Receptor for advanced glycation endproducts (RAGE) modulates glyoxalase-1 enzyme activity in mouse models.

by

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ABSTRACT

Alzheimer's disease (AD) is the most common cause of dementia in the world. AD is characterized pathologically by the presence of Amyloid-β (Aβ) plaques and tau neurofibrillary tangles (NFTs). Advanced glycation endproducts (AGEs), which are derived from alpha-dicarbonyls such as methylglyoxal (MG), form endogenously with physiological aging. These AGEs have been observed to co-localize with both the plaques and NFTs in AD patients' brains.

Receptor for advanced glycation endproducts (RAGE) has been implicated in the pathogenesis of AD. However, the exact mechanism by which RAGE contributes to AD pathology is only partially known. Glyoxalase-1(GLO1) is an important enzyme involved in the detoxification of precursors of AGEs, which serve as the highly reactive primary ligands for RAGE. GLO1 is found to be downregulated in the brains of advanced stage AD patients whereas RAGE is overexpressed in such brains.

Aging is associated with increased generation and deposition of AGEs, resulting from non-enzymatic glycation (or oxidation) of proteins and lipids. Higher AGE formation is associated with a multitude of cellular and synaptic disturbances. Thus, to see the direct effect of age on enzyme activity, we first performed a study of wild type (WT) animals from 3 to 30 months and found that beyond 12 months of age, GLO1 activity significantly decreases. This shows that aging can be a major factor contributing to AD pathology through the downregulation of GLO1 activity. For uniformity, we used 12 months old mice for all further studies.

In order to investigate whether RAGE directly modulates GLO1 enzyme activity and protein expression, we used previously generated multiple transgenic (Tg) non-AD mouse models by either genetic deletion of RAGE (RAGE knockout) or the introduction of signal deficient

dominant-negative mutant RAGE (DNRAGE). They were, in the past, characterized for RAGE and subsequent RAGE-mediated signal transduction in our lab. GLO1 enzyme activity was measured spectrophotometrically while GLO1 protein expression was determined with western blotting.

The Tg mice displayed either a) an increase in GLO1 enzyme activity, and/or b) an increase in GLO1 protein expression when compared with age-matched WT controls. Global RAGE knockout (RO) and neuronal RAGE knockout (nRKO) mice showed significantly higher GLO1 enzyme activity compared to WT controls. RO mice showed a significant increase in the protein expression but nRKO mice did not. Similarly, the mice with DNRAGE targeted to cortical neurons (neuronal DNRAGE) and to microglia (DNMSR) exhibited an increase in GLO1 enzyme activity compared with WT mice but showed no significant change in protein expression. This differential effect in protein expression can be due to the difference in post-translational modification such as disulfide bridge formation or presence of GLO1 variations. It could also be the effect of normal or higher GLO1 activity in non-neuronal or microglial cells.

Given the clear modulation of GLO1 by RAGE variation, we performed the GLO1 enzyme activity and expression assays in Tg mice modeling amyloid plaque development by expressing a mutant form of human APP leading to overproduction of Aβ (mAPP) to see if similar effects are observed in a mouse model showing AD-like pathology. We also crossed these mAPP mice with RO mice to develop RAGE-deficient mAPP mice (mAPP/RO). The mAPP mice showed a significant reduction in both GLO1 enzyme activity and protein expression. mAPP/RO mice and RO mice showed higher GLO1 activity and protein expression compared to mAPP mice. These findings highlight that RAGE-dependent signaling downregulates GLO1 enzyme activity and the

deletion of RAGE protects against this decrease. Also, it supports the hypothesis that loss of RAGE-mediated signaling leads to an increase in GLO1 activity, whereas specific APP mutation (and Aβ overproduction) contributes to the decrease in GLO1 activity.

Taken together, these data show that RAGE functions as an active modulator of GLO1 enzyme activity, thereby providing new insights into a mechanism by which the RAGE-dependent signaling cascade contributes to the pathogenesis of AD. Thus, RAGE deletion or blockade of RAGE signaling may be a potential target for developing treatments for preventing the progression of AD and related degenerative disorders through modulation of GLO1 function.

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LIST OF ABBREVIATIONS

AD- Alzheimer's disease
Aβ- Amyloid β protein
NFTs- Neurofibrillary tangles
AGEs- Advanced glycation endproducts
MG- Methylglyoxal
GSH- Glutathione
RAGE- Receptor for advanced glycation endproducts
GLO1- Glyoxalase-1 enzyme
WT- Wild type
mAPP- mutant amyloid precursor protein
RO- RAGE knockout
nRKO- neuronal RAGE knockout
DNRAGE- Dominant-negative form of RAGE lacking RAGE signaling targeted to neurons
DNMSR- Dominant-negative form of RAGE lacking RAGE signaling targeted to microglia
TBS- Tris buffer saline solution
TBST –Tris buffer saline solution with tween 20
BSA- Bovine serum albumin
Tg- Transgenic
CK2-CAMKIIA- Calcium-calmodulin-dependent kinase II
PDGF- Platelet-derived growth factor
PCR- Polymerase chain reaction
MSR- Macrophage scavenger receptor
MAPK-Mitogen-activated protein kinase

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INTRODUCTION

ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is a progressive, and irreversible neurodegenerative disorder. It is characterized by memory loss, personality changes, and cognitive impairment and affects 1 in 10 people of ages 65 or older, making it the most common form of dementia in older adults [1-6]. It is predicted to affect approximately 42 million people worldwide by the year 2020[7]. The main pathological features of AD are the formation of amyloid-β (Aβ) deposits in the anterior cerebral cortex and hippocampus as well as the formation of intracellular neurofibrillary tangles (NFTs) resulting in neuronal cell death [3, 5, 8-11]. Aβ, which is a 40–42 amino-acid peptide generated by proteolytic cleavage of the amyloid precursor protein (APP), and NFTs, which are composed of a hyper-phosphorylated microtubule-associated tau(τ) protein, are the major markers of progression of AD [3, 5, 6, 11-22].

ADVANCED GLYCATION ENDPRODUCTS (AGEs)

Advanced glycation endproducts (AGEs) are members of a heterogeneous class of molecules, which modify cellular function by distinct mechanisms including ligation and activation of signal transduction receptors. These AGEs are the products of non-enzymatic glycation (or oxidation) of proteins and lipids. The levels of AGEs increase during biological aging due to decreased efficiency of homeostatic processes. AGEs also further contribute to the normal physiological aging process [22, 23]. When their production is accelerated, they have a causative role in the complications of diabetes mellitus (DM) and several neurodegenerative diseases, including AD,

Parkinson's, and Huntington's diseases [22, 24-27]. Excess AGE accumulation is detrimental to neurons and is believed to be a key to the pathogenesis of cognitive decline in normal aging and specific chronic diseases of aging [28-31].

Long-lived proteins such as Aβ and hyper-phosphorylated tau that accumulate in AD brain are highly susceptible to AGE modification. AGE modified Aβ or tau protein result in increased oxidative stress and chronic inflammation, accelerating AD pathology and neuronal perturbation [32-40]. Moreover, Aβ or tau glycation results in increased aggregation and subsequent formation of senile plaques or NFTs, the major pathological feature of AD suggesting that AGE modification is an important risk factor for AD[24, 41-47]. Although increased accumulation of AGEs in brain, as seen in aging, diabetes, or neurodegenerative diseases, speeds up oxidative damage to neurons contributing to synaptic dysfunction and cognitive decline, the underlying mechanisms for this accumulation are not well understood [48].

These AGEs, formed by non-enzymatic glycation of proteins and lipids on exposure to sugars, play an important role in normal aging and in degenerative diseases such as AD, atherosclerosis and ALS (amyotrophic lateral sclerosis) [24, 47]. Receptor for these AGEs (RAGE) is a multi-ligand, transmembrane receptor expressed by neurons, microglia, astrocytes, cerebral endothelial cells, pericytes, phagocytes, cardiac myocytes, hepatocytes, and smooth muscle cells [49]. Increase in the expression of RAGE is seen in AD-affected brain regions [50-52]. RAGE and its isoforms play an important role in the regulation of metabolism, inflammation, and epithelial survival in stress conditions [14, 53-55].

In addition to AGEs' ability to directly alter the structure and function of targeted proteins within cells that causes cell or tissue damage, emerging evidence has also demonstrated AGEs as a signaling ligand, interacting with RAGE[22, 56-60]. AGEs elicit signal transduction changes that adversely affect numerous peripheral organs. Although AGE accumulation is increased in cortical neurons, hippocampal pyramidal neurons, astrocytes, and other glial cells in aging and AD brain[14, 45, 56, 59, 61, 62], the direct effect of AGEs–RAGE interaction on brain function, and on alteration in enzyme function and levels, still remains largely unknown.

RAGE

RAGE was first identified as a cell surface receptor of the immunoglobulin superfamily for AGEs [5, 63-68]. There are three major isoforms of RAGE. These different RAGE isoforms are generated as the result of alternative splicing and are defined as being the full-length RAGE, dominant negative RAGE (DNRAGE), and secretory RAGE (sRAGE) [53, 54, 68]. Increased expression of RAGE occurs in neuronal and non-neuronal cells in the peripheral and central nervous system in aging, diabetes, and AD affected individuals, where RAGE ligands are upregulated [43, 51, 64, 69-73].

RAGE and microglia

RAGE-dependent signaling in microglia contributes to neuroinflammation especially in mouse models of AD that ultimately impairs neuronal function and directly affects amyloid accumulation [74, 75]. Deposition of cross-linked insoluble protein aggregates such as amyloid plaques is characteristic for Alzheimer's disease. Microglial activation by these extracellular deposits has been proposed to play a crucial role in functional degeneration as well as neuronal cell death[76]. AGEs activate specific signal transduction pathways, resulting in the up-regulation of various proinflammatory signals such as cytokines [interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-a)] and nitric oxide synthase (NOS)[77] . The blockade of microglial RAGE (by using Cre/loxP technology) is found to have beneficial effect on Aβ-mediated neuronal changes relevant to AD pathogenesis [3, 10, 63, 64, 73, 74, 78-80].

RAGE and neuronal function

Genetic deficiency of neuronal RAGE protects against AGE-induced synaptic injury. RAGE and nuclear factor kappa B (NF-κB) have been related to neuronal death [81]. Interaction of Aβ with neuronal RAGE leads to NF-κB activation, indicating a possible role of neuronal RAGE in inflammation[72]. Aβ induces a RAGE-dependent pathway that involves activation of p38 MAPK, resulting in Aβ internalization and leads to mitochondrial dysfunction in cortical neurons[82]. RAGE blockade can be a target for development of interventions aimed at preventing progression of cognitive decline in aging and AGE-related neurodegenerative disorders[3, 5, 20, 27, 28, 48, 66, 82-85].

METHYLGLYOXAL

Methylglyoxal (MG) is a highly reactive dicarbonyl compound formed in vivo as a major byproduct of glycolysis. MG is an endogenous byproduct of the normal metabolism of carbohydrates along with lipids, and proteins, inevitably produced spontaneously or enzymatically [86, 87].

During glycolysis, MG is readily formed under alkaline conditions from glyceraldehyde or dihydroxyacetone[88]. Intermediate metabolites such as acetoacetate from lipids, and succinyl acetone and amino acetone from threonine and glycine metabolism, are also endogenous sources of MG . Accumulation of MG is highly toxic, since it readily reacts in vivo with basic phospholipids and nucleotides, and with lysine and arginine residues of proteins, leading to AGE formation [89-92]. MG can also impair the antioxidant system by depleting glutathione (GSH) levels[87]. It has been considered as a possible causative agent in a number of pathologies, such as diabetes [93, 94], hyperalgesia and inflammation, aging disorders[5, 59], Alzheimer's disease[95, 96], epilepsy, autism[97], and anxiety[98, 99] among others.

MG concentration in human plasma is in the range of 0.1–0.6 µM, and about 10- to 20 fold higher in the cerebrospinal fluid [100-102]. These elevated levels of MG can make nerve cells more susceptible to AGE formation, which is thought to be a relevant factor in the development of neurodegenerative diseases[103]. MG can induce irreversible loss of protein function, including cross-linking, as well as contributing to oxidative stress[104]. MG is the major precursor in the

formation of AGEs. Under normal physiological conditions, MG is detoxified by the glyoxalase system to give D-lactate, with GLO1 as the key rate-limiting enzyme in the process [86].

GLYOXALASE-1 (GLO1)

AGE precursor compounds are cellularly detoxified by the glyoxalase system, consisting of GLO1 and 2. GLO1 is a cytosolic enzyme which catalyzes the isomerization of hemithioacetal adducts, formed in a spontaneous reaction between a glutathionyl group (such as GSH) and aldehydes (such as MG). Since alpha-oxoaldehydes, such as MG are important intracellular AGE precursors [105], GLO1 has an important role in reducing cellular AGE levels. Binding of AGEs to RAGE causes suppression of GSH thus increasing the intracellular oxidative stress[60, 106]. Decrease in GSH leads to decreased GLO1 activity [107]. Also, decrease in GLO1 activity due to aging and oxidative stress results in increased glycation and tissue damage. In vitro studies with endothelial cell lines have shown that GLO1 over-expression prevents intracellular AGE formation [108]. GLO1 levels are diminished in brains of older adults and in AD patients' brains. However, the mechanism behind the change is still unknown. Understanding this mechanism of modulation will help in precise targeting of GLO1 for treatment of AD. [23, 46, 86, 90, 91, 93, 94, 98-100, 107-145]

As high levels of AGEs are involved in GLO1 downregulation, we hypothesized that RAGE decreases GLO1 activity in transgenic non-AD (RAGE knockout and DNRAGE) and AD (mAPP) mouse models. To test the proposed hypothesis, we divided the experiments in three parts to check:

1. effect of physiological aging 2. effect of RAGE deletion or signaling blockade and 3. effect of mutant APP on GLO1 enzyme activity and protein expression.

As RAGE is expressed in neurons and non-neuronal cells (i.e., glial, endothelial cells, and pericytes of the blood–brain barrier) [48], the results obtained from global RAGE knockout(RO) mice do not explain which cell types are important in AGE-induced impairments. Therefore, study of transgenic mice with neuronal RAGE knockout (nRKO) and DNRAGE mutant specifically in the microglia and neurons was done[28, 48, 146], to address the question of whether the activities of neuronal and microglial RAGE are responsible for AGE-induced GLO1 reduction [48].

The purpose of this thesis is to investigate the link between GLO1 activity and protein levels and presence of RAGE and provide a better understanding of their role in Alzheimer's disease pathophysiology.

MATERIALS & METHODS

ANIMALS

Generation and characterization of global knockout of RAGE mice

For our experiments, transgenic (Tg) RO mice generated previously were used. Homozygous RAGE null mice (generated in the 129 strain) were backcrossed three generations into C57BL/6; mating of heterozygous RAGE null males and females yielded heterozygous mice, as well as wild-type RAGE-bearing (RAGE$^{+/+}$) and homozygous RAGE null animals (RAGE$^{-/-}$). RAGE null mice are viable and display normal reproductive fitness [66]. In all cases, age-matched littermates (WT-wild type) were employed as controls. The mice were characterized for RAGE and signaling markers in our lab. The institutional Animal Care and Use Committee of the University of Kansas approved all protocols.

Generation and characterization of conditional neuronal knockout of RAGE mice

Previously generated nRKO mice were used for experiments. Our lab developed a model system in which neuronal expression of RAGE, in particular in the brain region responsible for learning and memory, is deleted so that consequences of receptor–ligand interaction could be assessed [48]. To this end, Cre recombinase neuronal RAGE null mice, termed as Tg nRKO mice were created by crossing neuronal-targeted and region-restricted Tg mice that express Cre recombinase in the entire forebrain, under the control of the forebrain-specific calcium-calmodulin-dependent kinase II (CAMKIIA, CK2) promoter to generate deletion of neuronal RAGE in the entire hippocampus and cortex. Cre-loxP system was used to generate the targeting vector and the RAGE/flox mice (performed by Ozgen Inc., Bentley DC, WA, and Australia).

The targeting vector consists of three fragments: the 5′ homology arm, the 3′ homology arm, and loxP arm. Two loxP sites flanking RAGE exons 2–4 allow for Cre-mediated deletion using Cre recombinase. Excision of exons 2–4 by Cre recombinase results in a frame shift and early stop codon from the mouse RAGE sequence, which block RAGE expression. Our lab first made homozygous RAGE floxed allele mice (*RAGE* $^{flox/flox}$); then *RAGE*$^{flox/flox}$mice were crossed with neuronal target CK2 mice to generate homozygous floxed and hemizygous Cre nRKO mice (*RAGE*$^{flox/flox}$*/CK2-Cre*), which were then verified by analysis of tail DNA with PCR amplification using primers for *flox* (700 bp) and *Cre* transgene (300 bp). Immunoblotting of cortical homogenates with anti-RAGE antibody showed significant reduction of RAGE expression levels in cerebral cortex of nRKO mice compared with non-Tg mice (~80% reduction *versus* non-Tg brain, $P<0.01$). The observed incomplete suppression of RAGE expression was due to the use of brain homogenates, which contained non-neuronal cells with intact RAGE. Confocal microscopy with double immunostaining of RAGE and MAP2(neuron specific protein marker) clearly demonstrated that there was virtually no RAGE signal in MAP2-positive neurons in cortex and hippocampus of nRKO mice, thereby verifying RAGE depletion in cortical neurons in the brain of nRKO mice.

Generation and characterization of transgenic mice with cytosolic dominant-negative RAGE (neuronal DNRAGE)

Previously generated neuronal DNRAGE mice were used for the experiments. We used the transgenic mice with signal-transduction-deficient mutants of RAGE in which the cytosolic domain of the receptor was deleted, thereby imparting a dominant-negative (DN)-RAGE effect, targeted to neurons (neuronal DNRAGE) driven by the platelet-derived growth factor-B (PDGF-B) chain promoter [66, 72]. These neuronal DNRAGE mice were characterized, demonstrating localization of DNRAGE in cortical neurons previously in our lab.

Generation of transgenic mice with Dominant-negative effect targeted to microglia (DNMSR-microglial DNRAGE)

Previously generated transgenic mice with signal-transduction-deficient mutants of RAGE in which the cytosolic domain of the receptor has been deleted, thereby imparting a dominant-negative RAGE effect, targeted to microglia (DNMSR) driven by the macrophage scavenger receptor (MSR) type A promoter, were used for this study[20, 75, 80]. Founders were identified by mouse tail biopsy; DNA was prepared, and Southern blot was performed using human RAGE cDNA as a probe and by PCR. Mice were generated and hemizygous mice backcrossed eight generations into C57BL/6 were used in our studies. Human DNRAGE transgene was identified by PCR using primers 4A-5'AGGATCAGGGCTGGGAACTCTA, and

4B-5' TCCCCCTGAACCTGAAACATAAAA.

Generation of transgenic mAPP AND mAPP/RO mice [28]

Previously generated transgenic mice with neuronal overexpression of a mutant human form of APP (Tg mAPP or mAPP, J-20 line, Jackson Lab) driven by the PDGF-B chain promoter were used in this study[28, 147]. These mAPP mice are a well-established mouse model of AD and exhibit many features of AD neuropathology[28, 72], and have been used in previously published studies [72, 73, 78, 147-150]. Homozygous RAGE null mice (RO mice) were backcrossed into C57BL/6 strain for more than 12 generations and were crossed with mAPP mice to generate mAPP/RO mice. Age-matched non-genetically modified WT littermates were used as controls in our studies. Mice were maintained on normal rodent chow and allowed free access to food and water. Genomic DNA was isolated from tail biopsies and subjected to PCR analysis to identify the deficiency of RAGE or human APP gene. The mice were anesthetized with ketamine (100 mg/kg) and Xylazine (10 mg/kg) and flush perfused transcardially with 0.9% saline. Brains were removed and divided sagittally. One hemibrain was post-fixed in phosphate-buffered 4% paraformaldehyde (pH 7.4) at 4°c for 26h and sectioned at 20 mm with a vibratome (Leica); the other hemibrain was dissected to hippocampus and cortex, snap frozen and stored at –80°c for protein analysis.

Animal models

Details of all animal models used are summarized in the following table: Table 1.

Table 1: Details of animal models used for experiments

No.	Genotype	Genetic Manipulation	RAGE (Receptor)	RAGE signaling	AD-like pathology
1	RO	Global RAGE knockout	-	-	-
2	nRKO	Neuronal RAGE knockout	- (in neurons)	-	-
3	Neuronal DNRAGE	Signal deficient mutant targeted to neurons	+	- (in neurons)	-
4	DNMSR	Signal deficient mutant targeted to microglia	+	- (in microglia)	-
5	mAPP	Overexpression of APP	+	+	+
6	mAPP/RO	APP crossed with RO	-	-	-

GLO1 ENZYME ACTIVITY KINETIC ASSAY

GLO1 catalyzes the isomerization of the hemithioacetal formed non-enzymatically from MG and GSH to S-D-lactoylglutathione. In this assay, the activity of GLO1 was measured spectrophotometrically by following the increase in OD240 for which the change in molar extinction coefficient $\Delta\varepsilon240=3.37\ mM^{-1}\cdot cm^{-1}$. The hemithioacetal was pre-formed in situ by incubation of MG and GSH in 50 mM sodium phosphate buffer (0.02 M Sodium phosphate dibasic heptahydrate, 0.03 M Sodium phosphate monobasic monohydrate, pH 6.6) at 37°C for 10 min. The tissue sample homogenate was then added, the OD240 was monitored over 10 min, and the initial rate of increase in OD240 and hence GLO1 activity was deduced with correction for blank. GLO1 activity is given in units per mg of protein where one unit is the amount of enzyme that catalyzes the formation of 1 μmol of S-D-lactoylglutathione per min under assay conditions.

Calculation of enzyme units

GLO1 enzyme activity was determined using the following set of equations:

$$\frac{\text{Units}}{\text{mL}}\text{enzyme} = \frac{\left[\left(\frac{\Delta\text{A240nm}}{\text{min for test}} - \frac{\Delta\text{A240nm}}{\text{min for blank}}\right)(\text{Total volume of assay})(\text{dilution factor})\right]}{[(\text{Extinction Coeff. of S} - \text{Lactoylglutathione})(\text{Volume of enzyme used})]}$$

Where. Total volume (in milliliters) of assay=0.2 (200 ul reaction)

Millimolar extinction coefficient of S-Lactoylglutathione at 240 nm= 3.37

Volume (in milliliter) of enzyme used= 0.0067 (6.7 ul protein samples)

$$\text{Units/ml enzyme} = \frac{(\Delta\text{A240nm/min Test} - \Delta\text{A240nm/min Blank})(0.2)(1)}{(3.37)(0.0067)}$$

$$\text{Units/mg protein} = (\text{units/ml enzyme})/(\text{mg protein/ml enzyme})$$

WESTERN BLOT

Total protein extracts were prepared from the dissected frontal cortical brain from snap-frozen hemi-brains by homogenization with 1× RIPA buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA,1% NP-40, 1% Sodium deoxycholate, 2.5mM sodium pyrophosphate,1 mM Na2VO4, Catalog #9806, Cell Signaling Technology, MA) containing 1× protease inhibitor cocktail (104 mM AEBSF, 80 µM Aprotinin, 4 mM Bestatin, 1.4 mM E-64, 2 mM Leupeptin and 1.5 mM Pepstatin A, Roche applied Science, IN) and 1 mM PMSF (Phenylmethylsulphonyl fluoride) OR with 0.01% Triton-X solution in DPBS (Dulbecco's Phosphate-buffered saline, KCl, KH_2PO_4, NaCl, $Na_2HPO_4.7H_2O$, Catalog #A14190144, Gibco-Thermo Fisher Scientific). Total protein extracts from cells were prepared using 1× cell lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5mM sodium pyrophosphate,1 mM Na2VO4, Catalog #9803, Cell Signaling Technology, MA) containing 1× protease inhibitor cocktail and 1 mM PMSF. Protein concentrations of the tissue or cell lysates were spectrophotometrically determined with Bio-Rad protein assay dye reagent (acidified Coomassie Brilliant Blue G-250) concentrate kit at 595nm. Equal amount of protein from each sample was loaded and then separated by SDS/PAGE (12% Bis–Tris gel, Catalog# NP0301, Invitrogen/Thermo Fisher Scientific, Austin, USA), and then electrophoretically transferred to 0.45 µm nitrocellulose membranes (Catalog# 162-0094, Bio-Rad Laboratories, Hercules, CA, USA) using NuPAGE running buffer (50 mM MES, 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.3, 50 ml 10 X buffer in 1 L milliQ H2O, Thermo Fisher Scientific, Cat. No. NP0002, Lot no. 1958831). Non-specific binding was blocked by 5% non-fat milk in TBS buffer (30 ml 5M NaCl and 20 ml 1M Tris-HCl in 1L milliQ H2O, 20 mM Tris–HCl, 150 mM NaCl, pH 7.6) for 1 h at room temperature. The membrane was incubated in diluted primary antibody in BSA

(bovine serum albumin) in TBST (TBS containing 0.1% Tween-20, pH 7.6) overnight with gentle shaking at 4 °C. Primary antibodies used for the reactions were as follows: rabbit anti-GLO1 (1:3000 Catalog #SC-67351, Santacruz biotechnology) and mouse anti-β-actin (1:5000, Catalog #A5316, Sigma, St. Louis, MO, USA). The membrane was washed with TBST and then incubated with the corresponding horseradish peroxidase-conjugated secondary antibody: goat anti-rabbit IgG or goat anti-mouse (1:10 000, Catalog #A6154 or #A4416, Sigma, St. Louis, MO, USA), for 1 h with gentle shaking at room temperature to identify sites of binding of each primary antibody. Finally, the chemiluminescent signal from immunoreactive band was detected with ECL using an enhanced chemiluminescent western blot system (Solution A: Luminol solution, Solution B: Peroxide solution, GE Healthcare, NJ). GLO1 protein expression was quantified as ratio of intensity of GLO1 band to the intensity of β-Actin band (loading control) immunoreactive bands by immunoblotting with anti-GLO1 and anti-β-Actin antibodies. Data from western blot was quantified using ImageJ (NIH) software and significance was analyzed using GraphPad Prism 8.

Antibodies

The antibodies used in the experiments are listed in following table: Table 2.

Table 2: Summary of antibodies used

No.	Antibody	Dilution	Cat. No.	Lot No.	Vendor
1	Rabbit polyclonal anti-glyoxalase-1 antibody	1:3000	Sc-67351	DO114	Santacruz Biotech.
2	Anti-rabbit IgG (whole molecule)–peroxidase antibody produced in goat	1:10000	A6154	SLBV9141	Sigma-Aldrich
3	Monoclonal anti-β-actin antibody produced in mouse	1:5000	A5316	SLBS6528	Sigma-Aldrich
4	Anti-mouse IgG (whole molecule)–peroxidase antibody produced in goat	1:10000	A4416	SLBW491 7	Sigma-Aldrich
5	Rabbit polyclonal anti-RAGE antibody	1:1000	GTX23611	41906	GeneTex

STATISTICAL ANALYSES

All statistical analyses were conducted using GraphPad Prism 8(GraphPad software Inc., La Jolla, CA). Shapiro-Wilk's test for normality and Brown-Forsythe-Levene test for homogeneity of variance were used to determine whether the data met the requirements for a parametric analysis of variance. Two-tailed student's t-test, one-way ANOVA, and two-way ANOVA were used for analyses (as indicated in the figure descriptions) followed by Tukey's or Dunn's test for post-hoc multiple comparisons where required. All results were reported as mean ± S.E.M. $^{*}p<0.05$ was considered significant. Other significant p-values were designated as $^{**}p<0.01$, $^{***}p<0.001$ and $^{****}p<0.0001$.

RESULTS

1) Effects of physiological aging on GLO1 activity and protein expression in 3 to 30-month-old WT mice

To investigate effect of physiological aging on GLO1 activity, we used WT mice of age: 3-30 months (n=5/age group). Spectrophotometric measurement of GLO1 enzyme activity revealed significantly increased GLO1 activity in the brains of 12 months old WT mice compared with WT mice at 3 and 6 months of age (Fig. 1A and C). We also found that beyond the age of 12 months, GLO1 activity significantly reduced up to 30 months of age (Fig. 1A and C). We observed that at 12 months of age, GLO1 activity increases with increase in total protein concentration (Fig.1B). Thus, we found that physiological aging contributes to significant decrease in GLO1 activity beyond 12 months. Thus, for uniformity, we used 12 months old mice for further experiments (except in case of RO mice).

2) Effects of global RAGE knockout on GLO1 activity and protein expression in 6- and 12-months old mice

In view of elevated expression of RAGE and reduced expression of GLO1 correlating to the severity of AD, we sought to detect the effect of RAGE on GLO1 activity and protein expression levels in RO mice. 6 month and 12-month-old homozygous RO mice were used (n=4-5/ genotype/age group). Spectrophotometric measurement of GLO1 enzyme activity revealed significantly increased GLO1 activity in the brains of RO mice compared with WT mice at 6 months (Fig. 2A) and 12 months of age (Fig. 3A). For 12- months- old RO mice, immunoblotting and quantification of GLO1 protein bands (Fig. 3B and 3C) revealed that the protein levels were significantly increased in RO mice compared with those of 12-months-old WT mice. But this effect

was not seen in 6 months old mice (Fig. 2B and 2C). Global deletion of RAGE contributes to increase in GLO1 activity in 6- and 12-months old mice and expression in 12 months old mice.

3) Effects of neuronal RAGE knockout on GLO1 activity and protein expression in 12-month old mice

Since RAGE is expressed in both neuronal and non-neuronal cells, we sought to detect the effect of conditional neuronal RAGE deletion on GLO1 activity and protein expression levels in homozygous nRKO mice. These nRKO mice were used along with age-matched WT controls. Spectrophotometric measurement of GLO1 enzyme activity revealed significantly increased GLO1 activity in the brains of nRKO mice compared with WT mice at 12 months of age (Fig. 4A). Immunoblotting and quantification of GLO1 protein bands (Fig. 4C) revealed that the protein levels were not increased significantly in nRKO mice compared with those of WT mice at 12 months of age (Fig. 4B and 4C). Unlike 12-months-old RO mice, nRKO mice did not show elevated GLO1 protein levels. This effect can be attributed to the specific cell types in which RAGE knockout was carried out. Here, we found that neuronal deletion of RAGE significantly contributes to increase in GLO1 activity but not protein expression.

4) Effects of neuronal DNRAGE mutant on GLO1 activity and protein expression in 12-month old mice

Since we found that RAGE deletion leads to increase in GLO1 activity and protein expression, we wanted to investigate the effect of neuronal DNRAGE on GLO1 activity and protein expression levels. Heterozygous neuronal DNRAGE (n=3-6/ genotype) mice were used along with age-matched WT controls. Spectrophotometric measurement of GLO1 enzyme activity revealed

significantly increased GLO1 activity in the brains of neuronal DNRAGE mice compared with WT mice at 12 months of age (Fig. 5A). Immunoblotting and quantification of GLO1 protein bands (Fig. 5B) revealed that the protein levels were not increased significantly in these mice compared with those of WT mice at 12 months of age (Fig. 5C). This pointed out the need to repeat the study in a higher number of experimental mice to confirm the results. Thus, we found that loss of signaling of neuronal RAGE significantly elevates GLO1 activity but not protein expression.

5) Effects of microglial DNMSR mutant on GLO1 activity and protein expression in 12 months old mice

As microglia have been implicated in the possible pathway of effect of RAGE in AD progression, we wanted to investigate the effect of microglial DNMSR mutant on GLO1 activity and protein expression levels. Heterozygous DNMSR (n=4/ genotype) mice were used along with age-matched WT controls. Spectrophotometric measurement of GLO1 enzyme activity revealed significantly increased GLO1 activity in the brains of microglial DNRAGE mice compared with WT mice at 12 months of age (Fig. 6A). Immunoblotting and quantification of GLO1 protein bands (Fig. 6B) revealed that the protein levels were not significantly increased in these mice compared with those of WT mice at 12 months of age (Fig. 6C). Thus, we found that loss of signaling of RAGE specifically in microglia significantly upregulates GLO1 activity but not protein expression.

6) Effects of mutant APP overexpression on GLO1 activity and protein expression in 12 months old mice

After checking the GLO1 activity and expression in the RAGE knockout and DNRAGE mouse models, we then determined whether RAGE had a direct role in modulating GLO1 activity and expression in mAPP mice overexpressing mutant APP/Aβ and the effect of deletion of RAGE in these mice.

Homozygous RAGE null mice (RO mice) were crossed with mAPP mice overexpressing mutant APP/Aβ to generate mAPP/RO mice (n=5/ genotype). We demonstrated that GLO1 activity and protein levels were significantly reduced in mAPP mice compared with WT mice. In contrast, GLO1 activity was found to be higher in both heterozygous mAPP/RO and homozygous RO mice due to global genetic deletion of RAGE (Fig. 7A).

Consistent with the enzyme activity results, immunoblotting and quantification of GLO1 protein bands (Fig. 7B and 7C) revealed that the protein levels were increased in both mAPP/RO and RO mice compared with those of mAPP mice at 12 months of age. Thus, global deletion of RAGE in mAPP/RO mice rescues the decrease in GLO1 activity and expression seen in mAPP mice.

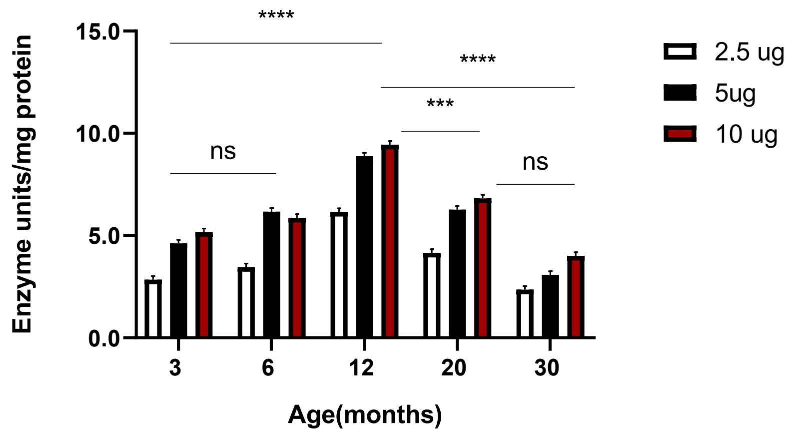
In summary, we provide substantial evidence of the protective effect of deletion of RAGE or loss of RAGE signaling on GLO1 enzyme activity. Genetic deletion of RAGE and blocked RAGE signaling in cortical neurons or microglia blocks AGE-mediated downregulation of GLO1 enzyme activity.

The results of enzyme activity and western blot assay are summarized in Table 3.

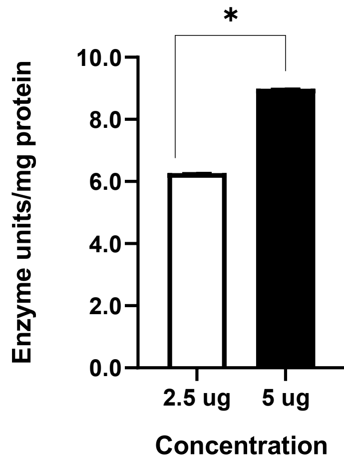
Table 3: Summary of GLO1 activity and expression in various mouse models compared to WT

No.	Genotype	Genetic Manipulation	GLO1 activity	GLO1 expression
1	RO	Global RAGE knockout	Increased	Increased
2	nRKO	Neuronal RAGE knockout	Increased	Unchanged
3	Neuronal DNRAGE	Signal deficient mutant targeted to neurons	Increased	Unchanged
4	DNMSR	Signal deficient mutant targeted to microglia	Increased	Unchanged
5	mAPP	Overexpression of APP	Decreased	Decreased
6	mAPP/RO	mAPP crossed with RO	Increased	Increased

1A.



1B.



1C.

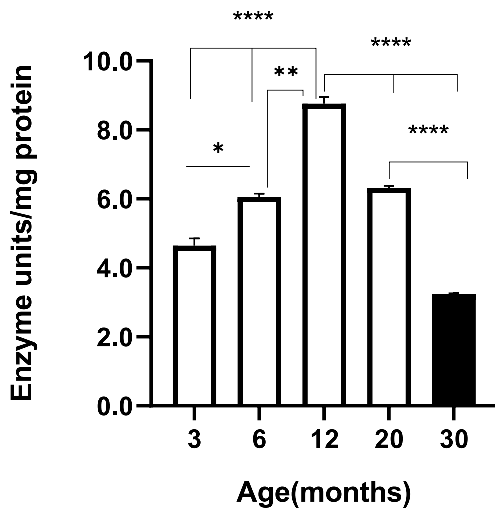
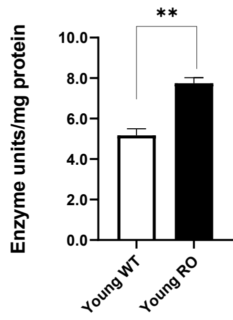


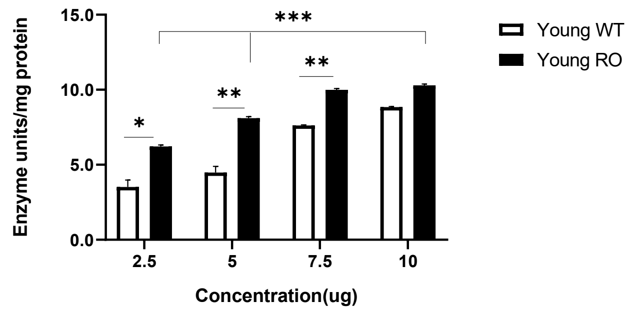
Figure 1: Effect of age on GLO1 enzyme activity in 3 to 30 months old WT mice.

(A)GLO1 enzyme activity was determined by spectrophotometric method in the cortex from 3 to 30-month-old WT mice (n=5/group) in an age-dependent manner at three different total protein concentrations [Two-way ANOVA with Tukey's post hoc multiple comparison tests, age: $F(3, 12) = 38.12$, concentration: $F(2, 3) = 114.2$ interaction: $F(8, 12) = 13.57$, $p<0.001$]. (B) Comparison of GLO1 activity at total protein concentration=2.5 and 5 ug at the age of 12 months (student's t test) (C)GLO1 activity of 3-30-month-old mice at total protein concentration=5 ug. (One-way ANOVA with Tukey's post hoc multiple comparison tests, $F(4, 10) = 225.7$ $P<0.0001$ *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$)

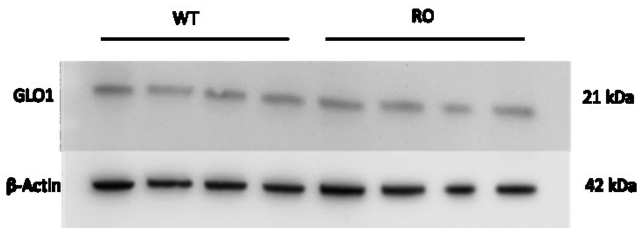
2A.



2B.



2C.



2D.

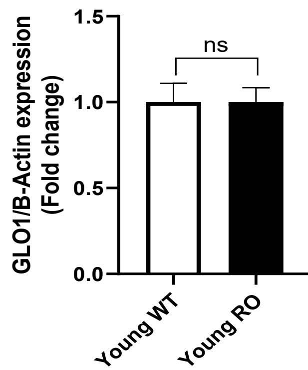
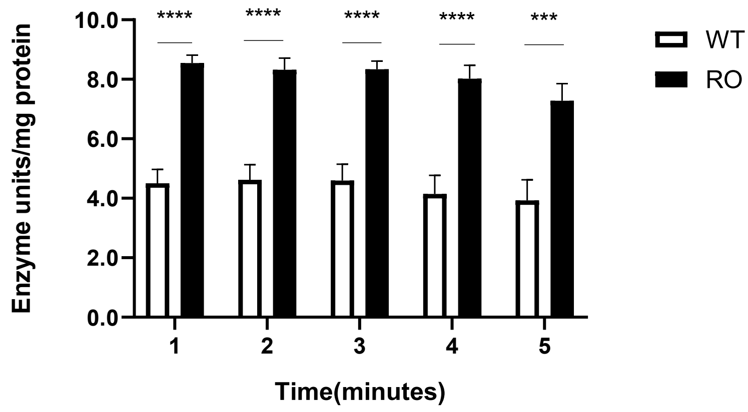


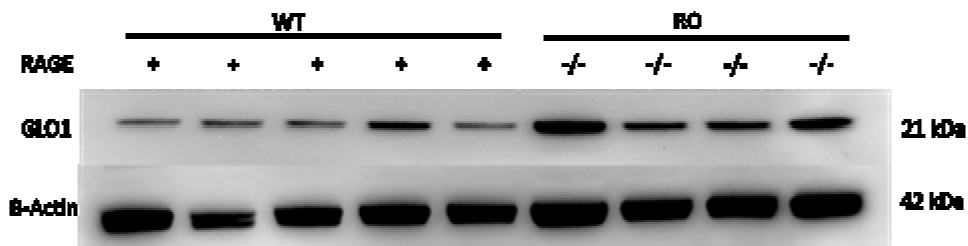
Figure 2: Effect of global RAGE knockout on GLO1 enzyme activity and protein expression in 6 months old WT and RO mice.

(A) GLO1 enzyme activity was determined by spectrophotometric method in the cortex from 6-month-old WT and RAGE Knockout (RO) mice (n=4/ genotype) at total protein concentration= 5ug and (B) at increasing total protein concentration levels (Two-way ANOVA- concentration: $F(3, 8) = 182.1$, genotype: $F(1, 8) = 246.1$, $p<0.0001$, interaction: $F(3, 8) = 7.690$, $P=0.0096$). (C) GLO1 protein expression was demonstrated by quantifying intensity of immunoreactive bands of GLO1 in the brain homogenates from indicated mice. (n=4/group). Representative immunoblots show immunoreactive bands for GLO1(21kda) as well as β-Actin protein used as loading control. (D) Quantification of GLO1 immunoreactive bands was performed blots in triplicate. (Student's t-test, ns=non-significant)

3A.



3B.



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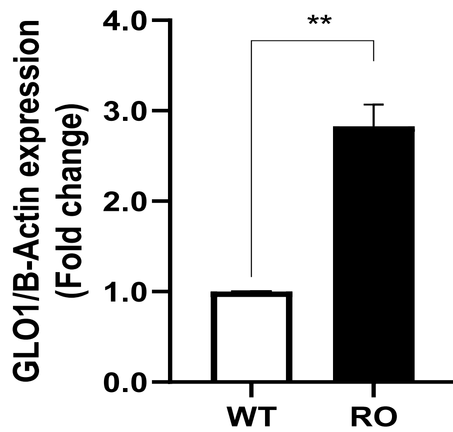
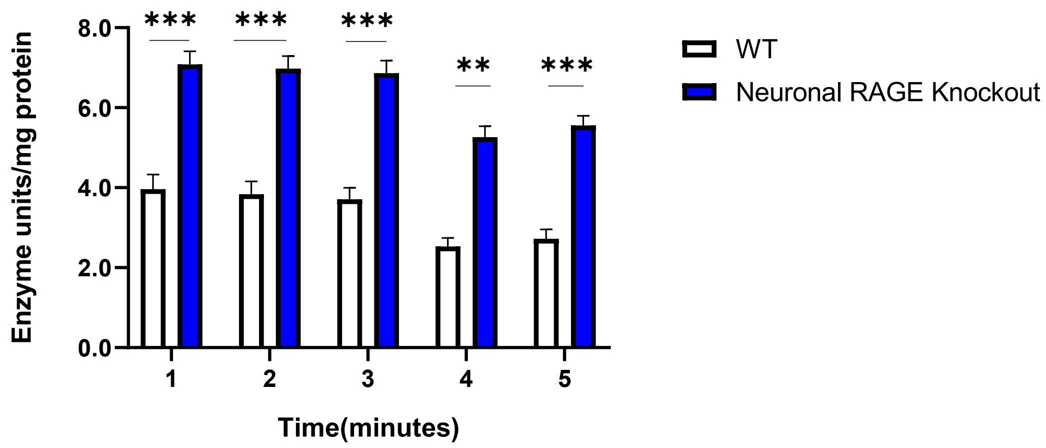


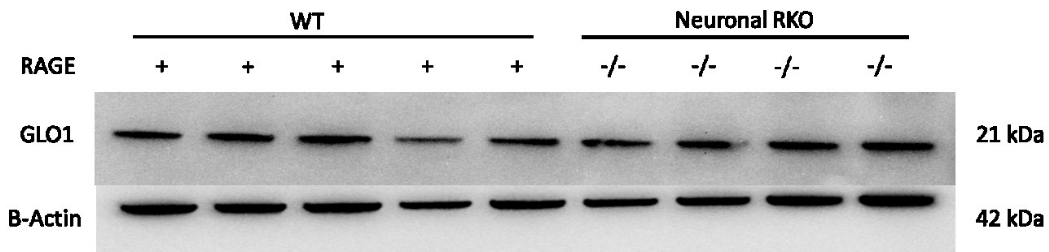
Figure 3: Effect of global RAGE knockout on GLO1 enzyme activity and protein expression in 12 months old WT and RO mice.

(A)GLO1 enzyme activity was determined by spectrophotometric method in the cortex from 12-month-old WT and RAGE Knockout (RO) mice (n=5/group) in a time-dependent manner. [Two-way ANOVA with Tukey's post hoc test, time: $F (4, 40) = 1.235$, genotype: $F (1, 40) = 140.2$, interaction: $F (4, 40) = 2.1335$, $P<0.0001$)] (B)GLO1 protein expression was demonstrated by quantifying intensity of immunoreactive bands of GLO1 in the brain homogenates from indicated mice. (n=4-5/group). Representative immunoblots show immunoreactive bands for GLO1(21kda) as well as β-Actin protein used as loading control. (C) Quantification of GLO1 immunoreactive bands was performed for blots in triplicate. (Student's t-test, **$p<0.01$ for RO compared with WT)

4A.



4B.



4C.

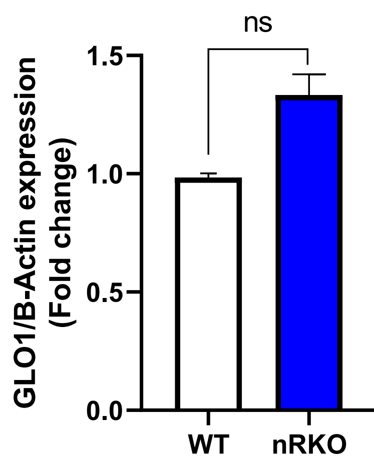
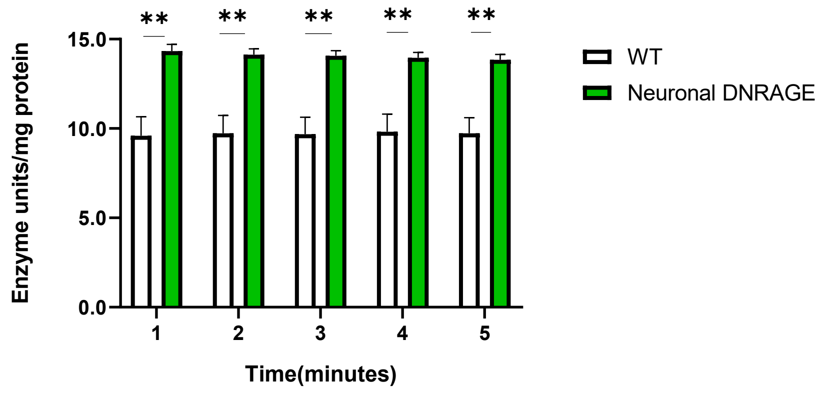


Figure 4: Effect of neuronal RAGE knockout on GLO1 enzyme activity and protein expression in 12 months old WT and nRKO mice.

(A)GLO1 enzyme activity was determined by spectrophotometric method in the cortex from 12-month-old WT and nRKO mice (n=4-5/group) in time-dependent manner [Two-way ANOVA, genotype: $F (1, 35) = 257.1$, time: $F (4, 35) = 13.55$, interaction: $F (4, 35) = 0.2272$, $P<0.001$]. (B) GLO1 protein expression was demonstrated by quantifying intensity of immunoreactive bands of GLO1 in the brain homogenates from indicated mice (n=4-5/group). Representative immunoblots show immunoreactive bands for GLO1(21kda) as well as β-Actin protein used as loading control. (C) Quantification of GLO1 immunoreactive bands was performed for blots in triplicate (Student's t-test, ns=non-significant).

5A.



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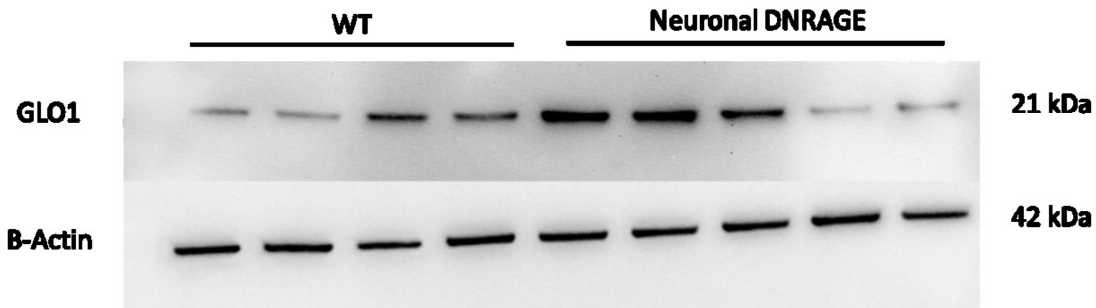


5C.

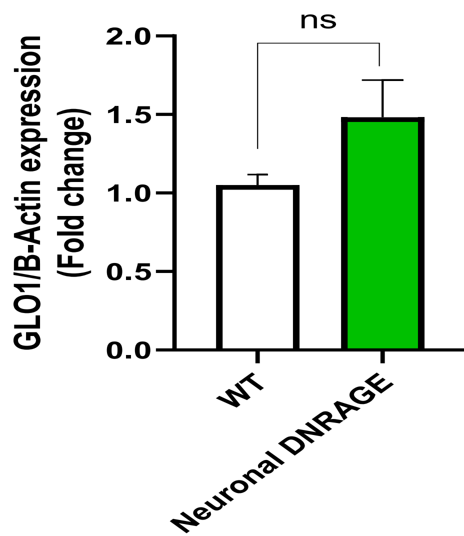
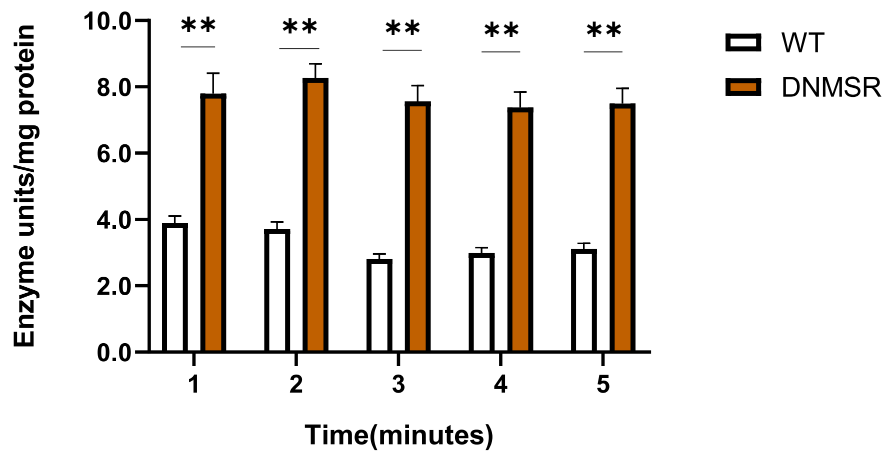


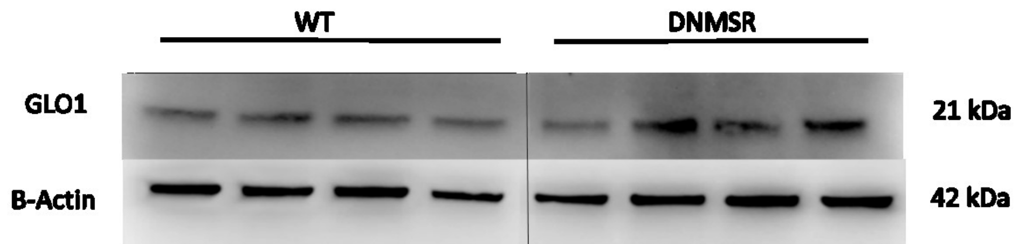
Figure 5: Effect of neuronal DNRAGE on GLO1 enzyme activity and protein expression in 12 months old WT and neuronal DNRAGE mice.

(A)GLO1 enzyme activity was determined by spectrophotometric method in the cortex from 12-month-old WT and neuronal DNRAGE mice (n=3-6/group) in time-dependent manner (Two-way ANOVA, time: $F (4, 50) = 0.01678$, genotype: $F (1, 50) = 89.30$, interaction: $F (4, 50) = 0.05961$, $P=0.0039$). (B)GLO1 protein expression was demonstrated by quantifying intensity of immunoreactive bands of GLO1 in the brain homogenates from indicated mice (n=4-5/group). Representative immunoblots show immunoreactive bands for GLO1(21kda) as well as β-Actin protein used as loading control. (C) Quantification of GLO1 immunoreactive bands was performed for blots in triplicate (Student's t-test, ns=non-significant).

6A.



6B.



6C.

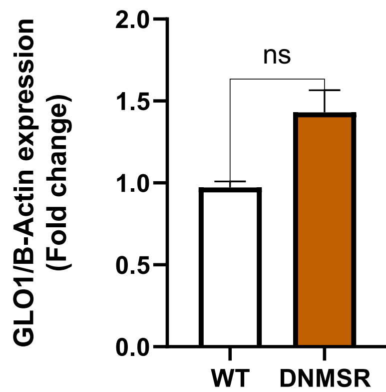
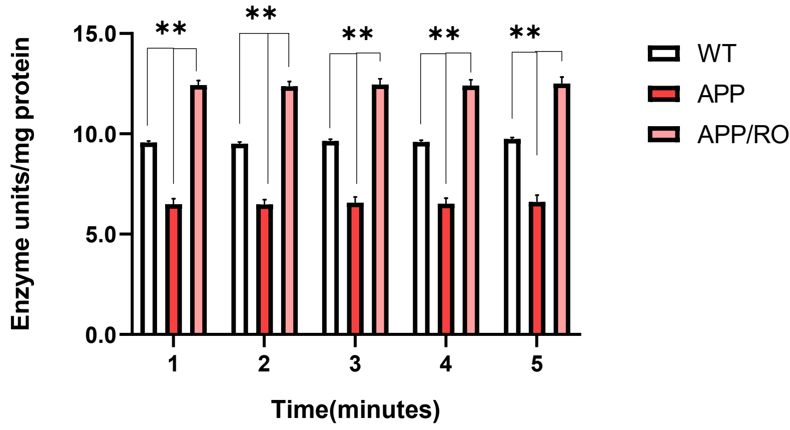


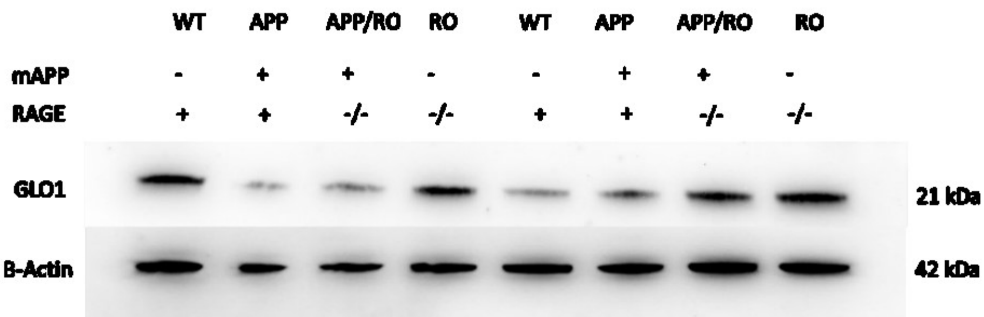
Figure 6: Effect of DNMSR mutant on GLO1 enzyme activity and protein expression in 12 months old WT and DNMSR mice.

(A)GLO1 enzyme activity was determined by spectrophotometric method in the cortex from 12-month-old WT and DNMSR mice (n=4/group) in time-dependent manner (Two-way ANOVA, time: $F (4, 16) = 36.85$, genotype: $F (1, 4) = 74.33$, interaction: $F (4, 16) = 6.103$, $P<0.0001$). (B)GLO1 protein expression was demonstrated by quantifying intensity of immunoreactive bands of GLO1 in the brain homogenates from indicated mice (n=4-5/group). Representative immunoblots show immunoreactive bands for GLO1(21kda) as well as β-Actin protein used as loading control. (C) Quantification of GLO1 immunoreactive bands was performed for blots in triplicate (Student's t-test, ns=non-significant).

7A.



7B.



7C.

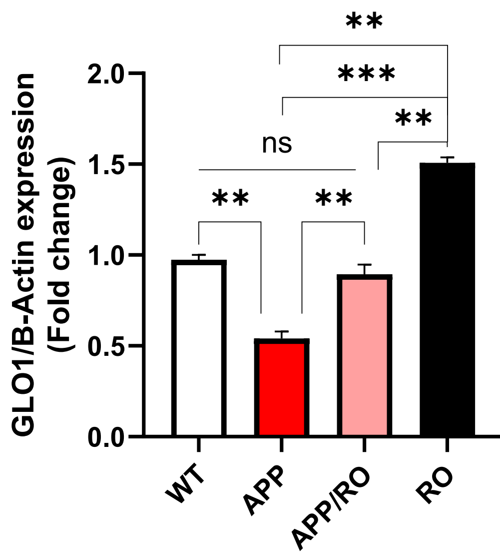


Figure 7: Effect of presence of mutant APP on GLO1 enzyme activity and protein expression in 12 months old WT, APP, APP/RO and RO mice.

(a)GLO1 enzyme activity was determined by spectrophotometric method in the cortex from indicated four genotypes of mice (n=5/group, age=12-15 months) in time-dependent manner [two-way ANOVA, genotype: $F(2, 3) = 163.1$, time: $F(3, 12) = 266.9$, interaction: $F(8, 12) = 0.5848$, $p<0.0001$]. (b)GLO1 protein expression was demonstrated by quantifying intensity of immunoreactive bands of GLO1 in the brain homogenates from indicated mice. (n=4-5/group). Representative immunoblots show immunoreactive bands for GLO1(21kda) as well as β-actin protein used as loading control. (c) quantification of GLO1 immunoreactive bands was performed for blots in triplicate (one-way ANOVA, Tukey's multiple comparison test, $F(3, 4) = 109.5$, $p=0.0003$, $^{**}p<0.01$, $^{***}p<0.001$ for RO compared with APP).

DISCUSSION

RAGE, a receptor for AGEs, is an immunoglobulin like cell surface receptor that is described as a pattern recognition receptor[3, 51, 55, 151]. RAGE is an important cellular co-factor for Aβ-mediated cellular changes relevant in pathogenesis of Alzheimer's disease. Interaction of RAGE with Aβ in neurons, microglia and vascular cells accelerates and exaggerates the detrimental effect on neuronal and synaptic function[42, 59, 81, 152]. RAGE-dependent signaling contributes to Aβ-mediated amyloid pathology and cognitive dysfunction observed in AD mouse model[3, 14, 28, 73, 85, 153-157]. On the other hand, RAGE deletion significantly attenuates neuronal and synaptic injury as previously shown[3, 28, 48]. RAGE inhibitors have been described previously to mitigate symptoms of AD[42, 57, 70, 158-160] and are found to be safe and effective orally in phase II clinical trials of AD[161].

MG is a major precursor of AGESs. As a metabolic byproduct continuously generated in vivo, MG can directly induce apoptosis[162-165] by generating oxidative stress. Moreover, MG is also found to be involved in the formation of Aβ and NFTs in AD[24, 31, 69, 166-173]. Furthermore, it is suggested that the decline in cognitive function in AD patients is closely related to the MG level as well as to the change in the glyoxalase system level or activity[23, 116, 122, 128, 174]. As one of the most important enzyme systems in vivo, the glyoxalase system exhibits detoxification of α-ketoaldehydes or dicarbonyl compounds including MG and glyoxal. However, the direct association between GLO1 and AD pathology still needed further exploration. Though various important signaling pathways possibly contribute to AD pathogenesis, here we focused our attention on the RAGE-signaling pathway.

Thus, the current study was designed to evaluate whether global deletion of RAGE along with the RAGE signaling blockade would have protective effects against changes in the GLO1 activity and protein expression due to AGEs-mediated disturbances and mAPP.

GLO1 enzyme activity is dependent on RAGE and is modulated in age-dependent manner.

Aging is characterized by physiological and functional deterioration across multiple organ systems. AGEs are found in abundance in the serum and accumulate in the tissues during normal aging. This accumulation is accelerated in pathological conditions such as diabetes and neurodegenerative diseases, such as AD[23, 59, 175]. Diabetes has been known to be a contributor to AD pathology[176, 177]. Increased levels of AGEs are reported in the cortical neurons of older adults and are linked to the severity of cognition impairment. The serum AGE levels are significantly increased in diabetic patients compared to healthy, non-diabetic controls. AGE-induced cellular perturbation and oxidative stress were blocked by antibodies to RAGE[178, 179]. Thus, increased levels of AGEs and RAGE play an important role in AGE-mediated cellular disturbances during the pathogenesis of aging-related disorders and diabetes.

To investigate the effects of physiological aging on GLO1 activity, we carried out an initial study in the WT mice of different ages. It is well supported in the literature that with increasing age, there is a consistent increase in the AGE levels which lead to RAGE-mediated changes in neuronal function which should lead to decrease in GLO1 activity according to our hypothesis [27, 100, 175, 180]. Corroborating that, we found significant decrease in the GLO1 activity of older WT mice compared to younger mice. We found that there was a slight increase in the activity from 3

months till the age of 12 months[100, 101] but beyond that, there was a significant reduction in GLO1 activity seen in 20- and 30-month old mice. These results support the minor elevation in GLO1 levels observed in young mice as reported in the previous literature[100, 101, 130]. Based on these preliminary studies, we used approximately 12-month-old mice for further comparative studies for uniformity and to remove the confounding factor of age.

Further, we wanted to look at the direct effect of genetic deletion of RAGE. Thus, we used previously generated RO mice. The spectrophotometric measurement of GLO1 enzyme activity revealed significantly increased GLO1 activity in RO mice compared with WT mice at 6 months and 12 months. In 12-month-old mice, the protein levels were significantly increased in RO mice compared with those of 12-month-old WT mice. But this effect was not observed in 6 months old mice. This difference in the effect can possibly be explained by the dissimilar changes seen in GLO1 levels in animals of different ages as found in the preliminary experiments in aging WT mice. This could be the effect of age on RAGE-modulated GLO1 expression and function.

RAGE is required both in neurons and microglia for regulating GLO1 activity.

Since RAGE is expressed in both neuronal and non-neuronal cells[14, 20, 27, 48, 54, 68, 69, 75, 80], the results from RO mice did not differentiate if the effect was due to a specific type of cell. Here, we wanted to look at the direct effect of other genetic manipulation of RAGE (conditional deletion or signaling blockade). Thus, we used two different previously generated transgenic mouse models: nRKO mouse model and DNMSR mouse model, to address the question of whether the activity of neuronal or microglial RAGE is responsible for AGE-induced reduction in GLO1.

We studied GLO1 activity and protein levels in these nRKO and DNMSR mice. These experiments revealed significantly increased GLO1 activity in the brains of both nRKO and DNMSR mice compared with age-matched WT control mice. The protein levels were slightly increased compared with those of WT mice, but the change was not statistically significant.
These results showed that absence of RAGE in both types of cells resulted in similar effect indicating that RAGE is required in both neuronal and microglial cells to modulate GLO1 activity. But these similar results do not necessarily point to a similar signaling mechanism.

Use of both global and neuronal knockout mice differs from cell-type specific DNRAGE mice. In case of DNRAGE mice, only the cytosolic tail is absent from the receptor leading to loss of signaling whereas for RAGE knockout mice, there is complete absence of receptor from the cell membrane. Thus, in case of DNRAGE mice, the receptor is available to bind ligands and modify them and reduce the unbound ligands from the extracellular fluid. That is not the case for knockout mice. Also, dimerization of RAGE is thought to be key for its signaling and other biological effects[181, 182], which is not possible in case of RAGE knockout.

To address this issue if the results seen in DNRAGE mice are due to sequestration of ligands (which is not possible in knockout mice), we further used neuronal DNRAGE mice. We found that neuronal DNRAGE mutation significantly upregulated GLO1 activity but did not alter protein expression, which is similar to the effect seen in earlier 2 models (nRKO and DNMSR). There were anomalies seen in the protein levels from western blot. Since the trend observed was of an increase in protein levels, we need to further increase the number of mice and repeat the

experiments to confirm the results. From these observations, we concluded that the effect in DNRAGE is not due to the difference between sequestration of ligands with the receptor. These results also show that the mechanism by which RAGE affects GLO1 activity and expression is similar for both types of cells. So, RAGE is an important modulator of GLO1 regardless of the cell type.

GLO1 protein levels are not altered in mice with cell-type specific RAGE mutations.

From the above experiments, we found that though RAGE deletion or DNRAGE leads to increased GLO1 enzyme activity in both global knockout and other 3 models, the protein levels are affected differently.

This difference can possibly be explained by various factors such as:

a) Difference in post-translational modification (PTM). GLO1 undergoes 4 different types of modifications namely (i) removal of the n-terminal methionine 1, (ii) n-terminal acetylation at alanine 2, (iii) a vicinal disulfide bridge between cysteine residues 19 and 20, and (iv) a mixed disulfide bond formed by reaction between thiolate of glutathione and disulfide bond of cysteine 139. MG also leads to PTM of proteins. Disulfide bridge formation impacts the enzyme activity while not impacting protein levels or enzyme stability. Different genotypes will possibly express different PTMs. So, if this particular modification i.e. disulfide bond formation occurs in the genotype, then it might be the reason of the higher enzyme activity but no change in protein levels[183].
b) Inherently, there can be differences in the conformation of the enzyme which may lead to variable binding causing the different effect on protein levels. This can also occur in case

of protein denaturation where the protein loses its function, which might lead to the protein levels to be lower or different. Since, the WT and Tg mice have similar conformation (being littermates), it possibly leads to insignificant difference/changes in protein levels.

c) For all the experiments, brain cortex homogenate was used. Thus, the homogenate contained both neuronal and non-neuronal cells. For cell-type specific mutants, some of the other cells in the homogenate without the knockout carried the intact RAGE exerting its effect, thus incomplete RAGE suppression may occur. This can possibly lead to no significant increase in the protein levels unlike the clear increase seen in the case of complete knockout models (RO and APP/RO).

d) In a yeast *Schizosaccharomyces pombe*, GLO1 activity is regulated in response to osmotic stress. But there is no change in mRNA or protein levels. This can be the case in mammalian GLO1 modulation as well[184]. In case of the experimental mice, different genotypes (WT and Tg) will experience different levels of osmotic stress which will cause enzyme activity to change but it might not affect the protein levels.

e) MG induces protein modification such as glycation which causes partial unfolding and thus, forms unstable proteins which degrade over time. This denaturation may cause non-significant differential change in the protein levels[185, 186]. Different degrees of denaturation might occur in animals based on gender and genotype. This will lead to differential influence on the protein levels. This might be the reason why we saw a variable degree of change in protein levels across multiple animal models.

f) The methods used such as enzyme activity assay and western blotting have different sensitivity to the GLO1 protein levels or activity measured during the experiments. In case of western blotting, the method measures the amount of static protein present in the cell

sample. In enzyme activity assay, the change in the OD measured tells us about the rate at which the enzyme reacts with the substrate and converts it to the product. In enzyme activity, the substrate binds to the enzyme leading to a signaling cascade which leads to signal amplification. This might be the main reason for the differences seen between the two methods, where we saw an increase in enzyme activity but no significant change in protein levels.

g) Gender differences and other variability among the mice. In literature, it has been found that there are sex differences in brain function, molecular changes and neurogenesis. Since our study included both male and female mice in different numbers (for each group), it is possible that the cumulative gender difference plays a role in the differential effect on protein expression [187]. It is also possible that an animal expresses higher or lower amount of protein leading to the different result.

APP mutation regulates GLO1 activity and protein level through RAGE signaling.

Amyloid plaques are characteristic lesions found in Alzheimer's disease (AD) and are composed of Aβ derived from APP [188]. Aβ formation involves sequential proteolytic cleavage of APP by β and γ-secretases [189-192]. β -secretase mediates APP cleavage to form the amino (N)-terminus of Aβ and yields the membrane bound C-terminal fragment CTFβ [190]. Next, γ-secretase cleaves CTFβ to release Aβ peptide and APP intracellular C-terminal domain (AICD), a 6-KD peptide also called CTFγ .

AGE accumulation has been demonstrated in senile plaques in different cortical areas such as entorhinal cortex[20, 75] and glial cells of AD brain [193]. We have previously published about RAGE-mediated Aβ accumulation in a mouse model of AD which was via modulation of β and γ-secretase activity through activation of GSK3β and P38 MAP kinase[28]. It has been demonstrated in vitro that aggregation of Aβ is significantly accelerated by AGE-mediated crosslinking[194]. Thus, AGEs may be one of the factors which accelerate Aβ deposition and plaque formation in AD[195].

Our previous studies demonstrated that increased cellular RAGE causes more Aβ-mediated perturbation in patients with AD and in an AD mouse model[3, 11, 13, 28, 155, 196, 197]. The literature mentions RAGE-dependent signaling in microglia contributes to neuroinflammation [74, 75]. However, deletion or selective deficiency of neuronal or microglial RAGE signaling by DNRAGE in the AD mouse model attenuated deterioration induced by AGEs or mAPP[21, 28]. It was also found that blockade of microglial RAGE is beneficial for Aβ-mediated neuronal changes [20, 69, 73].

We sought to study whether RAGE had a direct role in modulating GLO1 activity and expression in mAPP mice overexpressing mutant APP/Aβ (mAPP). We also determined the effect of deletion of RAGE in these mice(mAPP/RO). Tg mAPP (Swedish mutation: APP KM670/671NL, V717F) mouse model is a well-established animal model for AD. We demonstrated that GLO1 activity and protein levels were significantly reduced in homozygous mAPP mice compared with WT mice. In contrast, GLO1 activity was found to be higher in both heterozygous mAPP/RO and homozygous RO mice with global genetic deletion of RAGE. These effects seen in mAPP/RO

mice show that RAGE deletion has a protective effect against the downregulation of GLO1 activity seen in mAPP mice. Consistent with the enzyme activity results, the protein levels were also increased in both mAPP/RO and RO mice compared with those of 12-months-old mAPP mice.

Here, due to the presence of mutant APP in case of mAPP mice, the activity and protein levels decreased compared to WT controls. This mutation leads to overexpression of APP, which in turn leads to accelerated formation of Aβ. This Aβ formation causes plaque formation with increasing age. Since Aβ is known to bind to RAGE[14, 67, 151], this interaction causes RAGE-mediated signaling which leads to decrease in the GLO1 activity. In contrast, due to global knockout in RO and mAPP/RO mice, the activity and protein levels both showed significant improvement compared to mAPP mice. Thus, we concluded that global deletion of RAGE in mAPP/RO mice rescued the decrease in GLO1 activity and expression seen in mAPP mice.

Possible mechanism for effect of AGEs-RAGE interaction on GLO1 activity can be through multiple pathways such as formation of reactive oxygen species (ROS) leading to oxidative stress, activation of PI3K-AKT pathway [76] and stimulation of P38 or ERK signaling[66]. All these activations lead to upregulation of NF-κB, which is known to be involved in inflammation, DNA transcription and cytokine production[81, 106]. This NF-κB upregulation leads to pro-inflammatory cytokine release causing neuroinflammation, which is one of the major changes seen in case of AD. It has also been reported that NF-κB regulates APP and β and γ-secretases[198]. These pro-inflammatory changes might lead to downregulation of GLO1 activity[59].

So, to summarize, enzyme activity assay and western blot analysis indicated that in comparison to healthy controls, GLO1 activity and protein levels decreased with age and in the presence of mAPP expression. In transgenic non-AD models where RAGE was deleted or signaling was blocked, GLO1 enzyme activity increased though there was variable effect regarding the GLO1 protein levels possibly due to various factors discussed above.

The data presented herein clearly demonstrates, that genetic deletion of RAGE or loss of RAGE signaling significantly increased GLO1 activity in the four transgenic and mAPP mouse models. The GLO1 protein expression also increased in case of RO and mAPP/RO mice. Using our novel transgenic mouse models with RAGE deletion or conditional DNRAGE mutant for this evaluation, we provide convincing evidence to support the effects of AGEs–RAGE interaction on GLO1 enzyme activity and protein expression.

Our findings also suggest that RAGE is a strong potential target to limit AGEs-induced perturbations, which is in accordance with previous literature [3, 19, 28, 63, 72, 73, 79, 82, 153, 199, 200]. Also, the decrease of GLO1 expression with increasing age and pathology might be one reason for AGE or MG-induced neuronal impairment, apoptosis, and AGE formation in plaques and NFTs seen in AD. Thus, GLO1 appears to be an important enzyme target for protection against RAGE-mediated signaling[140, 201, 202]. So, restoring or enhancing GLO1 activity can provide new opportunities for AD treatment.

Several strategies, to prevent the AGEs-induced disturbances, are described in literature such as enhancement of GLO1 activity[108, 112, 114, 118, 119, 132-134, 203, 204], use of RAGE inhibitors [11, 57, 61, 124, 127, 160, 200, 205], inhibition of MG toxic reactions by using AGE antagonists[23, 127] which are antioxidants[133, 206] and decreasing glycation activity by using anti-diabetic drugs[16, 207-211]. All these approaches lead to direct or indirect enhancement or restoration of GLO1 activity. They are also efficient in alleviating some AD symptoms such as cognitive impairment and neuroinflammation.

Our study was a simple attempt at correlating certain factors such as RAGE in AD to the MG-detoxification process in the brain through the glyoxalase system. There are certain limitations to our studies because it may not be possible to always extrapolate animal data to humans since animal models are not a perfect predictor of human physiological or pathological conditions. There also needs to be a further extensive study in clinical settings as well as other animal models to have a better in vivo-in vitro correlation. We need a detailed study of significance of GLO1 enzyme activity using GLO1 genetic manipulations such as mice exhibiting GLO1 knockout or GLO1 overexpression [126].

To conclude, we show that GLO1 is directly upregulated by RAGE deletion or signaling blockade but the exact role of glyoxalase system in the AD pathogenesis and involved signaling pathways such as MAPK need further investigation. Also, GLO1 is downregulated in case of mAPP mutation featuring overproduction of Aβ. Blockade of AGEs-RAGE axis by genetic deletion of RAGE and by use of DNRAGE protects against this downregulation of GLO1 activity and further neurodegenerative changes seen in diseases such as AD.

Thus, it will be very crucial and valuable to further study the significance of glyoxalase system which will contribute to a better understanding of AD pathogenesis, as well as find new therapies for AD.

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