPK activates autophagy by inhibiting mTOR and directly activating ULK1.[289,316] Autophagy can regulate aging and age-related degenerative diseases by removing unnecessary and dysfunctional components from cells.[317,318] Autophagy induction through AMPK activation resulted in beneficial

effects on AD pathology, as autophagy has a critical role in the degradation of both soluble and insoluble forms of Aβ and tau.[319-321] Autophagic pathology, such as impaired clearance of autophagy vacuoles, is observed in AD.[322] Chronic disturbance of the autophagy-lysosomal system in neurons, such as dysfunctional autophagic pathway and accumulation of autophagic vacuoles, triggers AD neurodegeneration by accumulating Aβ and tau in diverse brain regions.[323,324]

G. AMPK abnormalities in AD

Diverse abnormal AMPK regulations are shown in AD. Aging, one of the major risk factors of AD, decreases the activity and basal levels of AMPK.[325] AMPK overactivation or activation via chronic stress is detrimental to the brain by triggering a 'metabolic failure-like' state.[326] AD pathology, such as Aβ, phosphorylated tau, and inflammation, generates chronic stress and brings out the detrimental effects of AMPK on AD brains, such as neuronal cell death via apoptotic or autophagic pathway and synaptic dysfunction.[327] Pathological disturbance of Ca^{2+} balance also causes synaptic and neuronal loss in AD.[328] Increased cytosolic Ca^{2+} levels might chronically activate AMPK through CaMKKβ and trigger neuronal cell death.[329]

Activated AMPK was abnormally accumulated in the AD brain.[312] The accumulation of activated AMPK was found in diverse brain regions with abundant neurons, such as the CA1 region of the hippocampus, entorhinal cortex, and temporal isocortex. The structures of activated AMPK accumulation were similar to tauopathy: granular intraneuronal (resembling pre-tangle aggregates of phosphorylated tau), tangle-like intraneuronal (resembling intraneuronal tau NFTs), and extraneuronal (resembling ghost tangles). In addition, the

accumulation of activated AMPK was colocalized to neuropil threads and dystrophic neurites surrounding amyloid plaques. Therefore, the atypical colocalization of activated AMPK accumulation mainly appeared in the neurons bearing pre-tangles and tangles.

The expression of AMPK α-subunit isoforms (α1 and α2) is highly associated with AD pathology. However, which isoform regulates AD pathology and how AD pathology is altered by the isoform are still unclear. Some studies showed that the AMPK α1-subunit is highly related to AD pathology. Zimmermann et al. (2020) showed that the AMPK α1-subunit is upregulated in AD.[330] Moreover, AMPK α1-subunit exacerbates AD pathology, such as reduced spine density, increased synaptic toxicity, and cognitive impairment.[142,331] However, Wang et al. (2020) showed that the AMPK α1-subunit is decreased in AD plasma.[332] Others showed that the AMPK α2-subunit has a critical role in AD pathology. The AMPK α2-subunit KO impaired hippocampal LTP and cognitive functions[333] and increased Aβ, sphingomyelin, and cholesterol levels.[308,334] On the other hand, Domise et al. (2016) showed that the AMPK α2-subunit KO rescued tau pathogenesis by decreasing tau phosphorylation at Ser202, Ser396/404, and Thr231.[217]

4. Aim of study

Metformin has been prescribed the most to T2DM patients for long-term duration.[335] T2DM is highly associated with AD as they share pathologies, such as inflammation, insulin resistance, and mitochondria dysfunction.[171] Moreover, T2DM can increase AD risk.[336] Therefore, metformin is a considered promising AD treatment and is currently in phase 3 clinical trial of AD treatment.

A number of studies have been conducted to elucidate the effects of metformin on AD pathology. However, their results are still conflicting: some showed that metformin is beneficial to AD pathology,[214,220] whereas others found that metformin exacerbates AD pathology.[210,226] If the detrimental results, especially Chen et al. (2009),[210] are revealed as fact, this might impede the prescription of metformin to AD patients and the drug repositioning research of metformin from T2DM to AD therapy.

There are some limitations in previous studies about the effects of metformin on AD pathology. First, the duration of metformin treatment varied by study and was mostly acute or sub-chronic (shorter than a year). Second, the dose of metformin varied by study. Third, the age of mice varied by study. Fourth, the AD mouse model, which only expresses one AD pathology (Aβ or tau), was used in most *in vivo* studies. Lastly, traditional behavioral assessments (e.g., Morris water maze and object recognition test) were conducted. They can only assess simple cognitive functions, such as recognition and working memory, and show inconsistent results due to the researchers' bias and environment. As the age of subjects, drug dosage, and treatment duration are important factors in AD and AMPK research, these variables might cause inconsistent results.

The effects of chronic metformin treatment on behaviors and AD pathology of C57BL/6J mice were assessed in the previous research of our lab.[337,338] In summary, chronic metformin treatment improved cognitive functions in an age-dependent manner. When the mice were young and middle-aged, cognitive functions were improved by metformin. However, cognitive functions were impaired in aged mice with metformin treatment. As C57BL/6J mice did not show AD pathology abundantly, the effects of chronic metformin treatment on AD pathology were not assessed thoroughly.

This research was designed to redeem the limitations of previous studies and this author's master's degree research. First, metformin was administered chronically to mimic the condition of patients who had been prescribed metformin for years.[335] Second, the dose of metformin was matched to that administered by Chen et al. (2009), which first found the detrimental effects of metformin.[210] Third, 3xTg-AD mice were used as transgenic AD model mice. This model is the most similar to AD patients, showing both Aβ and tau pathogenesis and cognitive impairments. Therefore, they are suitable for assessing the association between Aβ or tau pathogenesis and cognitive impairment. Fourth, the touchscreen operant system was used to evaluate diverse behaviors of 3xTg-AD mice throughout their age in this study. The touchscreen operant system is based on Cambridge Neuropsychological Test Automated Battery (CANTAB), the human touchscreen cognition assessment test. It can evaluate diverse domains of cognitive functions impaired in AD, such as attention, associative memory, and long-term memory, on the same subjects accurately and highly translational to human clinical trials (Table 3).[339,340] Lastly, behavioral assessment using the touchscreen operant system was conducted by matching the behavioral evaluation period to AD progression to assess diverse domains of cognitive functions impaired in AD, such as executive functions and long-term memory. Therefore, the following is the order of tasks performed in this study: fixed ratio (FR) and progressive ratio (PR) task → 5-choice serial reaction time (5-CSRT) task → paired associates learning (PAL) task → visual discrimination (VD) and reversal task.

This study assessed the following using 3xTg-AD, transgenic AD model mice. First, how chronic metformin treatment altered cognitive functions were assessed using the touchscreen operant system. Second, how chronic metformin treatment altered whole-body regulation was evaluated. Third, how chronic

metformin treatment altered AMPK regulation was assessed. Fourth, how chronic metformin treatment altered AD pathology was evaluated. Therefore, the genuine effects of metformin on behaviors and AD pathology were validated for the further safety and universal usage of metformin. Moreover, whether metformin worked as an AMPK regulator and caused any side effects under chronic treatment were also evaluated.

Table 3. Tasks of touchscreen operant system

Task	Cognitive function	Brain region	AD stage
FR and PR	Satiety Motivation	Amygdala Anterior cingulate cortex Dorsolateral prefrontal cortex Hypothalamus Orbitofrontal cortex Striatum	All stage
5-CSRT	Attention Inhibitory control	Prefrontal and parietal cortex	Early-stage - Attention - Inhibitory control (Inhibitory) Late stage - Inhibitory control
PAL	Object-location associative learning and memory	Hippocampus	Middle to late-stage
VD	Visual perception Basic non-hippocampal associative learning	Prefrontal and temporal cortex Visual area Striatum	Not affected
Reversal	Cognitive flexibility	Prefrontal cortex	Late-stage
Retention	Long-term memory	Hippocampus	Middle to late stage

Abbreviations: AD, Alzheimer's disease; FR, fixed ratio; PR, progressive ratio; 5-CSRT, 5-choice serial reaction time; PAL, paired associates learning; VD, visual discrimination.

II. MATERIALS AND METHODS

1. Animal

3xTg-AD [B6;129-Tg(APPswe, tauP301L)1Lfa Psen1tm1Mpm/Mmjax] mice were used as a transgenic AD mouse model to elucidate the effects of chronic metformin treatment on AD. The original line of 3xTg-AD mice was generated by Professor Frank M. LaFerla. Two transgene constructs containing APPswe and tauP301L were co-injected into single-cell embryos harvested from homozygous presenilin-1M146V knock-in mice to generate homozygous 3xTg-AD.[341] Male and female 3xTg-AD mice (Male, total: n = 26, control: n = 14, metformin: n = 12; Female, total: n = 11, control: n = 6, metformin: n = 5; MMRRC Stock No. 34830-JAX; The Jackson Laboratory, Bar Harbor, ME, USA) were bred and housed in groups of 1 – 4 per cage in a specific pathogen-free room with 12 hr light/dark cycle (lights on from 8:00 a.m. to 8:00 p.m.) and humidity- and temperature-controlled environment. Water was provided *ad libitum* throughout the experiment. Cages of animals were changed once a week by a researcher.

To ensure the effects of chronic metformin treatment on behaviors and AD pathology clearly, the experiments of this study were conducted on two cohorts. Cohort 1 was consisted of only male 3xTg-AD mice with food restriction to evaluate the alterations in behaviors via metformin as food restriction is obligatory for touchscreen-based cognitive function assessment. Moreover, different sexes could not be in the same touchscreen operant chamber via hormonal differences. The onset of cognitive function assessment was at 9-month-old when 3xTg-AD mice showed a cognitive decline.[342] All behavioral experiments were conducted once a day for 5-7 days a week. Cohort 2 was

consisted of female 3xTg-AD mice without food restriction to evaluate the alterations in AD pathology via metformin as AD pathology is more severe in females than males.[343] By maintaining a free-feeding condition, any possible alteration in AD pathology via caloric restriction could be excluded. All procedures were approved by Yonsei University Health System Institutional Animal Care and Use Committee (IACUC) and performed in accordance with National Institute of Health guidelines for the Care and Use of Laboratory Animals.

2. Drugs and antibodies

Metformin (1,1-dimethylbiguanide hydrochloride) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Following primary antibodies were used in this study. Anti-phospho-AMPKα (Thr172; Catalog No. 2535), AMPKα (Catalog No. 5831), AMPKα1 (Catalog No. 2795), AMPKα2 (Catalog No. 2757), phospho-ACC (Ser79; Catalog No. 11818), Aβ (D54D2; Catalog No. 8243), BACE1 (Catalog No. 5606), pGSK3β (Ser9; Catalog No. 9322), GSK3α/β (Catalog No. 5675), phospho-tau (Ser396; Catalog No. 9632), and tau46 (total tau; Catalog No. 4019) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-phospho-tau (Ser199/202; Catalog No. 44-768G) and phospho-tau (Thr231; Catalog No. 44-746G) antibodies were purchased from Invitrogen (Carlsbad, CA, USA). Anti-ACC (Catalog No. 05-1098) was purchased from Merck Millipore (Burlington, MA, USA). Anti-Aβ (H-43; Catalog No. sc-9129), ADAM10 (Catalog No. sc-28358), TACE (Catalog No.13973), phospho-tau (Ser262; Catalog No. sc-32828), phospho-tau (Ser356; Catalog No. sc-101814), and β-actin (Catalog No. 47778) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Anti-APP or Aβ (6E10; Catalog No. SIG-39320), Aβ (4G8; Catalog No.

SIG-39200), and sAPPβ (Catalog No. SIG-39138) antibodies were purchased from BioLegend (San Diego, CA, USA). Following secondary antibodies were used in this study. Goat-anti-rabbit linked with HRP (Catalog No. 7074) and horse-anti-mouse linked with HRP (Catalog No. 7076) antibodies were purchased from Cell Signaling Technology. Goat-anti-rabbit Alexa Fluor-488 conjugated (Catalog No. 111-546-144) and donkey-anti-goat Alexa Fluor-488 conjugated (Catalog No. 705-546-147) antibodies were purchased from Jackson Immunoresearch Lab, Inc. (West Grove, PA, USA).

Anti-phospho-AMPKα (Thr172), AMPKα, AMPKα1, AMPKα2, phospho-ACC (Ser79), ACC, APP or Aβ (6E10), sAPPβ, Aβ (H-43), ADAM10, TACE, BACE1, phospho-tau (Ser199/202), phospho-tau (Ser262), phospho-tau (Ser356), phospho-tau (Ser396), phospho-tau (Thr231), tau46, and β-actin primary antibodies and goat-anti-rabbit and horse-anti-mouse linked with HRP secondary antibodies were used in western blot analysis. Aβ (D54D2) and Aβ (4G8) primary antibodies and goat-anti-rabbit Alexa Fluor-488 conjugated secondary antibodies were used in immunohistochemistry.

3. Drug administration

Drug administration was started when the mice became 3-month-old. By diluting metformin into the drinking water, 2 mg/ml of metformin was orally treated in mice. For avoiding any aversive responses to metformin, the dose of metformin was gradually increased by week (0 mg/ml → 0.5 mg/ml → 1 mg/ml → 2 mg/ml). The dose of metformin in this study was chosen as 2 mg/ml as this dose is equivalent to 300 mg/kg/day in clinical usage.[210] Moreover, 300 mg/kg/day is comparable to 2,000 mg/day, the maximum daily defined dose

(DDD) of metformin, according to WHO Collaborating Centre for Drug Statistics Methodology.[222,344] Water and drug were changed twice a week.

4. Food restriction

Cognition-behavior assessment with a touchscreen operant system is based on the appetitive behaviors on the reward, and these appetitive behaviors can be modulated by restricting food. Food restriction was started when the mice became 4-month-old. After a one-week acclimatization period to the researcher, the weight of each mouse was measured under the free-feeding condition for three consecutive days. Mice maintained 90 - 85% of their average free-feeding weight by regulating the amount of food pellet every day. The amount of food pellet was designated by the change of daily weight of each mouse. Weight measurement and pellet distribution were conducted every day to maintain their weights accurately. The weight was reduced gradually, and the weight reduction per day should not exceed 5% of the free-feeding weight of each mouse. The number of pellets should be matched to the number of mice in each cage. Pellets were scattered on the bedding of the cage to let all the mice take the pellet equally.

5. Touchscreen operant system

A. Apparatus

Behavioral assessments were conducted on standard Bussey-Saksida mouse touchscreen chambers (Campden Instruments Ltd., Loughborough, UK) and associated software (ABETII and Whisker Control, Campden Instruments Ltd.). The touchscreen operant apparatus consisted of four standard modular testing chambers with a wooden sound- and light-attenuating box (56.5 × 54.5 × 53.2

cm, Campden Instruments Ltd.). The trapezoidal-shaped testing chamber consisted of a metal frame, Perspex sidewalls, and a stainless-steel grid floor (20 × 18 × 24 cm, Campden Instruments Ltd.). A touch-sensitive screen (12.1-inch, resolution: 800 × 600) and a reward receptacle (magazine) with a 3 W light bulb for illumination and an infrared beam for head entry detection were fitted at the front and rear sides of the chamber, respectively. The magazine was attached to a reward dispenser filled with the strawberry milkshake (Seoul Strawberry Milk®, SeoulMilk Dairy Cooperative, Seoul, Korea) and provided 20 μl of reward per trial. A 3 W house light, a tone generator, a tone generator, and an infrared camera were housed in the box. The house light was equipped above the chamber for the punishment signal against incorrect response. The tone generator was installed for the reward delivery signal. The fan was fitted for ventilation and external noise reduction. The infrared camera was housed above the chamber for recording the movement of subjects. Infrared beams were assembled around the testing chamber to detect subjects' movements. The front infrared beam was 6 cm away from the screen, and the rear beam was 3 cm away from the magazine. A black Perspex mask (24.3 × 28.0 cm, Campden Instruments Ltd.) with task-specific response windows was placed in the front of the screen. It reduced any unintended touches by the tail or other body parts of subjects and distinguished the location of stimuli by presenting them on each window divided by a certain gap (window size: for the 2-window mask, 7.0 × 7.5 cm; for the 3-window mask, 7.1 × 7.1 cm; for the 5-window mask, 4 × 4 cm). ABET II enables the environment, experiment, task setting, data visualization, and analysis. Stimulus delivery and detection, operant box input and outputs, and results recording are controlled by Whisker Control.[345]

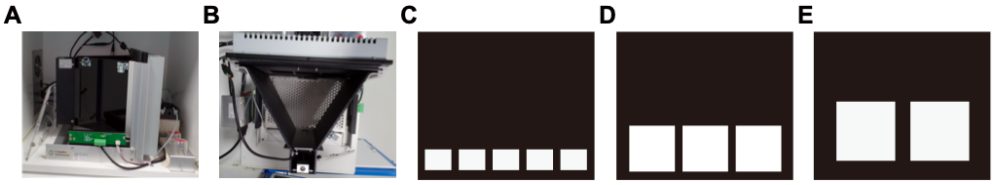

Figure 5. Touchscreen operant system apparatus. (A and B) Touchscreen operant system chamber. (C) 5-window mask. (D) 3-window mask. (E) 2-window mask.

B. Habituation and pretraining

(A) Habituation

Mice were acclimated to the researcher for one week. Before the cognitive function assessments using the touchscreen operant system, mice were habituated to the touchscreen chamber and the reward for at least two days. The touchscreen remained inactive during the habituation session. Before the onset of each session, 200 µl of reward was delivered in the magazine aperture. Mice freely explored the chamber and took the reward for 20 min. After the termination of each session, the movement of mice was evaluated by front and rear beam break to clarify any defect in the motor function and whether mice were habituated to the chamber. The reward consumption was also monitored to verify that mice were habituated to the reward. After all mice consumed the reward completely for two consecutive days, they proceeded to the next training.

(B) Pretraining

After completing habituation, mice have trained a series of pretraining procedures to operate touchscreen tasks. The following tasks were conducted to train the series in the Pavlovian stages in the mice: initial touch training (ITT), must touch training (MTT), and must initiate training (MIT). Before the onset of the FR and PR task, ITT was conducted first. During this stage, mice were trained to associate the stimulus offset with the reward delivery. A white square stimulus was presented in the middle window of the 5-window mask. The stimulus disappeared after 30 sec with a tone (1,000 ms, 3 kHz), and the magazine was illuminated with the delivery of the reward (20 μl). After the mouse collected the reward by putting its head into the magazine, the magazine light extinguished, and the subsequent trial began by presenting the stimulus on the screen after a 5 sec of inter-trial interval (ITI; house light off, no stimulus, inactive magazine). When the mouse responded to the stimulus by the nose-poking, a triple amount of the reward was delivered (60 μl). Mice should complete 30 trials within 60 min for one day. After each mouse accomplished this stage, they proceeded to the next stage, MTT. Mice were trained to associate the response to the stimulus with the delivery of reward during this stage. The stimulus remained on the screen until the mouse nose-poked it. When the mouse nose-poked the stimulus, the reward was delivered with the tone and the magazine illumination. The reward collection was followed by a 5 sec of ITI, and the subsequent trial began. Mice should complete 30 trials within 60 min for one day. After all mice completed this stage, mice proceeded to FR and PR tasks.

After the FR and PR task, MIT was conducted before the onset of the 5-CSRT task. Mice were trained to initiate a new trial. At the onset of each trial, the magazine was illuminated without the reward until the mouse nose-poked the

magazine and broke the infrared beam. After nose-poking, the magazine light disappeared, and the new trial commenced by presenting the stimulus on the screen. In this stage, the stimulus randomly appeared on one window of the 5-window mask. After the nose-poking to the stimulus and reward collection, the magazine was illuminated after 5 sec of ITI, which means the onset of the new trial. Mice should complete 30 trials within 60 min for two consecutive days. After all mice accomplished this stage, they proceeded to the 5-CSRT task.

C. 5-Choice serial reaction time (5-CSRT) task

(A) Acquisition

After the FR and PR tasks, mice conducted MTT before performing the 5-CSRT. Once all mice accomplished MTT for two consecutive days, the 5-CSRT task was started. For the 5-CSRT task, the 5-window mask was used (Fig 5E). A white square stimulus was presented on one of five spatially different windows pseudo-randomly (Fig 6A). The number of stimuli on each window was equal. One location was not repeated for more than two consecutive trials. The stimulus disappeared after stimulus duration (SD). However, the nose-poking to the screen was still recorded as a response for an extra 5 sec after the extinguishment of the stimulus; this period is called limited hold (LH). Therefore, mice should nose-poke the stimulus accurately during SD and LH.

Mice performed the task with 60 trials once a day for 60 min. Each trial commenced with the illumination of the magazine, and mice should nose-poke the magazine for the initiation of the trial. After a 5 sec of delay, the stimulus appeared. During this delay, the mice should focus on the screen. If the mouse nose-poked the screen before the stimulus presentation, it was recorded as

"premature," and the punishment time-out was activated for 5 sec with sudden illumination of light in the chamber. This punishment was followed by a 5 sec of ITI. This premature response was excluded from the total trials. When the mice responded to the stimulus during SD and LH, it could be recorded as correct when they responded to the stimulus or incorrect when they responded to the blank. A correct response was followed by a tone, magazine illumination, and reward delivery. After the reward collection, the magazine light turned off, and a 5 sec of ITI began. An incorrect response triggered a 5 sec of punishment time-out. This punishment was followed by a 5 sec of ITI. The correct and incorrect responses were recorded as "accuracy." If the mouse failed to respond to the stimulus or blank during SD and LH, it was recorded as "omission." After the omission response, a 5 sec of punishment time-out and a 5 sec of ITI were followed. After the ITI in every response, the magazine was illuminated for the subsequent trial initiation. The omission response did not affect the % of accuracy. If the mice kept responding to the correct or incorrect stimulus even after their responses were recorded, it was recorded as "perseveration."

The task was started with stage 9, which comprises 32 sec of SD and 5 sec of LH. If each mouse accomplished the stage for two consecutive days with ≥ 80% accuracy and ≤ 20% omission (the criterion of the 5-CSRT task), they moved to the next stage. As the stage proceeded, SD became shorter, while LH was fixed as 5 sec (e.g., stage 10, 16 sec of SD; stage 11, 8 sec of SD; stage 12, 4 sec of SD; stage 13, 2 sec of SD). The mice, which completed the criterion of the 5-CSRT task in stage 13 for two consecutive days, rested and had a reminder session once a week until all the other mice finished stage 13 successfully to prevent any over-training and match the acquisition levels of all mice. After all mice achieved the criterion of the 5-CSRT task in stage 13, they conducted stage

13 for two consecutive days to set the baseline. The number of sessions that took to accomplish stage 13 was measured.

(B) Probe test

After the baseline sessions, mice were moved to the within-session probe test, the more attentionally demanding test with different SD: 2.0 sec, 1.5 sec, 1.0 sec, 0.5 sec. This probe test was conducted for four consecutive days. Each probe session lasted for 60 min and had 60 trials. Four different SD appeared pseudo-randomly during each session, and the number of each SD was equal per session. Attention was analyzed by measuring accuracy [% of correct trials divided by total responded (correct and incorrect) trials] and omission (% of omission trials divided by total trials). Inhibitory control was assessed by measuring premature responses (the number of premature trials) and perseveration responses to correct and incorrect stimuli (the number of perseveration responses per choice). In addition, motor function was measured by beam break and latencies.

D. Paired associates learning (PAL) task

(A) dPAL task

After the 5-CSRT task, the dPAL task was begun. The 3-window mask was used (Fig 5D). The stimuli were presented only on the two spatially separated windows. The stimuli remained on the screen until the mouse nose-poked it.

Two different stimuli appeared on the two spatially different windows. One stimulus is in the correct location (S+), and the other is in the incorrect location (S-). The rest of one window remained blank. As line-shaped stimuli could

reduce the performance variability more than shape stimuli, three different line-shaped stimuli were used.[346] In this task, six different stimuli combinations were presented (Fig 6B). The number of each combination presentation was equal per session, and one combination was not repeated in more than two consecutive trials.

The magazine was illuminated before the onset of each trial. The mouse should nose-poke the magazine to initiate the trial. If the mouse nose-poked the correct stimulus, the reward was delivered with a tone and the magazine illumination. After the reward collection, 15 sec of ITI was commenced. After the mouse responded to the incorrect stimulus, a 5 sec of punishment time-out and a 5 sec of ITI were commenced. For the correct response, the subsequent trial with magazine illumination was given. However, for the incorrect response, the correction trial was given. The correction trial presents the same stimuli combination of the previous trial until the mouse chose the correct stimulus. This trial was excluded from the total trials and the analysis of accuracy. It only reduces the unwanted side-biases, not the main target of interest, during object-location associative learning. The number of trials per session was started at 24 and gradually increased up to 72 by 12 (e.g., 24, 36, 48, …), and each session lasted for 60 min. Mice were trained until they completed 72 trials in 60 min and their group average accuracy was ≥ 80% for at least three consecutive days. For object-location associative learning and memory, the accuracy % and the number of correction trials per block were analyzed. A block is combined with approximately 300 trials (range of 288-324).

(B) sPAL task

After the dPAL task acquisition, the baseline of the dPAL task (total number of trials: 60) was set for two consecutive days without the correction trials. After setting the baseline, mice moved to the sPAL task. The procedure of the sPAL task was the same as the dPAL task, except there were no correction trials, and the same stimulus was presented on two spatially different windows (Fig 6C). Therefore, one location was with the correct stimulus (S+), while the other was with the incorrect stimulus (S-). This task was conducted for three consecutive days, and each session lasted for 60 min with 60 trials. The purpose of the sPAL task is to elucidate whether the mice performed the dPAL task with object-location associative memory or conditional rule such as "if AC, select the left; if BC, select the right".[346]

(C) Retention session

After the sPAL task, mice conducted the sPAL retention sessions once a week for three weeks to confirm that the memory of reward contingency in the sPAL task was stably maintained.

E. Visual discrimination (VD) and reversal task

(A) VD task

After the retention sessions of the sPAL task, the VD task was conducted. For the VD task, the 2-window mask was used (Fig 5C). Two different stimuli shapes were presented on two spatially different windows (Fig 6D). The location of each stimulus was pseudo-random, and the number of the stimulus appearance

at each location was equal. The stimuli did not appear in the same location for more than three consecutive trials. The stimuli remained on the screen until the mouse nose-poked the stimulus.

Each session lasted for 30 min with 30 trials. After the mice nose-poked the magazine to turn off the light for initiating the trial, the stimuli were presented on the windows. One stimulus is the correct (S+) signal, and the other is the incorrect (S-) signal. Unlike the PAL task, the correct or incorrect signal only depended on the shape of the stimulus, not the location. When the mice nose-poked the correct stimulus, the reward was delivered with a tone and the magazine illumination. After the reward collection, 20 sec of ITI began. Then, the magazine was illuminated again for the initiation of the subsequent trial. However, the response to the incorrect stimulus was followed by a 5 sec of punishment time-out and a 20 sec of ITI. After the ITI, the correction trial began with the illumination of the magazine until the mouse responded to the correct stimulus. When the mice achieved ≥ 80% accuracy for two consecutive days (the criterion of the VD task), they rested and had reminder sessions once a week until all other mice reached the criterion. Total sessions, correct trials, and correction trials to the criterion were measured to analyze the visual discrimination. In addition, beam break and latencies were measured to assess the motor function.

(B) Reversal task

The 2-window mask was used for the reversal task (Fig 5C). Two different stimuli shapes were presented on two spatially different windows (Fig 6E). After all mice learned the VD task, they conducted it for two more consecutive days to set the baseline. A reversal task was conducted the following day of the

baseline sessions. The procedure of the reversal task was the same with the VD except that the correct and incorrect signals were switched. The former S+ signal became S-, and the S- signal became S+. Additionally, as the reversal task assesses the cognitive flexibility that requires sudden decision-making, the correction trial was excluded during this task. When each mouse achieved ≥ 80% of accuracy for two consecutive days (the criterion of the reversal task), it rested. Total sessions, correct trials, and accuracy were measured for elucidating cognitive flexibility. In addition, beam break and latencies were measured for monitoring the motor function.

(C) Retention session

Each mouse conducted one retention session ten days after the last day of the reversal task to confirm that the memory of reward contingency in the reversal task was stably maintained.

F. Fixed ratio (FR) and progressive ratio (PR) task

(A) FR task

For the FR and PR tasks, the 5-window mask was used (Fig 5E). A white square stimulus was presented only on the middle window of the mask (Fig 6A). The stimulus remained on the screen until the mouse nose-poked it.

Mice conducted the FR task once a day for 60 min. Between the trials, a 4.5 sec of ITI was given. Each mouse proceeded to the next stage after accomplishing whole trials of each stage for one day. FR1, FR2, FR3, and FR5 stages were proceeded in sequential. Mice were trained to collect a single reward

by a single nose-poking for 30 trials (total nose-poking: 30 times) during the FR1 stage. For the FR2 stage, mice should nose-poke twice to collect a single reward for 15 trials (total nose-poking: 30 times). To complete the FR3 stage, mice should nose-poke three times to get a single reward for ten trials (total nose-poking: 30 times). Finally, during FR5 sessions, the mice got a single reward by nose-poking the stimulus five times for 30 trials (total nose-poking: 150 times). When the mice completed the FR5 sessions, they rested and had a reminder session once a week until all the other mice finished FR 5 sessions successfully to prevent any over-training and match the acquisition levels of all mice.

To set the baseline, all mice should accomplish FR5 for two consecutive days. After setting the baseline, they moved to FR5-uncapped (FR5-UC), a probe test that evaluates satiety and motivation. FR5-UC was conducted for 60 min; however, there was no limit to the number of trials. FR5-UC sessions were performed for two separate days by maintaining the weight of the mice between sessions. Total trials, target touches, and blank touches were analyzed for evaluating satiety and motivation. In addition, front and rear beam break and response and reward collection latencies were analyzed to assess the motor function. The movements at the touchscreen side and the magazine side were measured by the front beam break and rear beam break, respectively. The time spent nose-poking the correct signal and reaching the magazine for getting the reward was measured by response latency and reward collection latency, respectively.

(B) PR task

After the FR task, the PR task was conducted once a day. Before the onset of the PR4 stage, the FR5 stage was conducted for two consecutive days to set the

baseline. Then, PR4 was performed for three consecutive days. The number of nose-poking on the screen per trial was fixed in the FR task. For PR 4, the response number per trial was gradually augmented on a linear +4 basis (e.g., 1, 5, 9, 13, …). Each session lasted for 60 min; however, if the mice did not touch the stimulus or collect the reward on the magazine for 5 min, the session was terminated. Between the trials, a 4.5 sec of ITI was given. Breakpoint, total target touches, and blank touches were analyzed for evaluating motivation. In addition, beam break and latencies were analyzed to assess the motor function. The breakpoint is defined as the number of nose-poking to stimulus during the last complete trial that the reward was successfully given within a session.

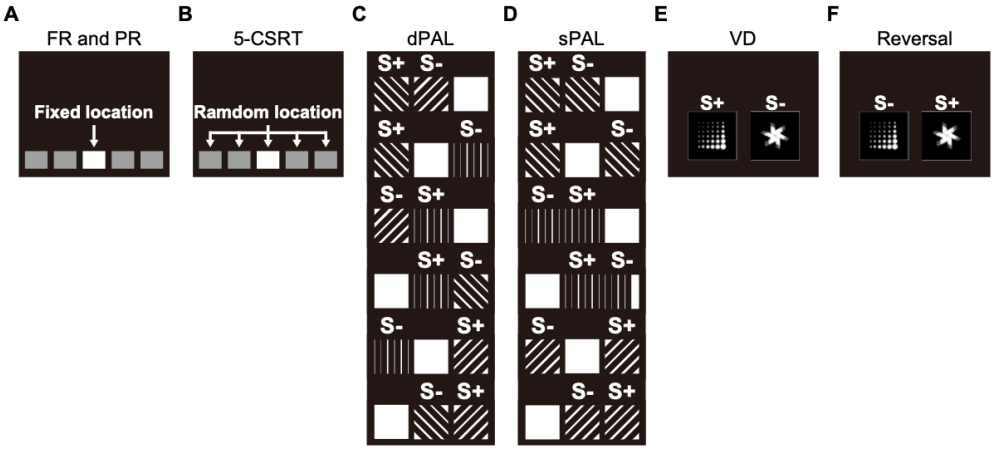

Figure 6. Stimuli of touchscreen operant system tasks. Stimulus for (A) FR and PR, (B) 5-CSRT, (C) dPAL, (D) sPAL, (E) VD, and (F) reversal task.

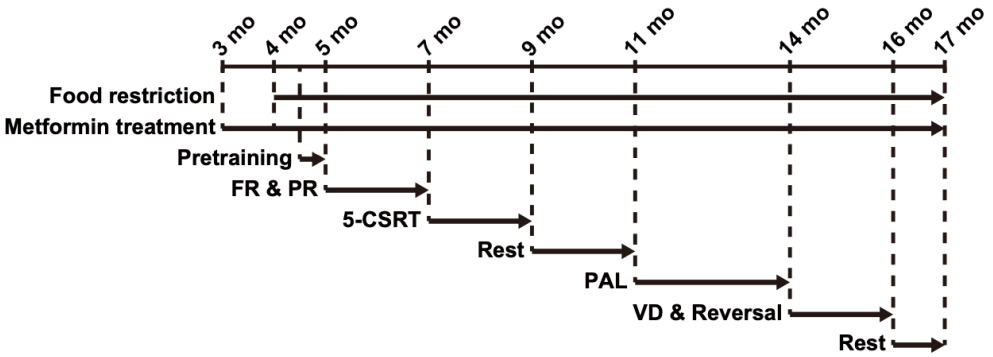

Figure 7. Experimental timeline of this study. Each task was performed in sequence to assess the cognitive function impaired in a certain age of 3xTg-AD mice.

6. Serum extraction

Whole blood was collected from the anesthetized mice by heart puncture. Blood samples were transferred to a BD Microtainer tube (Becton, Dickinson and Company, NJ, USA) and left in RT for 30 min to clot the blood. Then, the samples were centrifuged at 1300 ×g for 30 min. The serum left above the polymer barrier was harvested into the new tube. The serums were stored at -80 ℃ until further analysis.

7. Liquid chromatography-mass spectrophotometry (LC-MS)

The concentrations of metformin and vitamin B12 in mouse serum were measured by liquid chromatography-mass spectrophotometry (LC-MS). To detect metformin from the serums, 1400 µl of extraction solvent (methanol:isopropanol:distilled water=3:3:2, v/v/v) was added to 50 µl of serum samples. The solutions were kept on ice and sonicated for 15 min. After

centrifugation at 13,200 rpm and 4℃ for 15 min, supernatants of samples were collected into new tubes. The samples were dehydrated and concentrated by a ScanVac speed vacuum concentrator (Labogene, Seoul, Korea). The extracts were stored at -80℃ until further analysis.

The dried samples were reconstituted with 250 μl of distilled water containing 0.1% formic acid for LC-Orbitrap MS analysis. Chromatographic separation was performed on an Ultimate-3000 UPLC system (Thermo Fisher Scientific, MA, USA) and an Acquity UPLC BEH C18 Column (1.7 μm; 100 mm × 2.1 mm; Waters, MA, USA) equipped with UPLC BEH HILIC VanGuard pre-column (1.7 μm; 5.0 mm × 2.1 mm; Waters). The mobile phase consisted of two different buffers: buffer A (0.1% formic acid in distilled water) and buffer B (0.1% of formic acid in acetonitrile). The flow rate was set to 0.3 ml/min, and a gradient of buffer B phase was programmed as follows: 0–0.1 min, 0.5% B; 10 min, 80% B; 10.1–12 min, 99.5% B; 12.1–15 min, 0.5% B. Mass-spectrometric analysis was performed on a Q-Exactive plus instrument (Thermo Fisher Scientific) with positive ionization mode for detection. The acquisition method was conducted using a Full MS scan ranging from 120 to 1800 m/z. Data acquisition and preprocessing were managed by Xcalibur software (Thermo Fisher Scientific). RAW data files were processed using Compound Discoverer 2.0 software (Thermo Fisher Scientific). Compound annotation was performed against mzCloud node in which the MS2 mass tolerance and annotation threshold were set to 10 ppm and 70, respectively.

8. Blood glucose concentration measurement

The mouse tail was slightly cut for collecting a blood sample. Blood glucose concentration was measured from the blood sample by glucometer system Accu-

Chek Performa kit (Roche Diagnostics GmbH, Mannheim, Germany) twice before the onset of the behavioral assessment (initial) and after the end of the behavioral assessment (final). Both time points were under the food restriction condition.

9. Western blot analysis

Fresh hippocampi were dissected from the anesthetized mice and stored at -80 ℃ until the further experiment. Hippocampi were homogenized with lysis buffer containing 20 mM Hepes (pH 7.0), 1 mM EDTA, 1 mM EGTA, 10 mM KCl, 1.5 mM $MgCl_2$, 250 mM sucrose, phosphatase inhibitors, and protease inhibitors (Complete, EDTA-Free Protease Inhibitor Cocktail; Roche, Indianapolis, IN, USA) as the concentration of 10 μl per 1 mg wet weight of hippocampal tissues. Homogenates were incubated for 15 min on ice with vortexing every 2-3 min, and then samples were centrifuged at 8,000 ×g and 4℃ for 30 min. After collecting the supernatants from the homogenates, the concentrations of proteins were quantified by DC assay (Bio-Rad Laboratories, Hercules, CA, USA) and ELISA microplate reader (Versamax microplate reader, Molecular Devices, Sunnyvale, CA, USA). The software (SoftMax Pro, Molecular Devices, Sunnyvale, CA, USA) was used to create the standard curves and measure the concentration of hippocampus protein. Each sample with 20 μg protein was separated by 8% or 10% SDS-PAGE and transferred onto PVDF membranes (Merck Millipore). Membranes were washed with tris-buffered saline (TBS; 50 mM Tris-HCl, 140 mM NaCl, pH 7.3) containing 0.1% Tween-20 (TBS-T) and blocked nonspecific binding with 5% skim milk in TBS-T for 1 hr at RT. After blocking, membranes were incubated with primary antibodies (1:1,000-5,000) overnight at 4°C. After washing for 5 min three times with TBS-T, membranes were incubated with secondary antibodies conjugated with

horseradish peroxidase (1:5,000-10,000) for 1 hr at RT. After washing for 5 min three times with TBS-T, enhanced chemiluminescence solution (Amersham™ ECL™ western blotting detection reagent; GE Healthcare, Piscataway, NJ, USA) was applied on the membranes to detect target protein bands by a luminescent image analyzer (ImageQuant LAS 4000 mini; GE Healthcare). The intensities of detected target protein bands were normalized by β-actin.

10. Immunohistochemistry

Brain hemispheres were freshly embedded with OCT compound in cryomold (Sakura Finetek) and frozen. Fresh frozen brains were cut into 15 μm coronal sections on a cryostat (Leica biosystems, Buffalo Grove, IL, USA). The sections containing the cortex and hippocampus were immediately placed on gelatin subbed slides and stored at -80℃ until further analysis. The sections were airdried overnight and fixed with phosphate-buffered saline (PBS) containing 4% paraformaldehyde for 10 min at RT. For D56D2 immunostaining, the sections were permeabilized with PBS containing 0.3% Triton X-100 for 1 hr and blocked with PBS containing 5% bovine serum albumin (BSA) and 0.3% Triton X-100 for 1 hr. Then, the sections were incubated with D56D2 antibody (1:100) diluted in PBS containing 2% BSA and 0.3% Triton X-100 overnight at 4℃. After washing for 5 min three times with PBS, the sections were incubated with Alexa-488 conjugated secondary antibody (1:100; goat-anti-rabbit) diluted in PBS containing 2% BSA for 1 hr at RT.

For 4G8 immunostaining, 70% formic acid in PBS was treated for 20 min on the sections for antigen retrieval to detect Aβ.[347] The sections were washed with PBS for 20 min to discard formic acid completely. Next, the sections were permeabilized with PBS containing 0.3% Triton X-100 for 1 hr and blocked with

Mouse-On-Mouse blocking reagent (MOM kit, Vector Lab, Inc., Burlingame, CA, USA) for 1 hr. After washing with PBS twice for 2 min, the sections were activated by MOM kit diluent for 10 min and incubated with 4G8 antibody (1:100) overnight at 4℃. After washing for 5 min three times with PBS, the sections were incubated with MOM biotinylated anti-mouse IgG (1:300) diluted in the diluent for 1 hr at RT. After washing for 5 min three times with PBS, the sections were incubated with fluorescein avidin (1:300; Vector Lab, Inc.) diluted in the diluent for 30 min at RT.

All sections were washed for 5 min three times with PBS and counterstained with 6-diamidino-2-phenylindole (DAPI; 1:2,000; Sigma-Aldrich) in PBS. The slides were mounted with Vectashield mounting medium (Vector Lab, Inc.) and covered by the coverslip. The immunoreactivity was observed by fluorescence microscope (Olympus, Tokyo, Japan).

11. Statistical analysis

All statistical analyses were conducted by R version 3.6.3[348] and Graphpad Prism version 9 (Graphpad Software Inc., La Jolla, CA, USA) to analyze the significance of differences between control (Ctrl) and chronically metformin-treated groups (MET). Unpaired Student's two-tailed t-test was conducted on single comparisons. Log-rank test was conducted on the survival rate and sessions to reach the criterion of mice. One-way analysis of variance analysis (ANOVA) was conducted on the concentration of metformin diluted in drinking water by days. Mixed-effects model was conducted on RM data to identify the main effect (group, age, concentration, time, session, SD, and block) and the interaction (group by age, group by concentration, group by time, group by session, group by SD, and group by block). Tukey's and Sidak's multiple

comparison *post hoc* tests analyzed simple main effects. Pearson's correlation coefficient between the variables of the 5-CSRT task was calculated. All data were expressed as the mean ± standard error of the mean (S.E.M.). Significance was set at $p \leq 0.05$.

III. RESULTS

PART 1: The effects of chronic metformin treatment on behaviors in 3xTg-AD mice

Various touchscreen operant system tasks were conducted in cohort 1 to elucidate the effects of chronic metformin treatment on cognitive decline shown in AD.[4,349]

1. 5-CSRT task

Attention and inhibitory control, one of the executive functions, are impaired in AD patients.[146] 5-CSRT task was conducted to elucidate the effects of chronic metformin treatment on attention and inhibitory control in 3xTg-AD mice.

A. Chronic metformin treatment did not alter the acquisition ability of 3xTg-AD mice

In the acquisition phase, the number of sessions that took to accomplish the criterion of the 5-CSRT task at the final stage was not different between groups (Fig 8, Student's t-test, t = 0.602, df = 23, p = 0.553). Therefore, acquisition ability was not affected by chronic metformin treatment in 3xTg-AD mice.

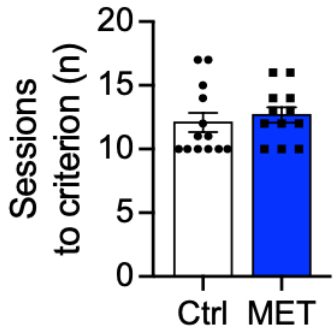

Figure 8. Effects of chronic metformin treatment on the acquisition of 5-CSRT task in 3xTg-AD mice. Acquisition levels were not altered by chronic metformin treatment in 7-month-old 3xTg-AD mice. Sessions to criterion are the number of sessions that took to achieve the 5-CSRT task criterion at the final stage. The graph of sessions to 5-CSRT task criterion of Ctrl (n = 13) and MET (n = 12). Data are presented as mean ± S.E.M. Unpaired Student's two-tailed t-test was used for statistical analysis. Ctrl; Control, MET; Metformin.

B. Chronic metformin treatment impaired the retrieval of attention in 3xTg-AD mice

The levels of accuracy were not different between groups of 3xTg-AD mice (Fig 9A, accuracy by SD, mixed-effects model, main effect of group: $F_{(1, 23)}$ = 0.043, p = 0.838, main effect of SD: $F_{(3, 369)}$ = 124.926, p < 0.0001, group by SD interaction: $F_{(3, 369)}$ = 0.268, p = 0.849; Fig 9C, accuracy by session, mixed-effects model, main effect of group: $F_{(1, 23)}$ = 0.060, p = 0.809, main effect of session: $F_{(3, 69)}$ = 1.233, p = 0.304, group by session interaction: $F_{(3.69)}$ = 0.903, p = 0.445)). However, the levels of omission were significantly increased by chronic metformin treatment as the probe sessions of 5-CSRT task were proceded (Fig 9B, omission by SD, mixed-effects model, main effect of group: $F_{(1, 23)}$ = 0.439, p = 0.514, main effect of SD: $F_{(3, 369)}$ = 99.622, p < 0.0001, group

by SD interaction: $F_{(3, 369)} = 0.083$, $p = 0.969$; Fig 9D, omission by session, mixed-effects model, main effect of group: $F_{(1, 23)} = 0.435$, $p = 0.516$, main effect of session: $F_{(3, 69)} = 2.784$, $p = 0.047$, group by session interaction: $F_{(3, 69)} = 2.881$, $p = 0.042$, simple effect of group in session 1: $F_{(1, 23)} = 7.865$, $p = 0.010$).

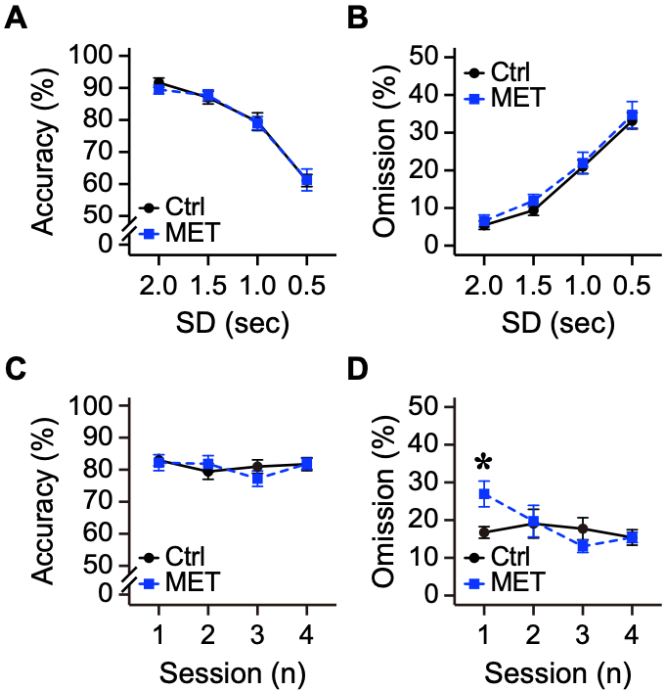

Figure 9. Effects of chronic metformin treatment on the attention in 3xTg-AD mice. Chronic metformin treatment delayed the regaining of the 5-CSRT task performance in 9-month-old 3xTg-AD mice. The 5-CSRT task was conducted to assess attention. (A) Accuracy and (B) omission of Ctrl (n = 13) and MET (n = 12) by SD randomly presented in the 5-CSRT probe test. (C) Accuracy and (D) omission of Ctrl and MET in each session of the 5-CSRT probe test. Data are presented as mean ± S.E.M. *$p < 0.05$ vs. Ctrl. Mixed-effects model with Tukey's multiple comparison test was used for statistical analysis. Ctrl, Control; MET, Metformin; SD, stimulus duration.

C. Chronic metformin treatment did not alter the inhibitory control in 3xTg-AD mice

Inhibitory control, such as impulsivity and compulsivity, was also measured by the 5-CSRT task. The impulsivity and compulsivity can be analyzed by premature responses and perseveration responses, respectively. The numbers of premature responses and perseverative responses to correct and incorrect stimuli wer not different between groups of 3xTg- AD mice (Fig 10A, the number of premature responses by SD, mixed-effects model, main effect of group: $F_{(1, 23)}$ = 0.120, p = 0.732, main effect of SD: $F_{(3, 369)}$ = 1.310, p = 0.271, group by SD interaction: $F_{(3, 369)}$ = 0.568, p = 0.636; Fig 10B, the number of perseveration responses to correct stimuli by SD, mixed-effects model, main effect of group: $F_{(1, 23)}$ = 0.497, p = 0.488, main effect of SD: $F_{(3, 369)}$ = 17.538, p < 0.0001, group by SD interaction: $F_{(3, 369)}$ = 0.195, p = 0.900; Fig 10C, the number of perseveration responses to incorrect stimuli by SD, mixed-effects model, main effect of group: $F_{(1, 23)}$ = 3.125, p = 0.090, main effect of SD: $F_{(3, 369)}$ = 1.690, p = 0.169, group by SD interaction: $F_{(3, 369)}$ = 1.264, p = 0.286; Fig 10D, the number of premature responses by session, mixed-effects model, main effect of group: $F_{(1, 23)}$ = 0.120, p = 0.732, main effect of session: $F_{(3, 69)}$ = 1.576, p = 0.203, group by session interaction: $F_{(3.69)}$ = 1.254, p = 0.297; Fig 10E, the number of perseveration responses to correct stimuli by session, main effect of group: $F_{(1, 23)}$ = 0.497, p = 0.488, main effect of session: $F_{(3, 69)}$ = 2.154, p = 0.101, group by session interaction: $F_{(3, 69)}$ = 0.264, p = 0.851; Fig 10F, the number of perseveration responses to incorrect stimuli by session, mixed-effects model, main effect of group: $F_{(1, 23)}$ = 3.125, p = 0.090, main effect of session: $F_{(3, 69)}$ = 1.186, p = 0.322, group by session interaction: $F_{(3, 69)}$ = 1.999, p = 0.122).

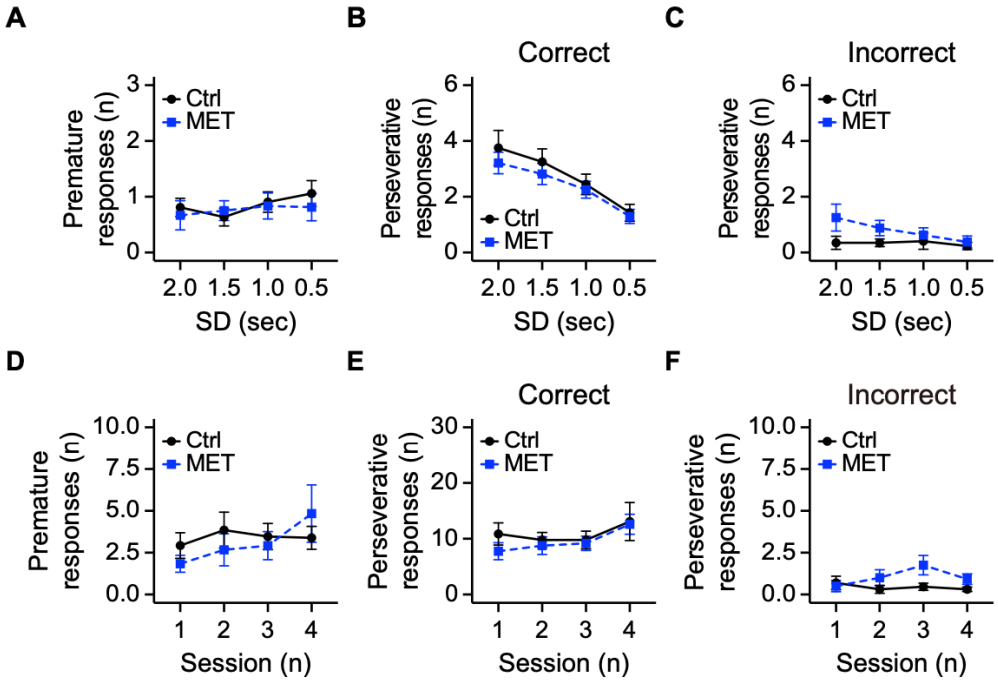

Figure 10. Effects of chronic metformin treatment on the inhibitory control in 3xTg-AD mice. Inhibitory control was impaired by chronic metformin treatment in 9-month-old 3xTg-AD mice. The 5-CSRT task was conducted to assess inhibitory control. The number of (A) premature responses, (B) perseveration responses to the correct stimulus, and (C) perseveration responses to the incorrect stimulus of Ctrl (n = 13) and MET (n = 12) by randomly presented SD in the 5-CSRT probe test. The number of (D) premature responses, (E) perseveration responses to the correct stimulus, and (F) perseveration responses to the incorrect stimulus of Ctrl and MET in each session of the 5-CSRT probe test. Data are presented as mean ± S.E.M. Mixed-effects model with Tukey's multiple comparison test was used for statistical analysis. Ctrl, Control; MET, Metformin; SD, stimulus duration.

D. Chronic metformin treatment did not alter the motor function in 3xTg-AD mice

As beam break rates and latencies were not different between groups during the 5-CSRT probe sessions, the motor function was not affected by chronic metformin treatment (Fig 11A, front beam break rate, mixed-effects model, main effect of group: $F_{(1, 23)} = 0.093$, $p = 0.763$, main effect of session: $F_{(3, 69)} = 3.265$, $p = 0.026$, group by session interaction: $F_{(3, 69)} = 1.031$, $p = 0.384$; Fig 11B, rear beam break rate, mixed-effects model, main effect of group: $F_{(1, 23)} = 0.102$, $p = 0.752$, main effect of session: $F_{(3, 69)} = 1.844$, $p = 0.147$, group by session interaction: $F_{(3, 69)} = 1.123$, $p = 0.346$; Fig 11C, response latency, mixed-effects model, main effect of group: $F_{(1, 23)} = 2.817$, $p = 0.107$, main effect of session: $F_{(3, 69)} = 0.271$, $p = 0.846$, group by session interaction: $F_{(3, 69)} = 0.516$, p = 0.673; Fig 11D, reward collection latency, mixed-effects model, main effect of group: $F_{(1, 23)} = 0.350$, $p = 0.560$, main effect of session: $F_{(3, 69)} = 0.587$, $p = 0.626$, group by session interaction: $F_{(3, 69)} = 0.744$, $p = 0.530$). Taken together, chronic metformin treatment impaired the attention restoration without altering the response control ablility and motor function of 3xTg-AD mice.

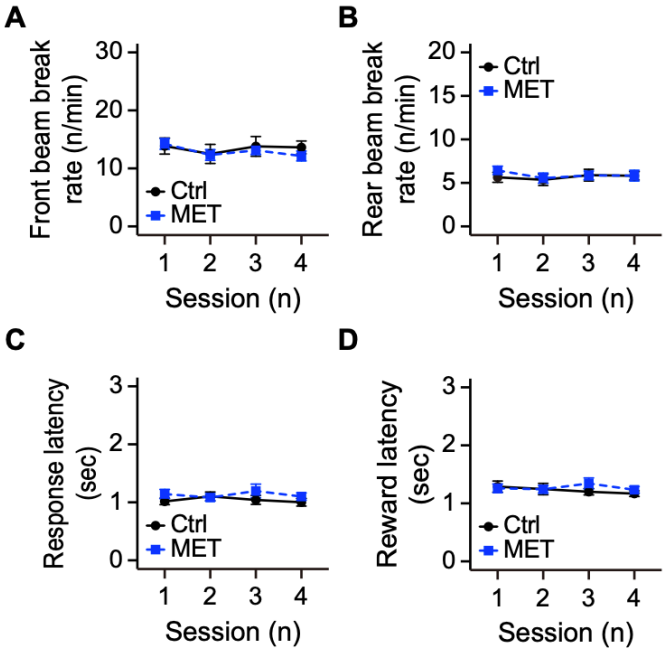

Figure 11. Effects of chronic metformin treatment on the motor function in 3xTg-AD mice. Motor function during the 5-CSRT task was not altered by chronic metformin treatment in 9-month-old 3xTg-AD mice. (A) Front beam break rate, (B) rear beam break rate, (C) response latency, and (D) reward collection latency of Ctrl (n = 13) and MET (n = 12) in the 5-CSRT probe test. Data are presented as mean ± S.E.M. Mixed-effects model with Tukey's multiple comparison test was used for statistical analysis. Ctrl, Control; MET, Metformin.

E. Chronic metformin treatment inversed the correlation between the variables of the 5-CSRT task in 3xTg-AD mice

The relationship between the variables in the 5-CSRT task was analyzed to evaluate how each variable is associated with each other and alters related behaviors. As attention and inhibitory control by SD were not altered by chronic metformin treatment (Fig 9A and B and 10A-C), there was no noticeable

alteration in the relationship between the variables (Fig 12). Chronic metformin treatment reversed the positive correlation in front beam break-premature responses, front beam break-perseverative responses to correct stimuli, and rear beam break-perseverative responses to correct stimuli into negative (Fig 12). Additionally, the negative correlation in response latency-perseverative responses to incorrect stimuli and reward collection latency-rear beam break was converted to positive by chronic metformin treatment (Fig 12).

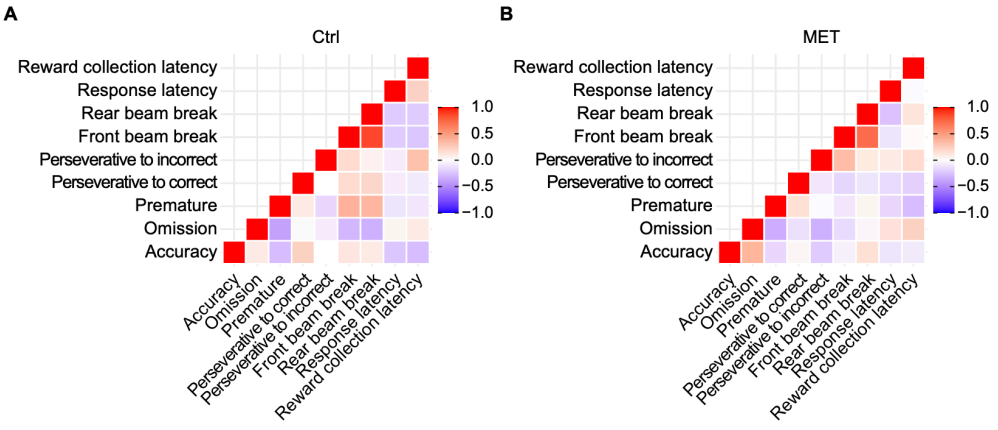

Figure 12. The relationship between the variables of the 5-CSRT task in 3xTg-AD mice. Correlation between the variables of the 5-CSRT task was reversed by chronic metformin treatment in 9-month-old 3xTg-AD mice (A-F). The heatmaps indicate the relationship between the variables in the 5-CSRT task of 8-month-old 3xTg-AD mice (E and F; Ctrl: n = 13, MET: n = 12). (A-F), Pearson's correlation coefficient was used for determining statistical significance. Ctrl, Control; MET, Metformin.

2. PAL task

After assessing executive functions via the 5-CSRT task, learning and memory were evaluated by the PAL task. Associative memory[350] and long-term memory[149] are impaired in AD patients. dPAL task, sPAL task, and sPAL retention sessions were conducted to elucidate the effects of chronic metformin treatment on object-location associative learning and memory, the usage of conditional rules, and long-term memory in the 3xTg-AD mice.

A. Chronic metformin treatment impaired the object-location associative learning and memory in 3xTg-AD mice

Chronic metformin treatment significantly delayed the increase of accuracy the decrease of the number of correction trials during the dPAL task (Fig 13A, response accuracy, mixed-effects model, main effect of group: $F_{(1, 14)} = 4.411$, $p = 0.054$, main effect of block: $F_{(9, 798)} = 171.409$, $p < 0.0001$, group by block interaction: $F_{(9, 798)} = 2.255$, $p = 0.017$, simple effect of group in block 6: $F_{(1, 14)} = 6.792$, $p = 0.021$, simple effect of group in block 7: $F_{(1, 14)} = 7.054$, $p = 0.019$, simple effect of group in block 9: $F_{(1, 14)} = 4.981$, $p = 0.043$; Fig 13B, the number of correction trial, mixed-effects model, main effect of group: $F_{(1, 14)} = 3.033$, $p = 0.104$, main effect of block: $F_{(9, 798)} = 40.114$, $p < 0.0001$, group by block interaction: $F_{(9, 798)} = 3.754$, $p = 0.00001$, simple effect of group in block 5: $F_{(1, 14)} = 5.129$, $p = 0.040$, simple effect of group in block 6: $F_{(1, 14)} = 5.905$, $p = 0.029$, simple effect of group in block 7: $F_{(1, 14)} = 6.953$, $p = 0.020$, simple effect of group in block 9: $F_{(1, 14)} = 5.392$, $p = 0.036$). Motor function was not affected by chronic metformin treatment as both response and reward collection latencies were not different in Ctrl and MET. (Fig 13C, response latency, mixed-effects model, main effect of group: $F_{(1, 14)} = 2.515$, $p = 0.135$, main effect of block: $F_{(9,}$

$_{798)}$ = 2.377, p = 0.012, group by block interaction: $F_{(9, 798)}$ = 0.415, p = 0.928; Fig 13D, reward collection latency, mixed-effects model, main effect of group: $F_{(1, 14)}$ = 1.147, p = 0.302, main effect of block: $F_{(9, 798)}$ = 1.664, p = 0.094, group by block interaction: $F_{(9, 798)}$ = 1.096, p = 0.363). These findings indicate that chronic metformin treatment impaired object-location associative learning and memory without altering motor function in 3xTg-AD mice.

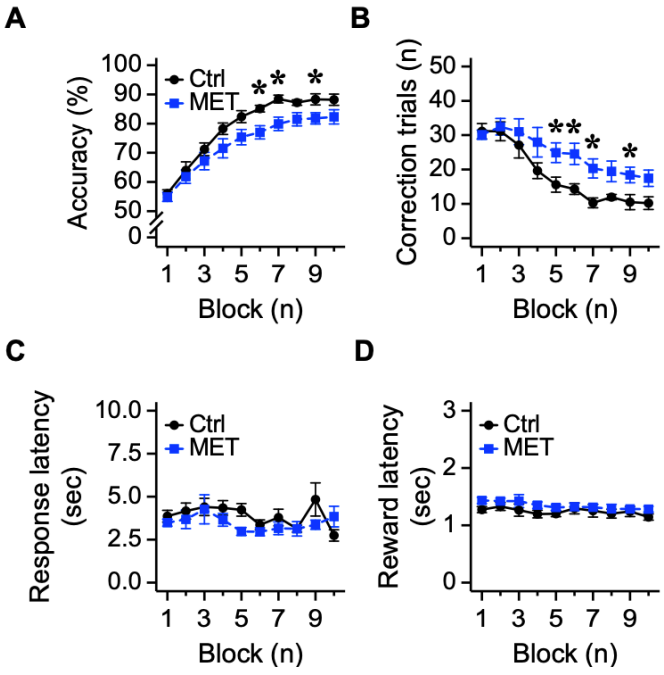

Figure 13. Effects of chronic metformin treatment on the object-location associative learning and memory in 3xTg-AD mice. Object-location associative learning and memory were impaired by chronic metformin treatment in 11-month-old 3xTg-AD mice. The dPAL task was conducted to assess object-location learning and memory. (A) Accuracy, (B) the number of correction trials, (C) response latency, and (D) reward collection latency of Ctrl (n = 6) and MET (n = 10). Data are presented as mean ± S.E.M. *p < 0.05 vs. Ctrl. Mixed-effects model with Tukey's multiple comparison test was used for statistical analysis.

Ctrl, Control; MET, Metformin. Block was combined with approximately 300 trials (range of 288-324).

B. Chronic metformin treatment blocked the consolidation of object-location associative memory in 3xTg-AD mice

Chronic metformin treatment significantly lowed the accuracy during the dPAL and sPAL sessions (Fig 14A, mixed-effects model, main effect of group: $F_{(1, 14)} = 6.703$, $p = 0.021$, main effect of session: $F_{(1, 14)} = 15.1$, $p = 0.002$, group by session interaction: $F_{(1, 14)} = 1.056$, $p = 0.322$). Motor function was not altered by chronic metformin treatment (Fig 14B, response latency, Student's t-test, t = 1.175, df = 16, $p = 0.221$; Fig 14C, reward collection latency, Student's t-test, t = 0.3755, df = 16, $p = 0.712$). Therefore, MET poorly attained the object-location associative memory than Ctrl.

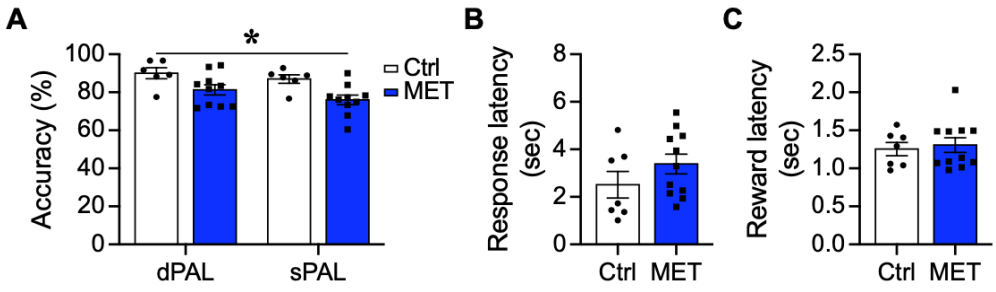

Fig 14. Effects of chronic metformin treatment on the consolidation of object-location associative memory in 3xTg-AD mice. Object-location associative memory was not attained properly by chronic metformin treatment in 13-month-old 3xTg-AD mice. The sPAL task was conducted to assess the usage of conditional rules and object-location associative memory. (A) Response accuracy, (B) response latency, and (C) reward collection latency of Ctrl (n = 6) and MET (n = 10) in dPAL and sPAL sessions. Data are presented as mean ±

S.E.M. *$p < 0.05$ Ctrl. Mixed-effects model with Sidak multiple comparison test was used for statistical analysis of (A). Unpaired Student's two-tailed t-test was used for statistical analysis of (B) and (C). Ctrl, Control, MET, Metformin.

C. Chronic metformin treatment impaired the long-term memory of the reward contingency of the PAL task in 3xTg-AD mice

In sPAL retention sessions conducted once a week for three weeks after the dPAL and sPAL sessions, MET showed significantly lower accuracy than Ctrl (Fig 15A, mixed-effects model, main effect of group: $F_{(1, 14)} = 19.084$, $p = 0.0006$, main effect of session: $F_{(3, 42)} = 7.817$, $p = 0.0003$, group by session interaction: $F_{(3, 42)} = 0.721$, $p = 0.545$). Even though MET took a longer time to respond to the correct stimuli during sPAL retention sessions, both groups took a similar time to take the reward. These findings indicates that motor function was not altered by chronic metformin treatment (Fig 15B, response latency, Student's t-test, t = 2.831, df = 16, $p = 0.012$; Fig 15C, reward collection latency, Student's t-test, t = 0.458, df = 16, $p = 0.653$). Taken together, chronic metformin treatment impaired the long-term memory about the association between the PAL task stimuli and the reward without altering motor function in 3xTg-AD mice.

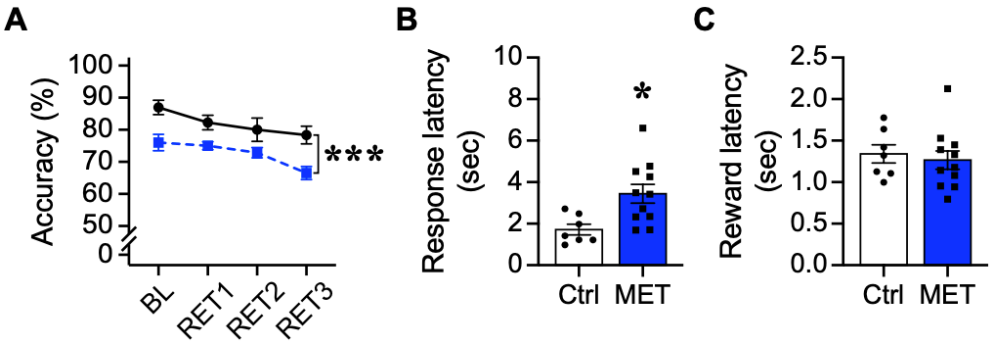

Fig 15. Effects of chronic metformin treatment on the long-term memory of the reward contingency of the PAL task in 3xTg-AD mice. Long-term memory of the reward contingency of the PAL task was impaired by chronic metformin treatment in 12-13-month-old 3xTg-AD mice. The sPAL retention sessions were conducted to assess long-term memory about the association between the PAL task stimuli and reward. (A) Response accuracy, (B) response latency, and (C) reward collection latency in sPAL retention sessions of Ctrl (n = 6) and MET (n = 10). Data are presented as mean ± S.E.M. *$p < 0.05$, ***$p < 0.001$ vs. Ctrl. Mixed-effects model with Tukey's multiple comparison test was used for statistical analysis of (A). Unpaired Student's two-tailed t-test was used for statistical analysis of (B) and (C). Ctrl, Control; MET, Metformin; BL, baseline; RET, retention.

3. VD and Reversal task

Even though visual discrimination is not altered in AD patients, cognitive flexibility is impaired.[351] For assessing cognitive flexibility by reversal task, the VD task was conducted first to train the reward contingency towards the stimuli. The VD task can elucidate the effects of chronic metformin treatment on visual discrimination in 3xTg-AD mice.

A. Chronic metformin treatment did not alter the visual discrimination in 3xTg-AD mice

In the VD task, both groups took the same number of sessions, trials, and correction trials to criterion (Fig 16A, the number of sessions to criterion, log-rank test; x^2: 0.627, df: 1, p = 0.428; Fig 16B, total trials to criterion, Student's t-test, t = 0.723, df = 16, p = 0.480; Fig 16C, total correction trials to criterion, Student's t-test, t = 0.906, df = 16, p = 0.379). Chronic metformin treatment did not alter motor function, as neither response nor reward collection latencies differed between groups (Fig 16D, response latency, Student's t-test, t = 0.288, df = 16, p = 0.777; Fig 16E, reward collection latency, Student's t-test, t = 0.794, df = 16, p = 0.439). Taken togehter, visual discrimination was not affected by chronic metformin treatment in 3xTg-AD mice.

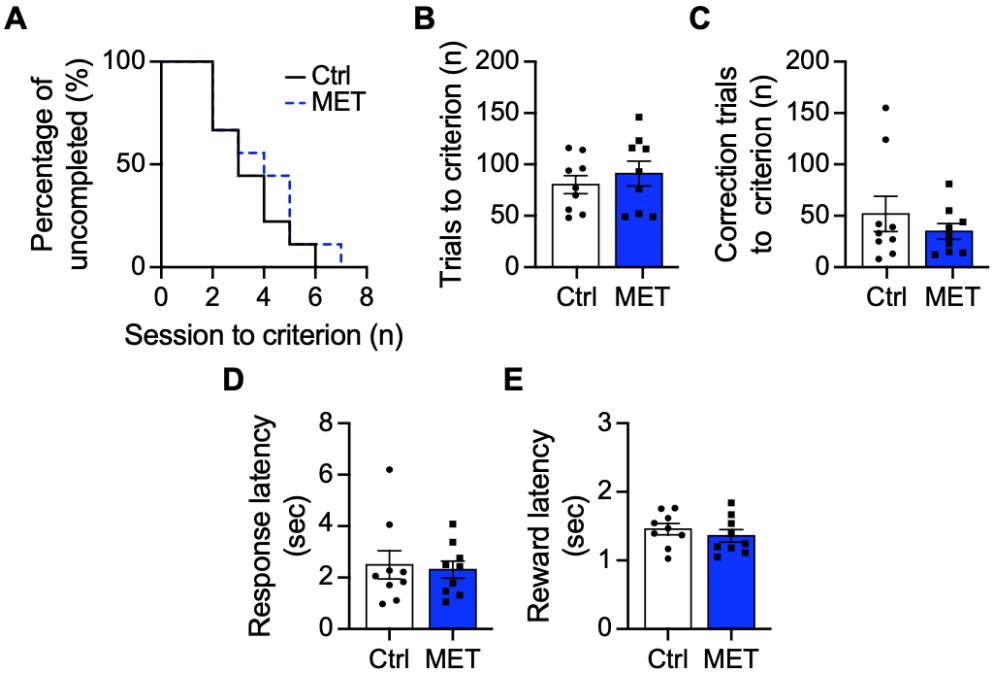

Fig 16. Effect of chronic metformin treatment on the visual discrimination in 3xTg-AD mice. Visual discrimination was not altered by chronic metformin treatment in 14-month-old 3xTg-AD mice. The VD task was conducted to assess visual discrimination. (A) Percentage of mice below the criterion of VD task, (B) total trials to accomplish criterion, (C) total correction trials to accomplish criterion, (D) response latency, and (E) reward collection latency of Ctrl (n = 9) and MET (n = 9). Data are presented as mean ± S.E.M. Log-rank test was used for statistical analysis of (A). Unpaired Student's two-tailed t-test was used for statistical analysis of (B)-(E). Ctrl, Control; MET, Metformin.

B. Chronic metformin treatment did not alter the cognitive flexibility in 3xTg-AD mice

In the reversal task, both groups took the same number of sessions and trials to criterion (Fig 17A, the number of sessions to criterion, log-rank test; x^2: 1.343,

df: 1, $p = 0.247$; Fig 17B, total trials to criterion, Student's t-test, t = 1.198, df = 11, $p = 0.256$). Besides, acquisition levels were not different between groups (Fig 17C). Chronic metformin treatment did not alter motor function as response and reward collection latencies were not different between groups (Fig 17D, response latency, Student's t-test, t = 1.403, df = 11, $p = 0.188$; Fig 17E, reward collection latency, Student's t-test, t = 1.805, df = 11, $p = 0.098$). These findings show that chronic metformin treatment did not alter cognitive flexibility in 3xTg-AD mice.

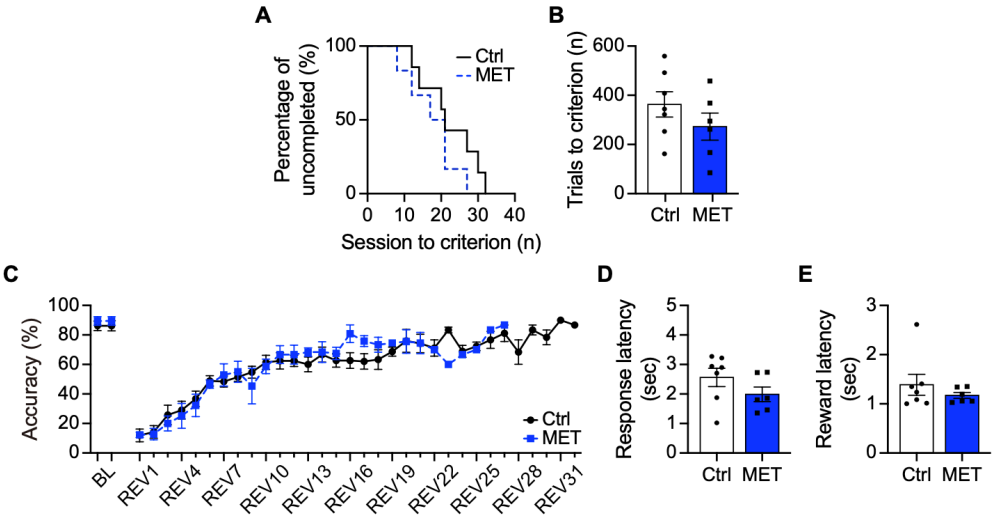

Fig 17. Effects of chronic metformin treatment on the cognitive flexibility in 3xTg-AD mice. Cognitive flexibility was not altered by chronic metformin treatment in 14-month-old 3xTg-AD mice. The reversal task was conducted to assess cognitive flexibility. (A) Percentage of mice below the criterion of reversal task, (B) total trials to accomplish criterion, (C) response accuracy, (D) response latency, and (E) reward collection latency of Ctrl (n = 7) and MET (n = 6). Data are presented as mean ± S.E.M. *$p < 0.05$ vs. Ctrl. Log-rank test was used for statistical analysis of (A). Unpaired Student's two-tailed t-test was used

for statistical analysis of (B), (D), and (E). Ctrl, Control; MET, Metformin; REV, reversal.

C. Chronic metformin treatment did not alter the long-term memory of the reward contingency of the reversal task in 3xTg-AD mice

Retention session was conducted ten days after each mouse reached the criterion of reversal task. The accuracy was not different between groups (Fig 18A, accuracy, mixed-effects model, main effect of group: $F_{(1, 11)} = 0.489$, $p =$ 0499, main effect of session: $F_{(1, 11)} = 91.538$, $p < 0.0001$, group by session interaction: $F_{(1, 11)} = 2.408$, $p = 0.149$). Both response and reward collection latencies were not different in Ctrl and MET (Fig 18B, response latency, Student's t-test, t = 0.699, df = 11, $p = 0.499$; Fig 18C, reward collection latency, Student's t-test, t = 0.903, df = 11, $p = 0.386$). Therefore, chronic metformin treatment did not alter the long-term memory about the association between the reversal task stimuli and the reward without altering motor function in 3xTg-AD mice.

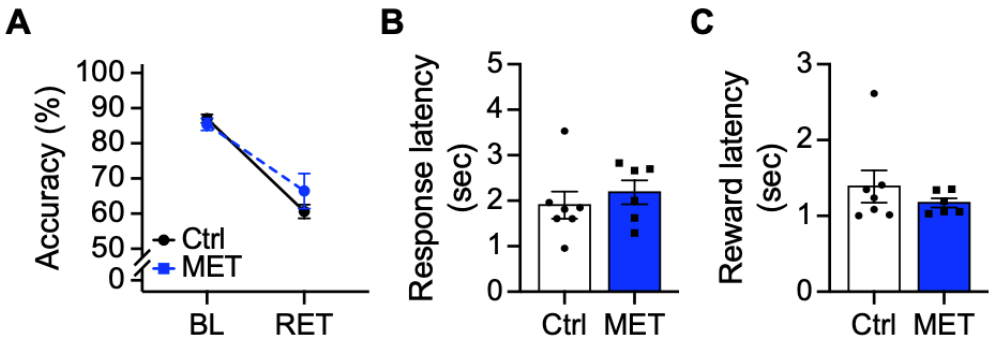

Fig 18. Effect of chronic metformin treatment on the long-term memory of the reward contingency of the reversal task in 3xTg-AD mice. Long-term memory of the reward contingency of the reversal task was not altered by chronic

metformin treatment in 16-month-old 3xTg-AD mice. The retention session of the reversal task was conducted to assess long-term memory about the association between the reversal task stimuli and the reward. (A) Response accuracy, (B) response latency, and (C) reward collection latency of Ctrl (n = 7) and MET (n = 6). Data are presented as mean ± S.E.M. Mixed-effects model with Tukey's multiple comparison test was used for statistical analysis of (A). Unpaired Student's two-tailed t-test was used for statistical analysis of (B) and (C). Ctrl, Control; MET, Metformin; BL, baseline; RET, retention.

4. FR and PR task

Cognitive functions were exacerbated by metformin in 3xTg-AD without altering the motor functions. However, decreased appetite and upregulated metabolism via metformin might alter the task performance of the mice as the behavioral assessment using the touchscreen operant system is based on the appetitive responses.[198,340] FR and PR tasks were conducted to clarify the alterations in satiety and motivation, which are lowered in AD (apathy) since the early stage,[352] via chronic metformin treatment.

A. Chronic metformin treatment did not alter the satiety in 3xTg-AD mice

In FR task, the number of total trials and blank touches were not different between groups (Fig 19A, total trials, Student's t-test, t = 0.015, df = 23, p = 0.988; Fig 19B, total blank touches, Students' t-test, t = 1.280, df = 23, p = 0.213). As beam breaks and latencies were not different between groups, chronic metformin treatment did not alter the motor function (Fig 19C, front beam break, Student's t-test, t = 0.081, df = 23, p = 0.936; Fig 19D, rear beam break, Student's

t-test, t = 0.079, df = 23, p = 0.945; Fig 19E, response latency, Student's t-test, t = 0.172, df = 23, p = 0.865; Fig 19F, reward collection latency, Students' t-test, t = 0.487, df = 23, p = 0.631). Taken together, chronic metformin treatment did not affect the levels of satiety in 3xTg-AD mice.

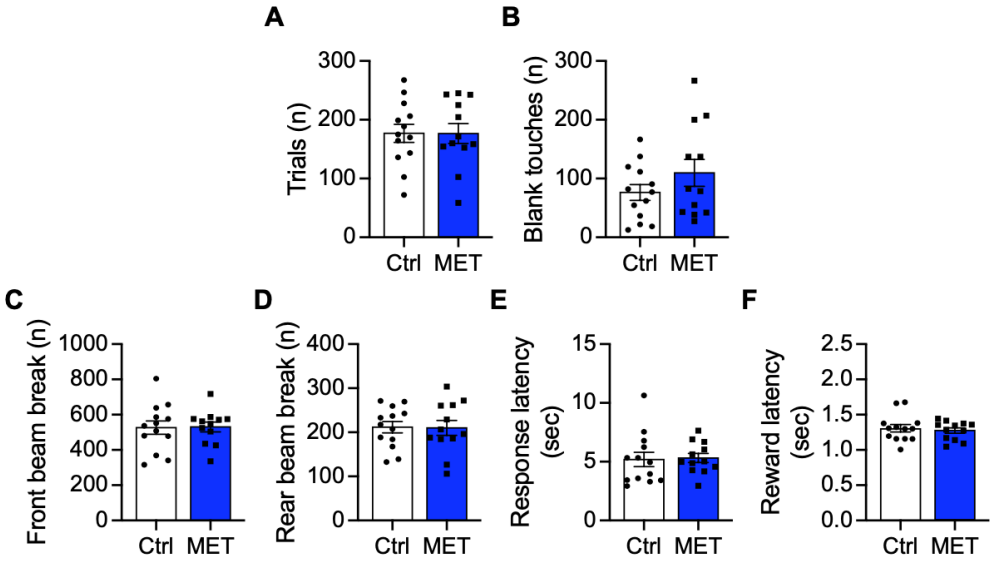

Figure 19. Effects of chronic metformin treatment on the satiety in 3xTg-AD mice. Satiety was not altered by chronic metformin treatment in 5-month-old 3xTg-AD mice. FR task was conducted to assess the satiety. (A) Total trials, (B) total blank touches, (C) front beam break, (D) rear beam break, (E) response latency, and (F) reward collection latency of Ctrl (n = 11) and MET (n = 12). Data are presented as mean ± S.E.M. Unpaired Student's two-tailed t-test was used for statistical analysis. Ctrl, Control; MET, Metformin.

B. Chronic metformin treatment did not alter the motivation in 3xTg-AD mice.

In PR task, the number of total breakpoint and blank touches were not altered by chronic metformin treatment (Fig 20A, total breakpoint, mixed-effects model, main effect of group: $F_{(1, 23)} = 0.926$, $p = 0.346$, main effect of session: $F_{(2, 46)} = 7.675$, $p = 0.001$, group by session interaction: $F_{(2, 46)} = 0.443$, $p = 0.645$; Fig 20B, total blank touches, mixed-effects model, main effect of group: $F_{(1, 23)} = 0.112$, $p = 0.741$, main effect of session: $F_{(2, 46)} = 5.806$, $p = 0.006$, group by session interaction: $F_{(2, 46)} = 0.567$, $p = 0.571$). As beam breaks and latencies were not different between both groups, the motor function was not affected by chronic metformin treatment (Fig 20C, front beam break, Student's t-test, t = 0.479, df = 23, $p = 0.636$; Fig 20D, rear beam break, Student's t-test, t = 0.217, df = 23, $p = 0.830$; Fig 20E, response latency, Student's t-test, t = 0.290, df = 23, $p = 0.774$; Fig 20F, reward collection latency, Student's t-test, t = 0.716, df = 23, $p = 0.481$). Together, chronic metformin treatment did not affect the motivation in 3xTg-AD mice. Additionally, the changes in behaviors via metformin were not caused by the appetite alteration.

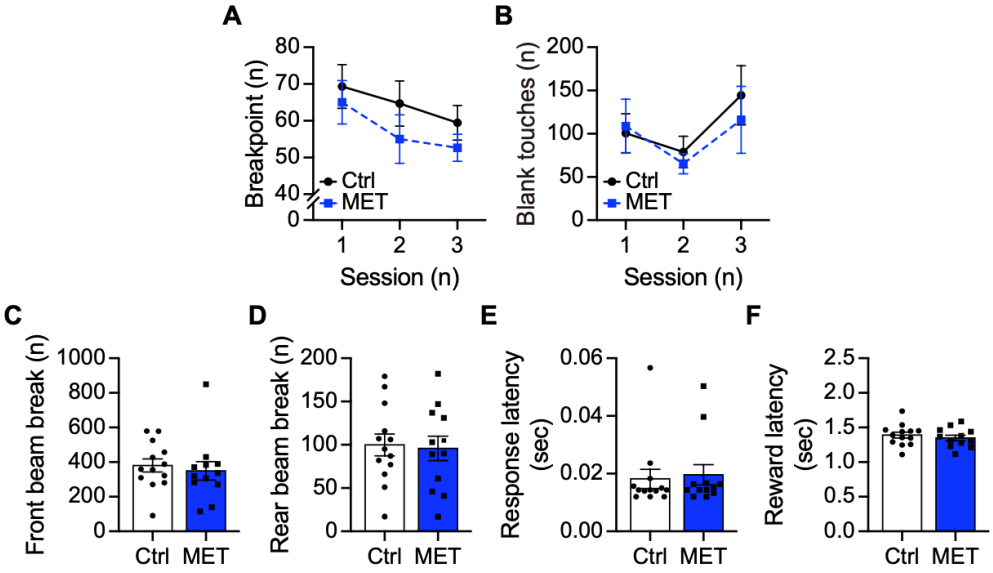

Figure 20. Effects of chronic metformin treatment on the motivation in 3xTg-AD mice. The motivation was not altered by chronic metformin treatment in 6-month-old 3xTg-AD mice. PR task was conducted to assess motivation. (A) Total breakpoint, (B) total blank touches, (C) front beam break, (D) rear beam break, (E) response latency, and (F) reward latency of Ctrl (n = 11) and MET (n = 12). Data are presented as mean ± S.E.M. Mixed-effects model with Tukey's multiple comparison test was used for statistical analysis of (A) and (B). Unpaired Student's two-tailed t-test was used for statistical analysis of (C)-(F). Ctrl, Control; MET, Metformin.

PART 2: The effects of chronic metformin treatment on whole-body energy metabolism regulation in 3xTg-AD mice

Metformin is a well-known systemic metabolism up-regulator.[353] Even though the patients take metformin for the long-term duration,[335] the whole-body energy metabolism effects of metformin with long-term treatment are poorly studied. Therefore, weight, water consumption, blood glucose levels, survival

rate, and vitamin B12 levels of 3xTg-AD were measured to elucidate the general effects of chronic metformin treatment in the body.

1. Metformin was administered into the body of mice via chronic oral treatment

Before analyzing the whole-body energy metabolism alteration via metformin, the concentration of metformin in the drinking water and mice serum were assessed to validate that metformin was delivered properly via chronic oral treatment. Metformin was stably maintained in the drinking water as its concentration was consistent for 14 days (Table 4, one-way ANOVA, $F = 2.859$, $p = 0.081$). Metformin was found in the serum of MET (Table 5). The existence of metformin in mice serums supports that the altered behaviors and AD pathology shown in this study were caused by metformin.

Table 4. The concentration of metformin in drinking water

	Day 0	Day 3	Day 4	Day 7	Day 14
Concentration, μM	633.95 ± 147.575	473.97 ± 32.120	428.01 ± 76.536	703.17 ± 36.581	402.35 ± 29.397

The concentration of metformin in drinking water was measured by LC-MS analysis. Data are presented as mean ± S.E.M (n = 3 per group). One-way ANOVA with Tukey's multiple comparison test was used for statistical analysis.

Table 5. The concentration of metformin in the mice serum

	Concentration, μM
Ctrl	BLOQ
MET	9.60 ± 3.239

The concentration of metformin in the serums of 3xTg-AD mice was measured by LC-MS analysis. Data are presented as mean ± S.E.M (n = 4 per group). BLOQ, below limit of quantitation. Ctrl, Control; MET, Metformin.

2. Chronic metformin treatment did not alter the weight of 3xTg-AD mice

As metformin inhibits food intake, weight loss might be found during the chronic metformin treatment.[198] The weight of both groups during the experiment was not different whether 3xTg-AD mice were under food restriction condition or not (Fig 21A, 3xTg-AD mice with food restriction, mixed-effects model, main effect of group: $F_{(1, 24)} = 0.878$, $p = 0.358$, main effect of age: $F_{(4, 79)} = 5.989$, $p < 0.001$, group by age interaction: $F_{(4, 79)} = 1.051$, $p = 0.386$; Fig 21B, 3xTg-AD mice without food restriction, mixed-effects model, main effect of group: $F_{(1, 9)} = 0.006$, $p = 0.941$, main effect of age: $F_{(4, 34)} = 110.700$, $p < 0.001$, group by age interaction: $F_{(4, 34)} = 1.842$, $p = 0.144$). These findings suggest that chronic metformin treatment did not cause weight loss in 3xTg-AD mice under both food restriction and free-feeding conditions.

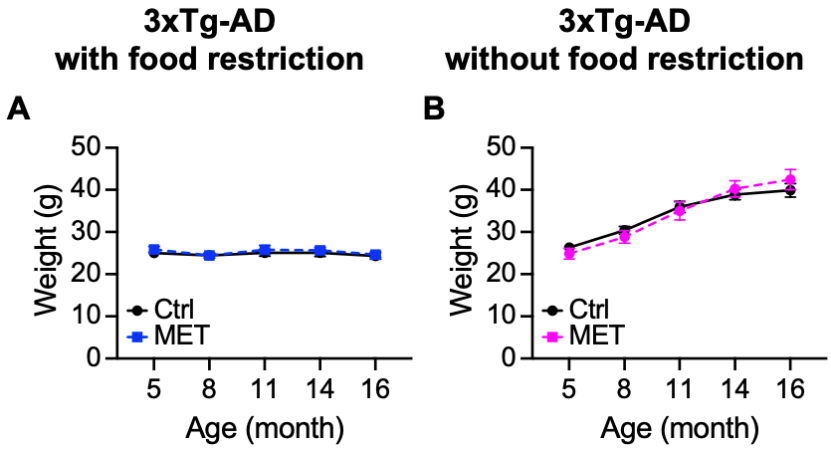

Figure 21. Weight of 3xTg-AD mice during chronic metformin treatment. Weight was not altered by chronic metformin treatment. (A) Weight of 3xTg-AD mice with food restriction (Ctrl: n = 14, MET: n = 12). (B) Weight of 3xTg-AD mice without food restriction (Ctrl: n = 6, MET: n = 5). Data are presented as mean ± S.E.M. Mixed-effects model with Tukey's multiple comparison test was used for statistical analysis. Ctrl, Control; MET, Metformin.

3. Chronic metformin treatment did not alter the water consumption of 3xTg-AD mice

Metformin might decrease water consumption following the lack of appetite.[198] The patients with metformin treatment often experience a lingering metallic taste in the mouth due to a consistent presence of metformin in saliva.[354] This discomfort can bring out a reduction in water consumption. Water consumption was assessed during chronic metformin treatment to evaluate that the mice drank the water with metformin without any aversive responses. Water consumption of both cohorts during the experiment was not different between groups (Fig 22A, 3xTg-AD mice with food restriction, mixed-effects model, main effect of group: $F_{(1, 11)} = 2.727$, $p = 0.160$, main effect of concentration: $F_{(1,}$

$_{10)}$ = 10.210, p = 0.010, group by concentration interaction: $F_{(1, 10)}$ = 2.329, p = 0.158; Fig 22B, 3xTg-AD mice without food restriction, mixed-effects model, main effect of group: $F_{(1, 4)}$ = 1.500, p = 0.288, main effect of concentration: $F_{(1, 4)}$ = 0.168, p = 0.703, group by concentration interaction: $F_{(4, 8)}$ = 0.006, p = 0.943). These findings indicate that water consumption of 3xTg-AD mice was not altered by chronic metformin treatment.

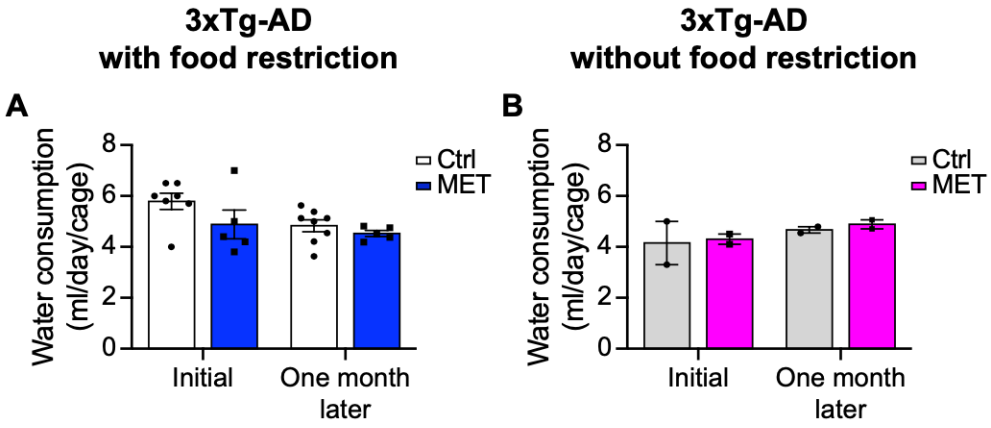

Figure 22. Water consumption of 3xTg-AD mice. Water consumption was not altered by chronic metformin treatment. (A) Water consumption of 3xTg-AD mice with food restriction. (B) Water consumption of 3xTg-AD mice without food restriction. Initial, when 2.0 mg/ml metformin was first administered to mice (Age: 4-month-old; 3xTg-AD mice with food restriction: Ctrl: n = 7, MET: n = 5; 3xTg-AD mice without food restriction: Ctrl: n = 2, MET: n = 2); One month later, after 2.0 mg/ml metformin was administered to mice for one month (Age: 5-month-old; 3xTg-AD mice with food restriction: Ctrl: n = 8, MET: n = 5; 3xTg-AD mice without food restriction: Ctrl: n = 2, MET: n = 2). The levels of mouse water consumption were presented with the average of each cage. Data are presented as mean ± S.E.M. Mixed-effects model with Sidak's multiple comparison test was used for statistical analysis. Ctrl, Control; MET, Metformin.

4. Chronic metformin treatment did not alter the blood glucose concentration of 3xTg-AD mice

Metformin ameliorates the progression of T2DM by lowering blood glucose levels.[180] Additionally, metformin might trigger hypoglycemia due to the long-term treatment.[355] However, Allard et al. showed that six months of metformin treatment did not alter the fasting glucose levels in C57BL/6J mice.[227] In this study, blood glucose concentrations of 3xTg-AD mice were measured before the onset of the behavior experiment (5-month-old) and after the end of the behavior experiment (16-month-old). During these measurements, the food restriction condition was maintained in cohort 1 to clarify the effect of chronic metformin treatment on blood glucose concentration. Blood glucose concentrations of both cohorts were not different between groups regardless of their age (Fig 23A, 3xTg-AD mice with food restriction, mixed-effects model, main effect of group: $F_{(1, 41)} = 0.037$, $p = 0.849$, main effect of time: $F_{(1, 41)} = 20.590$, $p < 0.001$, group by time interaction: $F_{(1, 41)} = 0.412$, $p = 0.525$; Fig 23B, 3xTg-AD mice without food restriction, mixed-effects model, main effect of group: $F_{(1, 17)} = 1.855$, $p = 0.191$, main effect of time: $F_{(1, 17)} = 19.450$, $p = 0.001$, group by time interaction: $F_{(1, 17)} = 0.038$, $p = 0.848$). These findings suggest that the blood glucose concentration of 3xTg-AD mice was not altered by chronic metformin treatment.

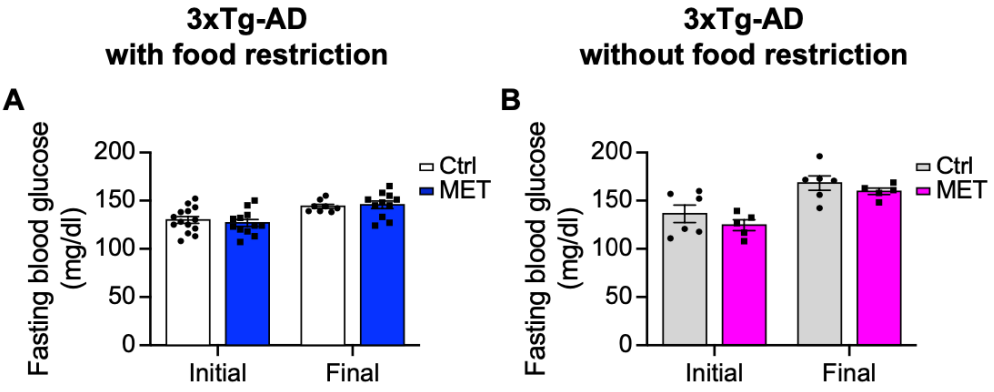

Figure 23. Blood glucose concentration of 3xTg-AD mice during chronic metformin treatment. Blood glucose concentration was not altered by chronic metformin treatment. (A) Blood glucose concentration of 3xTg-AD mice with food restriction. (B) Blood glucose concentration of 3xTg-AD mice without food restriction. Initial, before the onset of behavior experiment (Age: 5-month-old; 3xTg-AD mice with food restriction: Ctrl: n = 14, MET: n = 12; 3xTg-AD mice without food restriction: Ctrl: n = 6, MET: n = 5); Final, after the end of behavior experiment (Age: 16-month-old; 3xTg-AD mice with food restriction: Ctrl; n = 8, MET; n = 11; 3xTg-AD mice without food restriction: Ctrl: n = 6, MET: n = 5). Data are presented as mean ± S.E.M. Mixed-effects model with Sidak's multiple comparison test was used for statistical analysis. Ctrl, Control; MET, Metformin.

5. Chronic metformin treatment did not alter the vitamin B12 levels of 3xTg-AD mice

As vitamin B12 deficiency was found in patients with long-term metformin treatment,[205] the levels of vitamin B12 in male 3xTg-AD mice serums were measured. Cyanocobalamin (CNCbl), the synthetic vitamin B12, and hydroxocobalamin (HOCbl), the long-lasting vitamin B12, levels were not

different between groups in male 3xTg-AD mice (Table 6, CNCbl, Student's t-test, t = 0.530, df = 6, p = 0.615; HOCbl, Student's t-test, t = 1.083, df = 6, p = 0.321). Methylcobalamin (MeCbl) and adenosylcobalamin (AdoCbl) were not detected in the serums. Therefore, vitamin B12 deficiency was not found in 3xTg-AD mice with food restriction.

Table 6. The concentration of vitamin B12 in the mice serum

	CNCbl, nM	HOCbl, nM	MeCbl, nM	AdoCbl, nM
Ctrl	4.07 ± 1.477	0.74 ± 0.315	BLOQ	BLOQ
MET	5.29 ± 1.750	1.56 ± 0.692	BLOQ	BLOQ

The concentration of vitamin B12 in the serums of 3xTg-AD mice was measured by LC-MS analysis. Data are presented as mean ± S.E.M (n = 4 per group). BLOQ, below limit of quantitation. Ctrl, Control; MET, Metformin; CNCbl, Cyanocobalamin; HOCbl, Hydroxocobalamin; MeCbl, Methylcobalamin; AdoCbl, Adenosylcobalamin.

6. Chronic metformin treatment slightly increased the life span of 3xTg-AD mice

Metformin increases the life span in diverse strains of animals.[356,357] The survival rate in male and female 3xTg-AD mice was analyzed to evaluate the effects of metformin on the life span extension. The survival rate decreased from 100% to 57.1% in the control group (Ctrl) of males (6 of 14 mice died during behavioral assessment). However, the survival rate was only reduced to 91.7%

(1 of 12 mice were dead during behavioral assessment) in the metformin-treated group (MET) of males. There was a certain trend toward the significance of the increase in the life span of male 3xTg-AD mice with chronic metformin treatment (Fig 24A, log-rank test, x^2: 3.773, df: 1, p = 0.052). In contrast, there was no significant difference in the survival rate of females between groups (Fig 24B, log-rank test, x^2: 1, df: 1, p = 0.317). In Ctrl, 100% of mice survived (0 of 6 mice were dead), while 83.3% of mice survived in MET (1 of 6 mice were dead). These findings imply that chronic metformin treatment slightly increased the life span only in 3xTg-AD mice with food restriction.

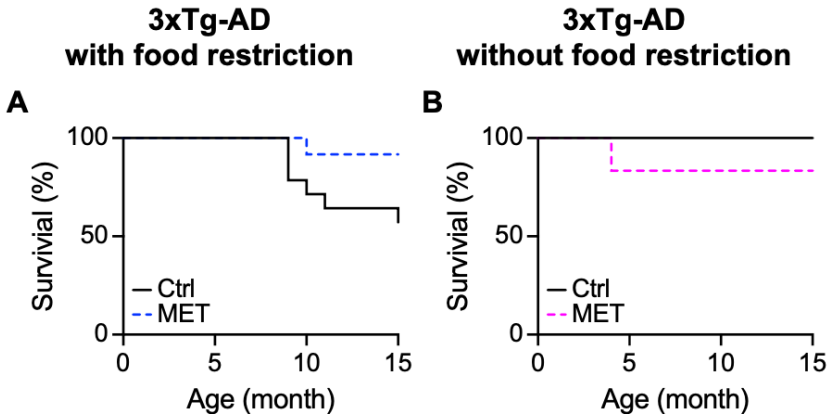

Figure 24. Survival rate of 3xTg-AD mice during chronic metformin treatment. A certain trend toward the significance of the increase in the life span was shown in male 3xTg-AD mice with chronic metformin treatment. Survival rate of (A) 3xTg-AD mice with food restriction and (B) 3xTg-AD mice without food restriction. At the end of the behavioral assessment, 57.1% of Ctrl and 91.7% of MET were left (The number of dead mice: Ctrl, 6 out of 14; MET, 1 out of 12) in 3xTg-AD mice with food restriction. In 3xTg-AD mice without food restriction, 100% of Ctrl and 83.3% of MET were left (Numer of dead mice: Ctrl, 0 out of 6; MET, 1 out of 6). Log-rank test was used for statistical analysis. Ctrl,

Control; MET, Metformin.

In conclusion, metformin did not alter the whole-body energy metabolism regulation except for a slight increase in the life span under long-term usage. Additionally, side effects, such as body mass reduction, lingering metallic taste, hypoglycemia, and vitamin B12 deficiency, were not found during chronic treatment of metformin.

PART 3: The effects of chronic metformin treatment on AMPK regulation and AD pathology in 3xTg-AD mice

The effects of chronic metformin treatment on AMPK regulation and AD pathology were assessed in cohort 2.

1. Chronic metformin treatment did not activate AMPK in 3xTg-AD mice

As metformin is an AMPK activator,[193] the effects of chronic metformin treatment on AMPK activation were assessed. The levels of phosphorylated AMPKα (pAMPKα) and AMPKα were not different between groups in female 3xTg-AD mice (Fig 25A and B, pAMPKα, Student's t-test, t = 1.065, df = 8, p = 0.318; AMPK, Student's t-test, t = 0.649, df = 8, p = 0.535; pAMPKα/AMPKα, Student's t-test, t = 1.159, df = 8, p = 0.280). The activity of ACC, the downstream of AMPK activation, was not also altered by chronic metformin treatment, as phosphorylated ACC (pACC), an inhibited form of ACC, and basal ACC levels were not different between groups (pACCSer79, Student's t-test, t = 0.387, df = 8, p = 0.709; ACC, Student's t-test, t = 0.563, df = 8, p = 0.589; pACC/ACC, Student's t-test, t = 0.664, df = 8, p = 0.525). These findings show

that metformin did not as an AMPK activator in 3xTg-AD mice under chronic treatment.

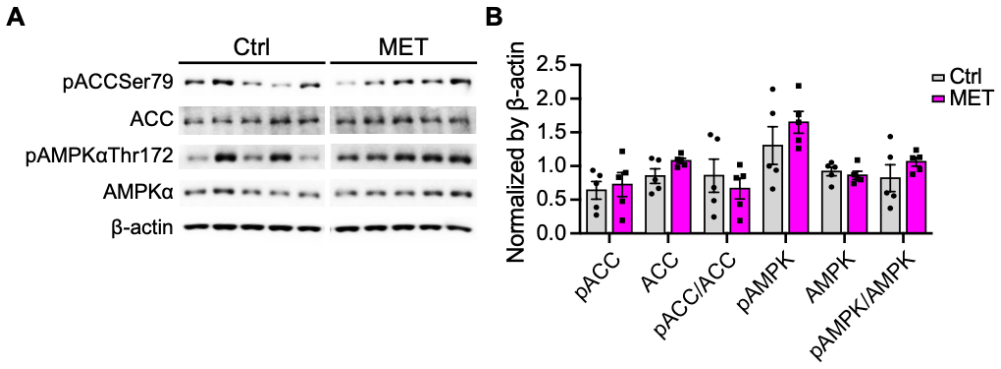

Figure 25. Effects of chronic metformin treatment on AMPK activation in 3xTg-AD mice. AMPKα was not activated by chronic metformin treatment in 17-month-old 3xTg-AD mice without food restriction. (A) Western blot analysis of AMPKα and ACC phosphorylation and expression in Ctrl (n = 5) and MET (n = 5). The bar graph of quantitative data of (A). Data are presented as mean ± S.E.M. *$p < 0.05$ vs. Ctrl. Unpaired Student's two-tailed t-test was used for statistical analysis of (B) and (C). Ctrl, Control; MET, Metformin.

2. Chronic metformin treatment increased AMPKα1-subunit in 3xTg-AD mice

The alteration of AMPK subunit expression, especially AMPKα-subunit, in AD is related to AD pathology and cognitive impairment.[330,333] However, which AMPKα-subunit isoform mainly regulates AD pathology, is still unclear. Therefore, the effects of chronic metformin treatment on the expression of AMPKα-subunit isoforms were assessed. AMPKα1-subunit was significantly increased by chronic metformin treatment in 3xTg-AD mice without food

restriction while the levels of AMPKα2-subunit were not altered (Fig 26A and B, AMPKα1, Student's t-test, t = 3.55, df = 8, p = 0.008; AMPKα2, Student's t-test, t = 0.625, df = 8, p = 0.550). As the previous studies found that AMPK α1-subunit is highly related to AD pathology and cognitive impairment,[330,332] AMPKα-subunit isoforms alteration might regulate AD pathology via chronic metformin treatment in 3xTg-AD mice.

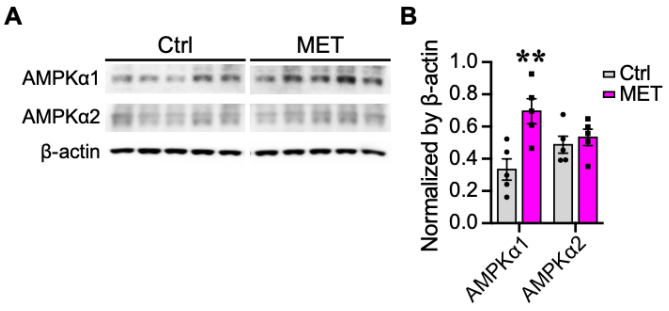

Figure 26. Effects of chronic metformin treatment on AMPKα-subunit isoforms expression in 3xTg-AD mice. AMPKα1-subunit was increased by chronic metformin treatment in 17-month-old 3xTg-AD mice without food restriction. (A) Western blot analysis of AMPKα1- and α2-subunit levels in Ctrl (n = 5) and MET (n = 5). (B) The bar graph of quantitative data of (A). Data are presented as mean ± S.E.M. **p < 0.01 vs. Ctrl. Unpaired Student's two-tailed t-test was used for statistical analysis of (B). Ctrl, Control; MET, Metformin.

3. Chronic metformin treatment exacerbated Aβ pathogenesis in 3xTg-AD mice

The burden of Aβ is the major pathology of AD. As Chen et al. (2009)[210] showed that metformin increased Aβ by upregulating BACE1, which increases sAPPβ through the amyloidogenic pathway, the effects of chronic metformin

treatment on Aβ pathogenesis were assessed in 3xTg-AD mice without food restriction. Aβ oligomers with the size of 70 kDa were significantly increased increased by chronic metformin treatment in 3xTg-AD mice without altering APP, sAPPβ, Aβ oligomers with the size of 25 kDa, ADAM10, and BACE1 levels (Fig 27A and B, APP, Student's t-test, t = 0.288, df = 8, p = 0.781; sAPPβ, Student's t-test, t = 0.513, df = 9, p = 0.622; Aβ (70 kDa), Student's t-test, t = 4.667, df = 8, p = 0.002; Aβ (25 kDa), Student's t-test, t = 2.056, df = 8, p = 0.074; ADAM10, Student's t-test, t = 0.507, df = 8, p = 0.626; BACE1, Student's t-test, t = 0.340, df = 8, p = 0.742). Aβ oligomers are distributed in the brains with a wide range of molecular weight, between 10 kDa to 100 kDa. When Aβ oligomers are bigger than 50 kDa, they are regarded as toxic-high soluble Aβ oligomers.[358] Therefore, chronic metformin treatment aggravated Aβ pathogenesis by increasing toxic Aβ oligomers. However, TACE levels were also significantly increased by chronic metformin treatment in 3xTg-AD mice (TACE, Student's t-test, t = 3.148, df = 8, p = 0.014). As TACE cleaves APP to sAPPα, which prevents the synthesis of Aβ, chronic metformin treatment increased the non-amyloidogenic pathway in 3xTg-AD mice regardless of the Aβ pathogenesis exacerbation.

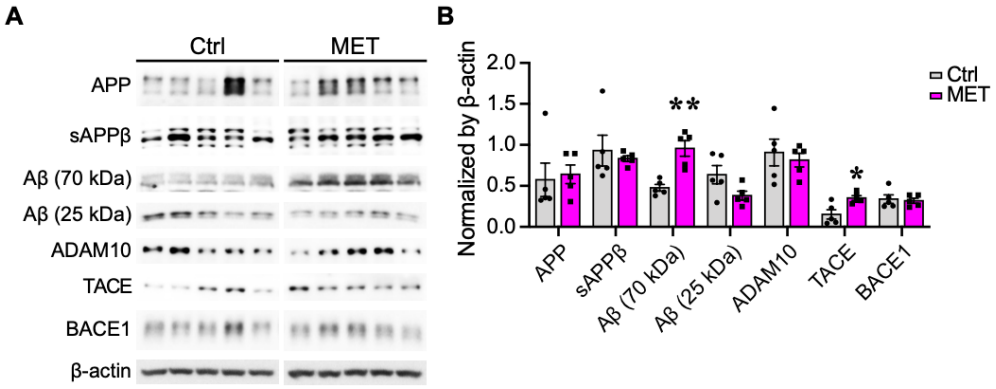

Figure 27. Effects of chronic metformin treatment on Aβ pathogenesis in 3xTg-AD mice. The expression of Aβ oligomers with the size of 70 kDa and TACE was increased by chronic metformin treatment in 17-month-old 3xTg-AD mice without food restriction. (A) Western blot analysis of APP, sAPPβ, Aβ, ADAM10, TACE, and BACE1 levels in Ctrl (n = 5) and MET (n = 5). (B) The bar graph of quantitative data of (A). Data are presented as mean ± S.E.M. *p < 0.05, **p < 0.01 vs. Ctrl. Unpaired Student's two-tailed t-test was used for statistical analysis of (B). Values of (B) were normalized by β-actin. Ctrl, Control; MET, Metformin.

Aβ plaques, the end-product of Aβ pathogenesis, were observed in 3xTg-AD mice.[341] As previous studies have shown that anti-Aβ antibodies may detect different forms of Aβ,[359] two different anti-Aβ antibodies were used in this study to detect Aβ plaques in 3xTg-AD mice without food restriction: D54D2 and 4G8. D54D2 detects several isoforms of Aβ, such as $Aβ_{37}$, $Aβ_{38}$, $Aβ_{39}$, $Aβ_{40}$, and $Aβ_{42}$.[360] 4G8 captures amino acid residues 17-24 of Aβ and detects both soluble and aggregated Aβ.[361] Aβ plaques-positive area in the hippocampus was measured with D54D2 immunostaining. The levels of Aβ aggregation in the hippocampus were evaluated with 4G8 immunostaining. The area of Aβ plaques in the hippocampus was significantly increased by chronic metformin treatment

(Fig 28A and B, the area of plaques in the hippocampus, Student's t-test, t = 3.852, df = 8, p = 0.005). Moreover, Aβ plaques were more aggregated in MET than Ctrl (Fig 28C and D). Referred to the previous studies,[23] the morphology of Aβ plaques in Ctrl were similar to diffuse plaques, and those in MET resembled compact plaques. These findings indicate that chronic metformin treatment aggravated Aβ pathogenesis in 3xTg-AD mice.

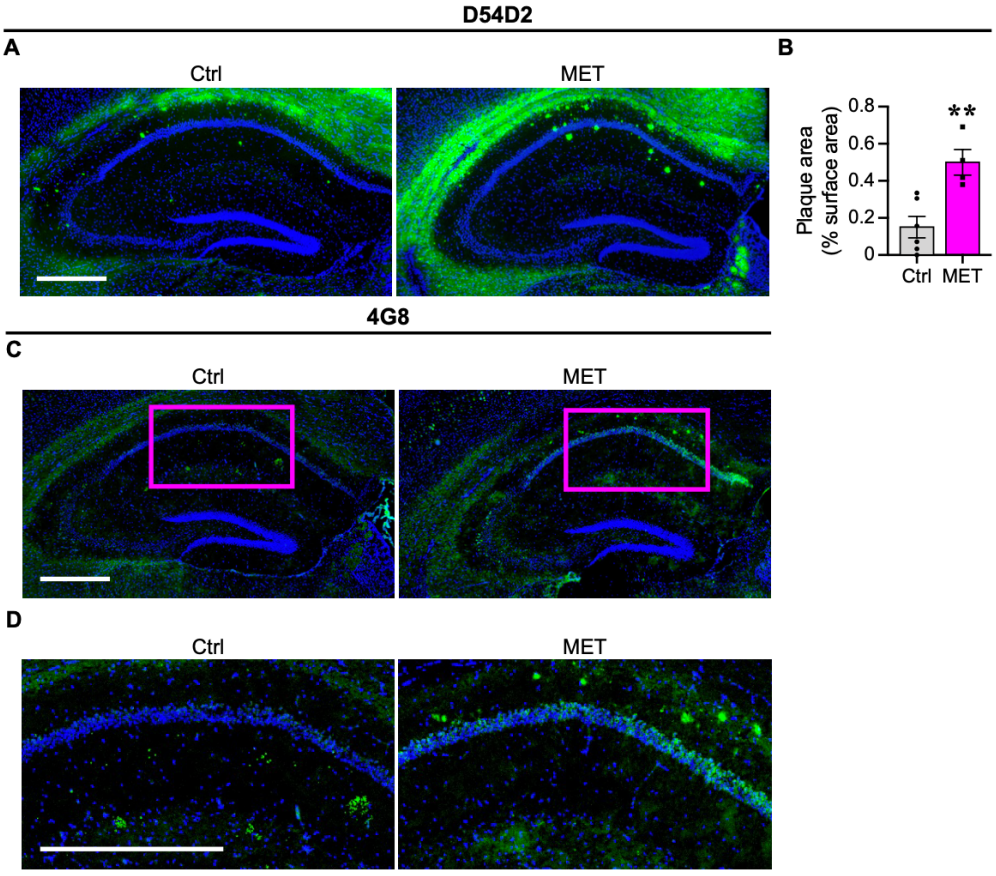

Figure 28. Effects of chronic metformin treatment on Aβ deposition in 3xTg-AD mice. Aβ plaques were increased and more aggregated by chronic metformin treatment in the hippocampus of 17-month-old 3xTg-AD mice without food restriction. (A) Representative immunohistochemistry images of

D56D2-positive Aβ deposition in the hippocampus of Ctrl (n = 6) and MET (n = 4). (B) Percent area of D56D2-positive Aβ deposition in the hippocampus. (C) Representative immunohistochemistry images of 4G8-positive Aβ deposition in the hippocampus of Ctrl (n = 6) and MET (n = 4). (D) Magnified representative immunohistochemistry images of (C). Scale bar: 500 μm. Data are presented as mean ± S.E.M. **$p < 0.01$ vs. Ctrl. Unpaired Student's two-tailed t-test was used for statistical analysis of (B). Ctrl, Control; MET, Metformin.

4. Chronic metformin treatment exacerbated tau pathogenesis in 3xTg-AD mice

Tau hyperphosphorylation is one of the main hallmarks of AD.[35] The effects of chronic metformin treatment on AD-associated tau phosphorylation sites, such as Ser199/202, Ser262, Ser356, Ser396, and Thr231, were assessed in 3xTg-AD mice. p-tauSer356 and p-tauThr231 were significantly increased by chronic metformin treatment in 3xTg-AD mice (Fig 29A and B, p-tauSer199/202, Student's t-test, t = 1.859, df = 8, p = 0.100; p-tauSer262, Student's t-test, t = 1.005, df = 8, p = 0.344; p-tauSer356, Student's t-test, t = 2.731, df = 8, p = 0.026; p-tauSer396, Student's t-test, t = 0.276, df = 8, p = 0.790; p-tauThr231, Student's t-test, t = 3.860, df = 8, p = 0.005; t-tau, Student's t-test, t = 0.124, df = 8, p = 0.905). p-tauSer356 and p-tauThr231 have crucial roles in microtubule stabilization.[362] p-tauSer356 is important to the onset of tau abnormality,[363] and p-tauThr231 is critical to tau hyperphosphorylation.[364] Among diverse tau kinases involved in AD pathology,[41] GSK3β expression levels were significantly increased by chronic metformin treatment without altering the levels of pGSK3β, inactivated form of GSK3β, and the ratio of pGSK3β/GSK3β (Fig 30A and B, pGSK3β, Student's t-test, t = 1.999, df = 8, p = 0.081; GSK3β, Student's t-test, t = 3.396, df = 8, p = 0.009; pGSK3β/ GSK3β, Student's t-test, t = 0.608, df = 8,

p = 0.560). As GSK3β phosphorylates tau sites associated with AD, such as Ser356 and Thr231,[365,366] the upregulation of GSK3β caused increased tau phosphorylation in 3xTg-AD mice. Taken together, chronic metformin treatment exacerbated tauopathy in 3xTg-AD mice.

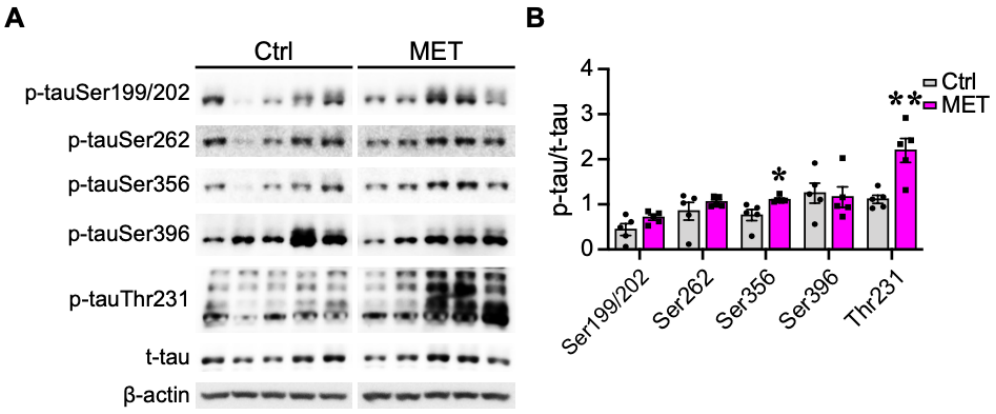

Figure 29. Effects of chronic metformin treatment on tau phosphorylation in 3xTg-AD mice. p-tauSer356 and p-tauThr231 levels were increased by chronic metformin treatment in 17-month-old 3xTg-AD mice without food restriction. (A) Western blot analysis of p-tau (Ser396, Ser199/202, Ser262, Ser356, and Thr231) and t-tau in Ctrl (n = 5) and MET (n = 5). (B) The ratios of p-tau/t-tau in Ctrl and MET. Data are presented as mean ± S.E.M. *p < 0.05, **p < 0.01 vs. Ctrl. Unpaired Student's two-tailed t-test was used for statistical analysis of (B). Ctrl, Control; MET, Metformin; p-tau, phosphorylated tau; t-tau, total tau.

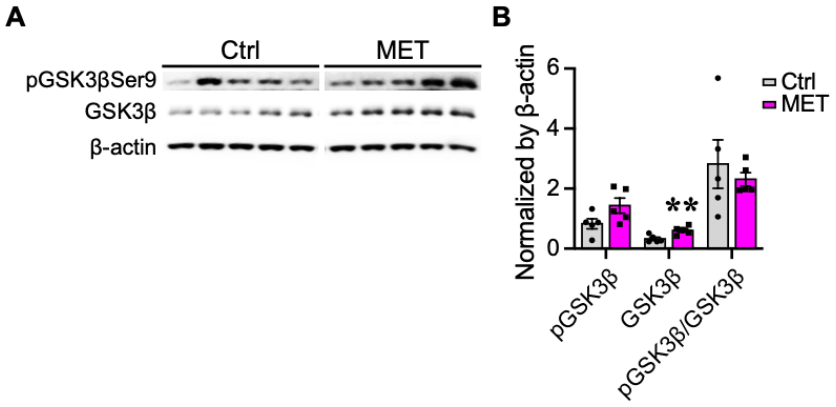

Figure 30. Effects of chronic metformin treatment on GSK3β expression in 3xTg-AD mice. GSK3β expression was upregulated by chronic metformin treatment in 17-month-old 3xTg-AD mice without food restriction. (A) Western blot analysis of GSK3β phosphorylation and expression in Ctrl (n = 5) and MET (n = 5). (B) The bar graph of quantitative data of pGSK3β and GSK3β and the ratio of pGSK3β/GSK3β. Data are presented as mean ± S.E.M. $^{**}p < 0.01$ vs. Ctrl. Unpaired Student's two-tailed t-test was used for statistical analysis of (B). Ctrl, Control; MET, Metformin.

IV. DISCUSSION

Metformin ameliorates T2DM progression by enhancing metabolism via AMPK activation. T2DM is highly associated with AD development by sharing pathologies and increasing the incidence of AD. Additionally, owing to the neuroprotective impacts of metformin,[206] many studies have been conducted to elucidate the effects of metformin on AD pathology to validate whether metformin is suitable for AD treatment. However, the results are still controversial. Besides, the detrimental effects of metformin on AD pathology have been reported.[210] These findings might impede the prescription of metformin to AD patients and the further drug repositioning of metformin into AD drugs. Therefore, genuine effects of chronic metformin treatment on behaviors, AD pathology, and AMPK regulation of 3xTg-AD mice, transgenic AD model mice, were assessed in this study to evaluate whether metformin is safe to be chronically prescribed to AD patients and an appropriate candidate for AD treatment.

First, this study has found that chronic metformin treatment was detrimental to behaviors in 3xTg-AD mice without affecting satiety and motivation. Learning and memory consolidation were impaired by metformin in 3xTg-AD mice as attention restoration, object-location associative learning and memory, and long-term memory were worsened. Different brain regions control different cognitive functions evaluated in the behavioral assessment of this study. Attention is regulated by the prefrontal cortex and parietal cortex.[367] Object-location associative learning and memory[368] and long-term memory[369] are regulated by the hippocampus. Therefore, chronic metformin treatment might impair the hippocampus and cortex-associated cognitive functions without altering the appetite.

Second, chronic metformin treatment did not alter the whole-body energy metabolism regulation regardless of age and caloric intake. Metformin generally has beneficial roles in whole-body energy metabolism regulation by reducing body weight and blood glucose concentration.[181] Unlike the previous studies, metformin did not alter weight, water consumption, blood glucose concentration, and vitamin B12 concentration while it only slightly extended the life span of 3xTg-AD mice with food restriction in this study. As the caloric intake of 3xTg-AD mice with food restriction was limited. their weight was not altered.

Few studies have found that metformin did not alter body regulation. In human studies, body weight was not altered via metformin in non-obese patients with T2DM[370] or polycystic ovary syndrome[371] and normal-weight healthy men.[372] In a mouse study, metformin did not alter the body weight, water, food consumption, and blood glucose levels of P301S mice which do not show insulin signaling dysfunction.[226] These findings suggest that health conditions, such as obesity and insulin resistance, might be a crucial factor in determining the effects of metformin on whole-body energy metabolism regulation. Even though 3xTg-AD mice show metabolic dysfunctions, the impairments are not severe enough as in diabetes models. 3xTg-AD mice have impaired glucose tolerance in 10 and 14-month-old females without affecting weight, insulin sensitivity, and glucose response.[373] In 9-month-old female 3xTg-AD mice, disrupted insulin signaling was found.[374] Therefore, lack of obesity and insulin resistance in 3xTg-AD mice might block the alteration in whole-body energy metabolism regulation via metformin. Furthermore, the blunted effects on whole-body energy metabolism regulation of metformin might trigger detrimental outcomes in behaviors and AD pathology.

In this study, 2 mg/ml of metformin was chronically treated in the mice. This dosage is equivalent to 300 mg/kg/day in clinical dosage and the maximum human DDD, 2,000 mg/kg.[210,222,344] However, the concentrations of metformin in the serum of 3xTg-AD mice were 9.60 ± 3.239 μM. As metformin concentrations in the plasma of humans and animals were between 10 μM to 40 μM, metformin existed in the mice body at a relatively low dosage.[193,375] Even though metformin was treated with the maximum clinical dose, its systemic serum concentration was quite low. Therefore, metformin might not enough to cause any possible side effects.

Third, chronic metformin treatment was involved in AMPK regulation in 3xTg-AD mice. Even though metformin is a well-known AMPK activator, it had no effects on AMPK phosphorylation under long-term treatment in this study. According to the previous studies, which found the blunted AMPK activity via aging,[325,376] AMPK activation of 17-month-old 3xTg-AD mice might also be blocked. Additionally, due to the body homeostasis system which controls the whole-body system into the normal state, AMPK activation via metformin might be diminished under the long-term administration. However, metformin worked as an AMPKα-subunit regulator in this study, as AMPKα1-subunit expression was upregulated by its chronic treatment.

Altered AMPK has negative impacts on the brain. Previous studies found that AMPK activation is detrimental to neuronal survival.[303,304] Especially, chronic or excessive AMPK activation has deleterious roles in brain functions by triggering a ‘metabolic failure-like’ state.[326] Over-activation of AMPK in aged or AD brains, in which environment has chronic stress, such as Aβ, phosphorylated tau, and inflammation, might exacerbate cognitive decline and AD pathology.[327] Not only AMPK activity but also AMPKα-subunit expression

affects AD pathology. Several studies showed that AMPKα1-subunit was upregulated in the AD hippocampus and involved in cognitive deficits and synaptic dysfunction.[142,330,331] According to these previous findings, chronic AMPK modulation via metformin, such as AMPKα1-subunit upregulation, might aggravate cognitive decline and AD pathology in this study.

Lastly, chronic metformin treatment also exacerbated AD pathology in 3xTg-AD mice. In Aβ pathogenesis, metformin increased toxic Aβ oligomers levels and Aβ plaques numbers and aggregation. In tau pathogenesis, metformin increased p-tauSer356 and p-tauThr231 levels via upregulating GSK3β expression. However, partial improvement of Aβ pathogenesis via chronic metformin treatment was found in 3xTg-AD mice simultaneously as metformin increased TACE levels. The increase of TACE might be the defensive response to the exacerbation of Aβ pathogenesis. Even though this study showed the detrimental effects of metformin on AD as Chen et al. did,[210] the functional mechanism of metformin was different. Metformin exacerbated AD pathology by increasing APP and Aβ oligomers expression, Aβ plaques synthesis, and tau phosphorylation, without altering BACE1 expression in this study. The differences in experimental conditions, such as treatment duration (acute or chronic) and the age of mice, might derive different findings.

Alteration of behaviors via chronic metformin treatment in C57BL/6J mice was found in the previous research of our lab.[337,338] Previous and current studies were conducted under identical experimental conditions, such as the dose and long-term treatment of metformin and the sequence of behavioral assessments, except for the mouse strain: C57BL/6J or 3xTg-AD. Metformin enhanced attention, inhibitory control, and learning of C57BL/6J mice when they were young and middle-aged. However, inhibitory control, memory consolidation,

and learning were impaired in C57BL/6J mice when they became old. In the case of male 3xTg-AD mice, metformin impaired cognitive functions throughout the age. Therefore, metformin might have deleterious effects on behaviors in old mice and transgenic AD model mice.

There are some limitations to this study. First, the mice were under food restriction conditions to motivate them to accomplish the behavioral tasks. As AMPK is activated via food restriction,[377] the alterations in behaviors, AD pathology, and AMPK regulation might not be solely due to the chronic metformin treatment. However, a previous study showed that caloric restriction to 80% did not activate AMPK.[306] Therefore, all alterations in this study were modulated by chronic metformin treatment, not AMPK activation via caloric restriction. Second, the behaviors of 3xTg-AD mice without food restriction (females) were not assessed. As the estrous cycle phases of females alter the behaviors,[378] the cognitive functions could not be evaluated accurately by the long-term behavioral assessment as males did. Therefore, further studies with short-term behavioral experiments, such as novel object recognition test and Y-maze test, in 3xTg-AD mice without food restriction will be needed to profoundly evaluate the effects of metformin on the behaviors. Third, even though metformin is an insulin sensitizer, how chronic metformin treatment altered insulin signaling was not assessed. Barini et al. (2016) showed that insulin signaling of P301S mice with eight months of metformin treatment was not altered.[226] The comparable results would be anticipated from this study as blood glucose concentration was not altered by chronic metformin treatment. Moreover, the concept (long-term metformin treatment) and result tendency (positive and negative results simultaneously) were similar to Barini et al. (2016).[226] Lastly, even though T2DM and AD are highly associated with each other,[171] this study only assessed the effects of metformin in a transgenic AD

mouse model. As metformin is a T2DM treatment, how metformin alters the behaviors and AD pathology also needs to be assessed in diabetes mouse models, such as $Lep^{ob/ob}$ or $Lepr^{db/db}$,[379] to elucidate the effects of metformin more deeply. AD pathology could be assessed in the T2DM model mouse, as it shows cognitive abnormalities and increased Aβ similar to the AD model mouse.[380]

These findings suggest that metformin prescription for familiar AD patients should be reconsidered. Additionally, drug repurposing of metformin as AD treatment might not be prospective. This study also implies that AMPKα1-subunit upregulation was highly related to cognitive decline and AD progression. Further studies are needed to elucidate the direct association of AMPK α-subunit alteration in behavior and AD pathology to evaluate the pathological mechanism of AD and the potential of AMPK as an AD diagnosis parameter.

V. CONCLUSION

This study assessed the effects of chronic metformin treatment on behaviors and AD pathology in 3xTg-AD mice to evaluate the therapeutic potential and safety of metformin as AD treatment. The alterations in behaviors via metformin were assessed by the touchscreen operant system in 3xTg-AD mice with food restriction. The alterations in AMPK and AD pathology via metformin were evaluated by western blot analysis and immunohistochemistry in 3xTg-AD mice without food restriction.

The followings are the key findings of this study.

1. Metformin impaired learning and memory consolidation in 3xTg-AD mice.
2. Metformin increased AMPKα1-subunit expression in 3xTg-AD mice.
3. Metformin exacerbated Aβ pathogenesis by increasing Aβ oligomers levels and Aβ plaques number and aggregation in 3xTg-AD mice.
4. Metformin exacerbated tau pathogenesis by increasing p-tauSer356 and p-tauThr231 levels via GSK3β upregulation in 3xTg-AD mice.

This study shows that chronic metformin treatment had deleterious effects on behaviors and AD pathology by upregulating AMPKα1-subunit expression in 3xTg-AD mice. Therefore, long-term metformin prescription for familiar AD gene-containing people should be reconsidered. This study also suggests that metformin is not a promising candidate for AD treatment.

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ABSTRACT (IN KOREAN)

메트포르민 장기 투여가 유전변이 알츠하이머 모델 쥐의 인지행동과 치매 병리에 미치는 영향

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조 소 연

목적: 알츠하이머 치매(Alzheimer's disease; AD)는 다영역 인지기능 장애를 동반하는 가장 흔한 신경 퇴행성 질환 중 하나이다. 메트포르민은 제 2 형 당뇨병(type 2 diabetes mellitus; T2DM)을 치료하는 대표적인 1 차 치료제로서 핵심 에너지 조절자인 AMP-activated protein kinase (AMPK)을 활성화하여 고혈당증과 인슐린 저항증과 같은 대사 기능 장애를 개선한다. T2DM 은 여러 연구를 통해 AD 진행과 밀접한 관련이 있음이 밝혀지면서, 메트포르민은 유망한 AD 치료제로 여겨지고 있다. 메트포르민이 AD 병리에 미치는 영향을 평가하기 위해 많은 연구가 진행되었지만, 결과는 아직까지 명확하지 않다. 특히 몇몇 연구는 메트포르민이 인지 기능 저하 및 AD 병리를 악화시킨다는 것을 발견하였다. 만약 이 결과가 사실이라면, 이는 AD 환자에게 메트포르민을 사용하는 것과 메트포르민의 T2DM 에서 AD 치료제로의 용도 변경 연구에 지장을 줄 수 있다. 따라서 본

연구에서는 메트포르민이 인지행동과 AD 병리에 미치는 영향을 확인하여, 메트포르민의 AD 치료제로서의 가능성 및 안전성을 평가하였다.

방법: 수컷 (n = 26) 및 암컷 (n = 12) 3xTg-AD 마우스를 유전변이 AD 모델 마우스로 사용하였다. 생후 3 개월부터 2 mg/ml 메트포르민을 마우스에게 경구 투여하였다. 터치스크린 시스템은 인지-행동 평가로서 식이를 제한한 수컷 마우스에서 4 개월부터 전 연령에 걸쳐 AD 에서 손상된 다양한 인지기능 영역에 장기 메트포르민 투여가 미치는 영향을 확인하기 위해 사용되었다. 장기 메트포르민 투여가 식이를 제한하지 않은 암컷 마우스의 해마에서 아밀로이드 베타 (Amyloid beta; Aβ) 및 타우 발병기전과 같은 AD 병리와 AMPK 조절에 미치는 영향을 웨스턴 블롯 분석과 조직면역염색을 통해 확인하였다.

결과: 장기 메트포르민 투여는 3xTg-AD 마우스의 학습 및 기억 응고화를 손상시켰다. 메트포르민은 식욕이나 전신 대사 조절에 영향을 미치지 않고, 집중력 회복, 물체-위치 연상 학습 및 기억, 장기 기억을 손상시켰다. 메트포르민은 장기투여 시 3xTg-AD 마우스의 AMPKα1-서브유닛 발현을 증가시킴으로서 AMPKα-서브유닛의 조절자로서 기능하였다. 장기 메트포르민 투여는 3xTg-AD 마우스의 AD 병리 또한 손상시켰다. 메트포르민은 Aβ 올리고머의 양, Aβ 플라크의 갯수 및 응집, 인산화 타우(p-tau)Ser356 및 p-tauThr231 의 양과 글리코겐 생성효소 인산화효소 3β (Glycogen synthase kinase 3β; GSK3β) 발현 증가를 통해 Aβ 및 타우 발병기전을 악화시켰다.

결론: 장기 메트포르민 투여는 3xTg-AD 마우스의 AMPKα1-서브유닛 아이소폼 발현 증가를 통해 인지행동 및 AD 병리를 악화시켰다. 따라서, 가족성 AD 유전자를 가진 사람들에게

메트포르민을 처방하는 것은 재고되어야 한다. 추가적으로, 메트포르민은 유망한 AD 치료제 후보가 아닐 수도 있다.

핵심 되는 말: 알츠하이머 치매, 메트포르민, 인지기능, 터치스크린 시스템, Aβ, p-tau, AMPK

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