Genetic deficiency of neuronal RAGE protects against AGE-induced synaptic injury

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Synaptic dysfunction and degeneration is an early pathological feature of aging and age-related diseases, including Alzheimer's disease (AD). Aging is associated with increased generation and deposition of advanced glycation endproducts (AGEs), resulting from nonenzymatic glycation (or oxidation) proteins and lipids. AGE formation is accelerated in diabetes and AD-affected brain, contributing to cellular perturbation. The extent of AGEs’ involvement, if at all, in alterations in synaptic structure and function is currently unknown. Here we analyze the contribution of neuronal receptor of AGEs (RAGE) signaling to AGE-mediated synaptic injury using novel transgenic neuronal RAGE knockout mice specifically targeted to the forebrain and transgenic mice expressing neuronal dominant-negative RAGE (DN-RAGE). Addition of AGEs to brain slices impaired hippocampal long-term potentiation (LTP). Similarly, treatment of hippocampal neurons with AGEs significantly decreases synaptic density. Such detrimental effects are largely reversed by genetic RAGE depletion. Notably, brain slices from mice with neuronal RAGE deficiency or DN-RAGE are resistant to AGE-induced LTP deficit. Further, RAGE deficiency or DN-RAGE blocks AGE-induced activation of p38 signaling. Taken together, these data show that neuronal RAGE functions as a signal transducer for AGE-induced synaptic dysfunction, thereby providing new insights into a mechanism by which the AGEs–RAGE-dependent signaling cascade contributes to synaptic injury via the p38 MAP kinase signal transduction pathway. Thus, RAGE blockade may be a target for development of interventions aimed at preventing the progression of cognitive decline in aging and age-related neurodegenerative diseases.

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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. The products of non-enzymatic glycation (or oxidation) of proteins and lipids, AGEs contribute to the normal aging process and when accelerated have a causative role in the vasculature complications of diabetes mellitus and several neurodegenerative diseases, including Alzheimer’s (AD), Parkinson’s, and Huntington’s diseases.1–6 In diabetic patients, the concentration of circulating AGEs (serum AGE level) has been reported at 7.2–22 mU/ml (equivalent to 30–88 µg/ml AGE-BSA), which is significantly higher than that of non-diabetic patients (3 mU/ml, equivalent to 12 µg/ml AGE-BSA).6,7 The brain AGE level was also increased to 5-6 µM (equivalent to 325–390 µg/ml AGE-BSA) in the diabetic animal model.8 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. For example, in a recent clinical study, peripheral AGE levels were associated with cognitive decline in older adults with and without diabetes.10 Diabetes complications affect the brain, increasing risk for depression, dementia, and AD. In fact, patients with type 2 diabetes are at twofold to threefold increased relative risk for AD11–18 and accelerated cognitive dysfunction.

Long-lived proteins such as β-amyloid peptide (Aβ) and hyperphosphorylated tau protein that accumulate in AD brain are highly susceptible to AGE modification.19–22 AGE-modified Aβ or tau protein results in increased oxidative stress and chronic inflammation, accelerating AD pathology and neuronal perturbation.19,20,22–25 Moreover, Aβ or tau glycation results in increased aggregation and subsequent formation of senile plaques or neurofibrillary tangles, the major pathological feature of AD.19,22 suggesting that AGE modification is an important risk factor for neurodegenerative diseases.26 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, its underlying mechanisms are not well understood.

Receptor for advanced glycation endproduct (RAGE) was first identified as a cell surface receptor of the immunoglobulin superfamily for AGEs.27,28 Increased expression of RAGE

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Abbreviations: aCSF, artificial cerebrospinal fluid; AD, Alzheimer’s disease; AGEs, advanced glycation endproducts; CK2, calcium-calmodulin-dependent kinase II; CML, Nε-carboxymethyl-lysine; DN-RAGE, dominant-negative RAGE; fEPSP, field-excitatory post-synaptic potential; JNK, c-Jun N-terminal kinase; LTP, long-term potentiation; nRKO, neuronal RAGE selective knockout; p38 MAPK, p38 mitogen-activated protein kinase; RAGE, receptor for advanced glycation endproduct; RKO, global RAGE knockout

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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. Although it has been shown that AGEs–RAGE interaction contributes to cellular perturbation relevant to the pathogenesis of the cardiovascular disease and the diabetes vascular complications, little is known about the role of AGEs and its interaction with RAGE on synaptic dysfunction. To understand the mechanisms involved in AGE-mediated synaptic damage, the following questions need to be addressed: (1) ‘Do AGEs alter synaptic structure and function? If so, are these changes dependent on RAGE signaling?’; (2) ‘Does RAGE blockage by genetic depletion protect from AGE-induced synaptic dysfunction and loss? ’; and (3) ‘What is the impact of neuronal RAGE in AGE-induced aberrant synaptic function?’ Thus it is important to evaluate the impact of AGEs–RAGE interaction on synaptic dysfunction and to explore the mechanism underlying AGE–RAGE-dependent signal transduction and its contribution to synaptic damage.

Here we investigate neuronal RAGE signaling in AGE-induced synaptic injury using our novel conditional RAGE knockout mice targeting cortical neurons as well as transgenic mice that overexpress signal transduction-deficient mutants of RAGE in neurons. Given that neuronal and non-neuronal cells in the brain may contribute to AGE-induced sustained neuronal and synaptic stress and dysfunction, we assessed the impact of global RAGE deletion in this setting and further delineated the mechanism by which neuronal RAGE-dependent activation of p38 MAP kinase potentiates AGE-insulted synaptic injury.

Results

Generation and characterization of conditional neuronal RAGE selective knockout (nRKO) mice. We 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 in an AGE-rich environment. To this end, we have recently created Cre recombinase RAGE null mice, termed Tg nRKO mice, 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. We used two Cre-loxP systems to generate the targeting vector and the RAGE/flox mice (performed by Ozgen Inc., Bentley DC, WA, Australia). Figure 1a demonstrates a targeting vector containing 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 (Figure 1b). 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. We first made homozygous RAGE floxed allele mice (RAGEflox/flox); then RAGEflox/flox mice were crossed with neuronal target CK2 mice to generate nRKO mice (RAGEflox/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) (Figure 2a). 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) (Figure 2b). Incomplete suppression of RAGE expression was due to brain homogenates, which contained non-neuronal cells. RAGE expression levels were not altered in cerebellum homogenates (Figure 2c). Confocal microscopy with double immunostaining of RAGE and MAP2 clearly demonstrated that there was virtually no RAGE signal in MAP2-positive neurons in cortex (Figures 2d–e) and hippocampus (Figure 2f) of nRKO mice, thereby verifying RAGE depletion in cortical neurons in the brain of nRKO mice.

Immunohistochemical characterization of Nε-carboxymethyllysine (CML) in AGE preparation. Of all forms of AGEs, CML is a major glycation product known to be stable and accumulate progressively in vivo in aging, AD, and
diabetes-associated complications. To verify whether CML was a major component in our AGE preparation, we prepared AGEs by incubation of bovine serum albumin (BSA) with glucose 6-phosphate for 6–8 weeks at 37°C and then performed immunochemical characterization using a monoclonal antibody targeting CML adducts. Using Coomassie blue dye for rapid reversible staining of protein bands in gel, we observed co-migration of BSA and AGE-modified BSA at ~66 and ~70 kD, respectively (Figure 3a). The increase in molecular weight of glycated BSA is due to AGE modification, which results in intramolecular crosslinking. Immunoblotting of the same preparation of BSA and AGE-BSA using specific antibody to CML showed prominent CML immunoreactivity only in the presence of AGEs, suggesting that BSA was glycated. In contrast, such immunoreactivity was absent on BSA without glucose 6-phosphate (Figure 3a). These results indicate that CML is a major component of our AGE preparation, used in all our experiments.

AGEs impair hippocampal long-term potentiation (LTP). To determine direct effect of AGEs on synaptic function, we examined synaptic transmission under basal condition and during LTP, a form of synaptic plasticity that is widely studied as a cellular model for synaptic function, in hippocampal slices from non-Tg mice treated with AGEs or BSA as a control. In vehicle-treated slices, BSA alone did not alter LTP (228.8 ± 17.04% versus 223.0 ± 7.95% in BSA-treated slices, P > 0.05, Figures 3b and c) or basal synaptic transmission (BST) as measured by input–output measurement (Figure 3d). AGE treatment significantly reduced LTP in a concentration-dependent manner (180.9 ± 15.36%, 139.1 ± 10.42%, and 124.9 ± 7.96% for 50, 100, and 200 μg/ml AGEs, respectively, versus 220.9 ± 20.75% for 100 μg/ml BSA, P < 0.01, Figures 3e and f). There were no significant differences of field-excitatory post-synaptic potential (fEPSPs) in CA1 stratum radiatum between AGE- and BSA-treated slices, indicating that AGEs did not affect normal BST in hippocampal synapses (Figure 3g). These results demonstrate that AGEs impair long-term synaptic plasticity.

RAGE deficiency rescues AGE-induced LTP deficits. To directly determine the impact of RAGE on AGE-mediated reduction of LTP, we first recorded LTP in hippocampal slices from global RAGE depletion (RKO) and non-Tg mice treated with AGEs. We found similar potentiation in RAGE-deficient
slices compared with non-Tg slices in the presence of BSA (224.3 ± 17.81% versus 208.8 ± 19.50%, P > 0.05, Figures 4a and e). However, RAGE deficiency largely protected hippocampal slices against LTP decreases with AGEs exposure (184.5 ± 9.73% versus 131.1 ± 7.53%, P < 0.01, Figures 4a and e), suggesting that RAGE deficiency improves synaptic transmission. Because RAGE is expressed in neuronal and non-neuronal cells in the brain, we next examined the contribution of neuronal RAGE to AGE-mediated synaptic function. We treated neurons from hippocampal slices from nRKO mice (with depletion of RAGE) cortices with AGEs and BSA for at least 60 min, and then we recorded LTP. Slices from both nRKO or non-Tg mice showed similar LTP in the presence of BSA (240.2 ± 9.41% versus 208.8 ± 19.50%, P > 0.05, Figures 4b and e). In contrast, nRKO slices significantly suppressed LTP decline induced by AGEs as compared with non-Tg slices (191.6 ± 15.55% versus 131.1 ± 7.53%, P < 0.01, Figures 4b and e). These results indicate that successful blockade of neuronal RAGE protects from AGE-impaired synaptic function.

RAGE functions as a signal transduction receptor for AGEs and Aβ, activating multiple downstream intracellular pathways. Thus, we evaluated the effect of RAGE signaling in neurons on AGE-induced LTP using hippocampal slices from DN-RAGE mice expressing signal transduction-deficient mutants of RAGE (which were targeted to neurons to determine RAGE-dependent signaling). Notably, DN-RAGE slices were significantly resistant to AGE-mediated reduction of LTP as compared with non-Tg slices (167.0 ± 12.66% versus 131.1 ± 7.53%, P < 0.01, Figures 4c and e). Figure 4d shows representative traces taken 1 min before (black line) and 60 min after LTP induction (gray line). BST was not affected in RAGE knockout or mutant mice (Figure 4f). These results indicated that RAGE-dependent signaling in neurons contributes to AGE-induced synaptic malfunction.

Mitogen-activated protein kinase 38 (MAPK p38) activation is involved in AGE-induced synaptic deficit. To determine which molecular players underlie AGE effects on hippocampal LTP, we evaluated the role of p38 and c-Jun.
N-terminal kinases (JNK) in hippocampal slices upon exposure to AGEs, because these kinases are activated (increased phosphorylation levels) in several pathological conditions and synaptic plasticity. Further, p38 is a key mediator of RAGE-mediated signal transduction. Using antibodies to phosphorylated forms of p38 and JNK with hippocampal extracts after exposure to AGEs and high frequency stimulation, we estimated activation of these two kinases. Addition of AGEs to non-Tg hippocampal slices significantly increased p38 phosphorylation compared with BSA addition, but treatment with AGEs did not affect total p38 levels (Figure 5a). Densitometry analysis of the combined immunoreactive bands revealed a significant increase in p38 phosphorylation by 1.5–1.7-fold when normalized to total p38 versus that of BSA-treated controls (Figure 5b). AGEs treatment did not affect JNK phosphorylation (Figure 5c).

To further evaluate the effect of p38 activation on AGE-mediated LTP impairment, we perfused hippocampal slices with SB203580, a specific p38 inhibitor, along with AGEs. Clearly, SB203580 application significantly blocked induction of p38 phosphorylation in the presence of AGEs compared with the AGE treatment alone without p38 inhibitor (SB203580) (Figure 5d). Consequently, AGE-impaired hippocampal LTP was restored by inhibition of p38 with SB203580 perfusion (Figures 5e and f). These results suggest that AGE-mediated p38 activation contributes to synaptic injury.

RAGE deficiency blocks AGE-induced p38 activation. To determine whether RAGE is a key player in AGE-mediated p38 activation, we assessed the effect of RAGE deletion on p38 phosphorylation induced by AGEs. Slices from RKO mice showed completely suppressed p38 activation as shown by decreased p38 phosphorylation compared with non-Tg slices with AGEs (Figures 6a and b). Further, RAGE depletion specifically targeted to cortical/hippocampal neurons or neuron-targeted expression of a signal transduction-deficient mutant of RAGE abolished AGE-induced p38 phosphorylation. Quantification of all immunoreactive bands

**Figure 4** Effect of RAGE depletion on AGE-induced LTP impairment. (a–c) LTP was recorded in hippocampal slices from non-Tg, RKO (a), nRKO (b), and DN-RAGE (c) mice after 1 h perfusion with BSA or AGEs (100 μg/ml). RAGE deletion alone did not alter LTP in the presence of BSA. In contrast, LTP was increased in slices from RKO, nRKO, and DN-RAGE mice compared with non-Tg slices in the presence of AGE. (d) Representative traces of fEPSP in slices treated with vehicle or 100 μg/ml BSA before θ-burst stimulation (black line) and after 1 h (gray line). (e) Residual potentiation from the fEPSP slopes occurring over the last 5 min of LTP recordings. (f) There were no significant differences in BST among indicated groups. N = 8–11 slices from 3 to 5 mice.
for phospho-p38 normalized to total p38 verified significantly reduced phospho-p38 immunoreactive bands in slices from RKO, nRKO, and DN-RAGE compared with non-Tg slices with AGE treatment (Figures 6a and b). These data indicate that RAGE deficiency prevents AGE-induced p38 activation.

p38 activation of signal transduction is a consequence of AGEs–RAGE interaction on neurons, contributing to neuronal and synaptic perturbation in an AGE-enriched environment.
Effect of AGEs-RAGE interaction on synaptic density.

To determine the extent of AGE-induced synaptic damage in AGE-insulted neurons, we first analyzed the effect of AGEs on synaptic density. Synapses were identified as synaptophysin-positive clusters attaching to dendrites labeled with MAP2 marker. Recruitment of the presynaptic protein, synaptophysin, to form synaptophysin-positive clusters is rapid and synaptic activity-dependent. Two hours after AGEs treatment, synaptophysin density in hippocampal neurons was not altered compared with BSA-treated control cells, whereas hippocampal neurons exposed to AGEs for 48 h showed synaptophysin-positive cluster reduced by 50–60% (Figure 7a). Importantly, RAGE-deficient neurons largely reversed AGE-induced decrease in synaptic density (Figure 7b). These data indicate that RAGE depletion rescues AGE-induced synaptic loss.

Discussion

In addition to its 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; 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, the direct effect of AGEs–RAGE interaction on brain function, in particular on changes in synaptic structure and function, remains largely unknown. Using our novel transgenic mouse model with neuronal expression of RAGE signaling and lacking neuronal RAGE in the forebrain for evaluation of synaptic transmission and plasticity (almost every brain function relays on synaptic transmission), we provide convincing evidence to support a pivotal role of neuronal AGES–RAGE interaction on MAPK P38 activation, hippocampal plasticity deficit, and synaptic injury.

We first demonstrated detrimental effect of AGEs on synaptic structure and function. The addition of AGEs significantly impaired synaptic plasticity by reducing LTP and synaptic morphology. LTP is important to long-term synaptic plasticity, and altered synaptic morphology (synaptic loss), which likely contributes to the learning and memory deficits seen in age-related cognitive decline. Aging is characterized by anatomical and functional decline across multiple organ systems. AGEs are found in the serum and accumulate in the tissue during aging; this accumulation is accelerated in pathological conditions such as diabetes and neurodegenerative diseases, including AD. Increased levels of AGEs are reported in the cortical neurons of older adults and are positively correlated with the severity of cognition impairment. The serum AGE levels were significantly increased in diabetic patients (equivalent to 30–88 μg/ml AGE-BSA versus 12 μg/ml AGE-BSA in non-diabetic patients). Our previous studies demonstrated that AGE albumin (1 μM, equivalent to 66.5 μg/ml) resulted in oxidant stress and activation of transcription factor NF-kB in vitro and in vivo, a process involving the AGE-binding protein RAGE. Notably, AGE-induced cellular perturbation and oxidant stress were blocked by antibodies to RAGE. Thus, increased levels of AGEs and of their receptor, RAGE, play an important role in AGE-mediated cellular perturbation during the pathogenesis of age-related processes and diabetes. The concentration of AGEs used in the present study correlates with that of the pathological condition found in human disease.

In our study, perfusion with AGEs in hippocampal slices impaired LTP in a concentration-dependent manner, consistent with findings observed in the rat model that exogenous
AGE treatment by stereotaxic injection into the brain led to synapse and memory impairments. However, the impact of neuronal RAGE on AGE-induced synaptic damage is unclear. Given that AGE formation and accumulation is accelerated in the brain of diabetic and AD subjects, AGE–RAGE interaction could be an important target in the search for agents that halt disease acceleration or exacerbation relevant to diabetes- and AD-related cognitive decline and dementia.

Next, using our genetic RAGE depletion mouse model, we investigated the effect of RAGE-dependent signaling on AGE-insulted synaptic injury. AGE perfusion-induced LTP reduction may be caused by varying factors; for example, one cause involves AGE binding to RAGE receptors, and another is via protein crosslinking-induced cellular malfunction. Genetic deletion of RAGE offers us the ability to identify AGEs-RAGE interaction in AGE-induced synaptic impairment. We recorded normal LTP readings in brain slices of our global RAGE null mice treated with vehicle compared with non-transgenic littermates, indicating that genetic RAGE deletion does not significantly alter synaptic structures important for LTP induction and expression under normal physiological conditions. Notably, RAGE deficiency protects against AGE-induced LTP deficits. Similarly, our data demonstrate the protective effect of RAGE depletion on AGE-induced synaptic loss. Although our results did not exclude other effects of AGEs such as protein crosslinking, we clearly demonstrated that AGEs-RAGE interaction is involved in AGE-induced synaptic injury.

As RAGE is expressed in neurons and non-neuronal cells (i.e., glial, endothelial cells, and pericytes of the blood–brain barrier), the results obtained from global RAGE knockout (i.e., glial, endothelial cells, and pericytes of the blood–brain barrier) indicate a role for RAGE in AGE-induced synaptic dysfunction. Neurons and non-neuronal cells may be involved in AGE-induced synaptic dysfunction. Future studies will be required to investigate the role of non-neuronal RAGE in synaptic failure induced by AGES. Regardless, our studies delineate the mechanisms involving neuronal RAGE-mediated signal transduction in AGE-induced synaptic dysfunction and degeneration. Neurons are susceptible to AGE insults via direct AGES–RAGE interaction contributing to synaptic dysfunction and degeneration.

Finally, our results suggest that the MAPK P38 signaling pathway is critical for AGES–RAGE-induced synaptic damage. AGE-induced LTP decline was attenuated in DN-RAGE slices with a signal transduction-deficient mutant of RAGE, indicating the contribution of RAGE-dependent signaling to AGE-induced synaptic impairment. Signaling cascades activated after ligand–RAGE interaction include pathways those of p21ras, extracellular signal-regulated kinase 1/2 (ERK1/2), p38, and JNK, Rho GTPases, phosphoinositol-3 kinase, GSK-3β, and the JAK/STAT (signal transducer and activator of transcription). Activation of different protein kinase cascades may represent a principal target of RAGE activation for control of synaptic plasticity.

In particular, we considered the potential involvement of two different kinases, JNK and p38 in LTP inhibition based on our previous study showing that p38 and JNK activation are required for LTP inhibition induced by Aβ–RAGE interaction. Hippocampal slices perfused with AGEs showed increased p38 activity but no change in JNK, suggesting the involvement of p38 activation in AGE-induced synaptic plasticity deficits. Indeed, the addition of p38 inhibitor prevented AGE-induced p38 activation and LTP impairment. Note, RAGE depletion blocked induction of p38 phosphorylation and restored defects in synaptic plasticity and density from AGE exposure. Our results indicate that neuronal AGES–RAGE interaction activates p38 MAK kinase, which in turn leads to synaptic dysfunction.

We further observed that if AGES level remained chronically high, synapses become structurally damaged. Using hippocampal neuronal cultures, we showed that short-term AGES exposure (for 2 h treatment) barely affected synaptic structure, whereas long-term treatment of AGES (for 48 h) caused significant changes in synaptic density. Importantly, RAGE deletion rescues AGE-induced synapse loss. These data indicate that neuronal AGES–RAGE interaction has functional (long-term plasticity) as well as structural effects on synapses, which may be important in the learning and memory deficit present in age-related dementia and age-associated neurodegenerative disease.

In summary, we provide substantial evidence of the protective effect of RAGE depletion on AGE-induced alterations in synaptic plasticity and density. Genetic deletion of RAGE and RAGE signaling in cortical neurons blocks AGE-mediated activation of p38 MAP kinase signal transduction with LTP deficits. We propose that AGES-RAGE-dependent activation of p38 MAP kinase is responsible for AGE-induced synaptic damage. Blockade of AGES-RAGE axis may protect against the impaired cognitive function in aged subjects with and without neurodegenerative diseases, including those with diabetes and AD.

Materials and Methods

Transgenic mice

Targeted disruption of mouse RAGE gene. We used the Cre-loxP system to generate the targeting vector and the RAGE/flox mice (performed by Ozgen Inc.). The targeting vector consists of three fragments, namely, the 5′ homology arm, 3′ homology arm, and loxP arm. Two loxP sites flank RAGE exons 2–4 to allow for Cre-mediated deletion using Cre recombinase. Excision of exons 2–4 by Cre recombinase led to a frame shift and early stop codon from the mouse RAGE sequence. A PGK-neo selection cassette was inserted downstream of exon 1. The PGK-neomycin resistance selection cassette is flanked by FRT sites and was deleted using FLPe recombinase (Figure 1, targeting vector).

Generation and characterization of conditional neuronal knockout of RAGE mice

Targeted disruption of mouse RAGE gene. We used the Cre-loxP system to generate the targeting vector and the RAGE/flox mice (performed by Ozgen Inc.). The targeting vector consists of three fragments, namely, the 5′ homology arm, 3′ homology arm, and loxP arm. Two loxP sites flank RAGE exons 2–4 to allow for Cre-mediated deletion using Cre recombinase. Excision of exons 2–4 by Cre recombinase led to a frame shift and early stop codon from the mouse RAGE sequence. A PGK-neo selection cassette was inserted downstream of exon 1. The PGK-neomycin resistance selection cassette is flanked by FRT sites and was deleted using FLPe recombinase (Figure 1, targeting vector).
Generation of neuronal deletion of RAGE mice (rRKO). After deletion of the PKG-neo cassette by crossing with FpE-deleter C57BL6 mice (Oxygen Inc.) to eliminate the neomycin resistance selection cassette (PKG-neo), the floxed RAGE allele was generated. To generate Cre recombinase RAGE null mice (RAGEfrx/o;CFK-Cre, namely, nRKO mice), selective for the cortical and hippocampal neuron under the control of the Camk2a (OK2) promoter, RAGE/flx mice were bred with neuronal-targeted and region-restricted transgenic mice that express Cre recombinase in the entire forebrain (L7Ag13 line) under the control of the forebrain-specific CAMK II promoter.55 Cre mice (L7Ag13 line) were kindly provided by Dr. Eric Kandel (Columbia University, New York, NY, USA). Floxed RAGE mice were identified by PCR with primers (forward: 5′-TACCGAGTCC GAGTCTACCGTAAG-3′ and reverse: 5′-TCTCCGTGTCCTCCTCCTCAGAG-3′) to amplify a 551-bp fragment from the wild-type (WT) allele and a 700-bp fragment from the flox/neo allele. The Cre transgene was identified by PCR primers (forward: 5′-ATCTCATGTCCGTCTCCG-3′ and reverse: 5′-ACATTTCTCCACCGTC-3′) to amplify a 300-bp fragment. Specific neuronal RAGE knockout was further confirmed by western blotting of cortical and cerebellum homogenates using rabbit anti-RAGE antibody (generated in our lab and used in our previous study).55 All transgenic mice were of the C57BL6 strain. The Institutional Animal Care and Use Committee of the University of Kansas approved all protocols. Male transgenic mice and their littermate controls were used for the in vitro electrophysiology experiments.

Homozgyous RAGE-null mice (RKO) and transgenic mice with cytosolic dominant-negative RAGE (DN-RAGE) We generated and characterized homozygous RAGE-null mice (RKO) as described previously.33,55 In addition, we used 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 (DN-RAGE) driven by the platelet-derived growth factor-B chain promoter. DN-RAGE mice were previously characterized, demonstrating localization of DN-RAGE in cortical neurons.54

Preparation of AGEs We generated AGEs by incubating BSA (25 mg/ml) with 250 mM glucose 6-phosphate at 37 ºC for 6–8 weeks in 0.1 M phosphate buffer containing 1.5 mM PMSF, 0.5 mM EDTA, and 1 mM azide.57 The control preparation (BSA) was treated identically with the exception that glucose 6-phosphate was omitted. We dialyzed preparations with phosphate buffer to remove free glucose, EDTA, PMSF, and azide after incubation. The degree of glycation was determined by immunoblotting using antibody to CML (TransGenic Inc., Kobe, Japan). Coomassie blue staining of gel was used as a protein-loading control.

Electrophysiological studies Electrophysiological studies were performed as described.54,58 Transverse hippocampal slices (400 µm) were cut from the mouse brain, maintained in an interface chamber at 29 ºC, and perfused with artificial cerebrospinal fluid (aCSF) continuously bubbled with 95% O2 and 5% CO2. The aCSF composition (mM) was: 124.0 NaCl, 4.4 KCl, 1.5 NaH2PO4, 25.0 NaHCO3, 2.0 CaCl2, 2.0 MgSO4, and 10.0 glucose. IEPS was recorded for the CA1 region of the hippocampus by placing the stimulating electrode at the level of the Schaeffer collateral fibers and recording electrode in the CA1 stratum radiatum. BST was assayed by plotting the stimulus voltage (V) against slopes of IEPSP to generate input–output relations. For LTP experiments, a test pulse was applied every minute at an intensity evoking ~35% of the maximum evoked response. LTP was induced using 5-burst stimulation (4 pulses at 100 Hz, with bursts repeated at 5 Hz and each tetanus, including three 10-burst trains separated by 15 s). Responses were recorded for 60 min after tetanization and measured as IEPSP slope expressed as the percentage of baseline.

Immunoblotting analysis Brain slices were lysed in extraction buffer (10 mM Tris-HCl (pH 7.4), 100 mM sodium chloride, 1 mM EDTA, 1 mM sodium fluoride, 20 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, 1 mM PMSF) containing protease inhibitor mixture (set V, EDTA-free; Calbiochem, Billerica, MA, USA). Protein extracts were separated by SDS/PAGE (12% Bis-Tris gel; Invitrogen, Grand Island, NY, USA), and then transferred to a nitrocellulose membrane (Amer sham, Pittsburgh, PA, USA). After blocking in TBST buffer (20 mM Tris-HCl, 150 mM sodium chloride, 0.1% Tween-20) containing 5% non-fat dry milk (Santa Cruz, Dallas, TX, USA) for 1 h at room temperature, we incubated the membrane, and gently shaking overnight (at 4 ºC) with primary antibodies, followed by incubation with the corresponding secondary antibody for 1 h at room temperature. We used antibodies to phosphorylation and total of p38 and JNK (Cell Signaling, Boston, MA, USA) and tubulin (Sigma, St. Louis, MO, USA); ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used for analysis of scanned blots and to quantify intensity of the immunoreactive bands.

Immunohistochemical analysis Mice were anesthetized by intraperitoneal injection of a mixture of ketamine (50 mg/kg) and xylazine (10 mg/kg) and perfused transcardially with 0.9% sodium chloride for 10 min. Mouse brains were fixed in 4% paraformaldehyde at 4 ºC for 2 days. Floating coronal sections of 30-μm thicknesses were cut by vibratome (Leica VT 1000S, Wetzlar, Germany), collected, and immersed in wash buffer (0.1 M sodium phosphate, 0.5 M sodium chloride, Triton X-100, pH 7.4) for 30 min. After preincubation for 1 h in blocking solution (10% normal goat serum, 0.3% Triton X-100 in PBS), we incubated sections overnight at 4 ºC with primary antibodies, using rabbit anti-RAGE IgG (3.5 μg/ml, generated in our Lab) and mouse anti-MAP2 (1:10,000, Chemicon, Billerica, MA, USA) for cortex and hippocampus staining. Sections were then incubated with Alexa Fluor 594-conjugated goat anti-rabbit IgG and 488 goat anti-mouse IgG secondary antibodies (1:1,000, Invitrogen) for 1 h at room temperature. Nuclei were stained by DRAQ5 (5 μM, Cell Signaling) for 5 min at room temperature.

Measurement of synaptic density Neuronal culture. We prepared hippocampal neurons from day 1 non-Tg or RKO mice in the C57/BL6 background as described previously,59 culturing neurons in neurobasal medium supplemented with 1 X B27, 600 μM L-Glutamine and penicillin-streptomycin. At day 10 in vitro, neurons from both Tg mice were treated with 100 μM of AGEs; BSA was used as a control in neurobasal medium supplemented with 0.5 X B27 for 2 and 48 h, respectively.

Immunostaining of cultured neurons. After the various treatments, neurons were fixed with 4% ice-cold paraformaldehyde for 5 min and then permeabilized with PBS containing 0.1% Triton and 5% goat serum for 1 h at room temperature followed by incubation with primary antibodies (rabbit anti-synaptophysin IgG (1:10,000, Dako) and mouse anti-MAP2 IgG (1:10,000, Chemicon)) to visualize dendrites at 4 ºC for 16 h. Neurons were incubated with both Alexa Fluor 488-conjugated goat anti-rabbit IgG and 594 goat anti-mouse IgG (1:1,000, Invitrogen) for 1 h at room temperature. After washing with PBS, neurons were covered with Vectashield mounting medium (H-1000, Vector Laboratories, Burlingame, CA, USA). Images were taken at equal exposure for all the different groups at ×63 oil lens under a confocal microscopy (Leica) and analyzed by the Universal Metamorph Image Program (Molecular Devices, Sunnyvale, CA, USA).

Synaptic density of cultured neurons. After the various treatments, neurons were fixed with 4% ice-cold paraformaldehyde for 5 min and then permeabilized with PBS containing 0.1% Triton and 5% goat serum for 1 h at room temperature followed by incubation with primary antibodies (rabbit anti-synaptophysin IgG (1:10,000, Dako) and mouse anti-MAP2 IgG (1:10,000, Chemicon)) to visualize dendrites at 4 ºC for 16 h. Neurons were incubated with both Alexa Fluor 488-conjugated goat anti-rabbit IgG and 594 goat anti-mouse IgG (1:1,000, Invitrogen) for 1 h at room temperature. After washing with PBS, neurons were covered with Vectashield mounting medium (H-1000, Vector Laboratories, Burlingame, CA, USA). Images were taken at equal exposure for all the different groups at ×63 oil lens under a confocal microscopy (Leica) and analyzed by the Universal Metamorph Image Program (Molecular Devices, Sunnyvale, CA, USA).

Statistical analysis One-way ANOVA was used for repeated measure analyses followed by Fisher’s protected least significant difference for post hoc comparisons. We used StatView 5.0.1 Windows software (SAS Institute, Cary, NC, USA), and results are reported as means ± S.E.M. P < 0.05 was considered significant.

Conflict of Interest The authors declare no conflict of interest.

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et al.

26. Grillo MA, Colombatto S. Advanced glycation end-products (AGEs): involvement in aging and Alzheimer’s disease. Brain Res Brain Res Rev 1997; 23: 134–143.

25. Necula M, Kuret J. Pseudophosphorylation and glycation of tau protein enhance but do not hyperphosphorylate via promoting AGEs formation. J Biol Chem 1994; 269: 21614–21619.

24. Lee VM, Kim Y, Liu J, Aoki S, Ohnishi K, Yasuda T, Aoki S, Yasuda T, Kawano K, Tsukada Y. Increased serum levels of advanced glycation end-products and diabetic complications. Diabetes Res Clin Pract 1998; 41: 131–137.

23. Li XH, Xie JZ, Jiang X, Lv BL, Cheng XS, Du LL. Advanced glycation end-products and diabetic complications. Diabetes Res Clin Pract 1998; 41: 131–137.

22. Loske C, Gerdemann A, Schepl W, Wycislo M, Schinzel R, Palm D. Characterization of two binding proteins for advanced glycosylation end products from cerebellar cortex. J Neurosci Lett 1999; 261: 17–20.

21. Yan SD, Yan SF, Chen X, Fu J, Chen M, Kuppusamy P. Advanced glycation end product level, diabetes, and accelerated cognitive aging. Neurology 2011; 77: 1351–1356.

20. Yan SD, Chen X, Schmidt AM, Brett J, Godman G, Zou YS et al. Advanced glycation end products and diabetic complications. Diabetes Res Clin Pract 1999; 41: 131–137.

19. Vitek MP, Bhattacharya K, Glendening JM, Stopa E, Vlassara H, Bucala R. Metal-mediated glycoxidation accelerates cross-linking of beta-amyloid peptide. Proc Natl Acad Sci USA 2003; 100: 1635–1645.

18. Petot GJ, Traore F, Debanne SM, Lerner AJ, Smyth KA, Friedland RP. Interactions of RAGE-dependent neointimal expansion in arterial restenosis. J Investig Med 2003; 51: 1635–1645.

17. Reddy S, Bicher J, Wells-Knecht K, Torpe SR, Baynes JW. N epsilon-carboxymethyllysine is a dominant advanced glycation product (AGE) antigen in tissue proteins. Biochemistry 1995; 34: 10927–10987.

16. Origna N, Arancio O, Domenici L, Yan SS. MAPK beta-amyloid and synaptic dysfunction: the role of RAGE. Expert Rev Neurother 2009; 9: 1635–1645.

15. Kilander L, Nyman H, Boberg M, Hansson L, Lithell H. Hypertension is related to increasing prevalence of adult-onset diabetes mellitus: a 15-year longitudinal study of blood pressure and dementia. J Intern Med 1997; 238: 149–155.

14. Leibson CL, O’Brien PC, Atkinson E, Palumbo PJ, Melton LJ 3rd. Relative contributions of apoipoprotein E genotype and dietary fat intake of healthy older persons during mid-adult life. J Gerontol A Biol Sci Med Sci 1997; 52: 171–177.

13. nonenzymatically glycated tau in Alzheimer’s disease induces neuronal oxidant stress resulting in cytokine gene expression and release of alpha-amyloid peptide. Nat Med 1995; 1: 693–694.

12. Loske C, Gerdemann A, Schepl W, Wycislo M, Schinzel R, Palm D et al. Transition metal-mediated glycoxidation accelerates cross-linking of beta-amyloid peptide. Eur J Biochem 2000; 267: 4171–4178.

11. U XH, Xie JZ, Jiang X, Lv BL, Cheng XS, Du LL et al. Methylation induces tau hyperphosphorylation via promoting AGEs formation. Neurochemical Molecular Med 2012; 14: 338–348.

10. Lee VM, Kim Y, Liu J, Aoki S, Ohnishi K, Yasuda T, Aoki S, Yasuda T, Kawano K, Tsukada Y. Increased serum levels of advanced glycation end-products and diabetic complications. Diabetes Res Clin Pract 1998; 41: 131–137.

9. Necula M, Kuret J. Pseudophosphorylation and glycation of tau protein enhance but do not hyperphosphorylate via promoting AGEs formation. J Biol Chem 1994; 269: 21614–21619.

8. Necula M, Kuret J. Pseudophosphorylation and glycation of tau protein enhance but do not hyperphosphorylate via promoting AGEs formation. J Biol Chem 1994; 269: 21614–21619.

7. Shimoike T, Inoguchi T, Umeda F, Nawata H, Kawano K, Ochi H. The meaning of serum advanced glycation end-products in aging and Alzheimer’s disease brain. Cereb Cortex 2005; 15: 211–220.

6. Ono Y, Aoki S, Ohnishi K, Yasuda T, Kawano K, Tsukada Y. Increased serum levels of advanced glycation end-products and diabetic complications. Diabetes Res Clin Pract 1999; 41: 131–137.

5. Necula M, Kuret J. Pseudophosphorylation and glycation of tau protein enhance but do not hyperphosphorylate via promoting AGEs formation. J Biol Chem 1994; 269: 21614–21619.

4. Lee VM, Kim Y, Liu J, Aoki S, Ohnishi K, Yasuda T, Aoki S, Yasuda T, Kawano K, Tsukada Y. Increased serum levels of advanced glycation end-products and diabetic complications. Diabetes Res Clin Pract 1999; 41: 131–137.
beta-amyloid-induced synaptic depression and long-term impairment in entorhinal cortex. J Neurosci 2010; 30: 11414–11425.

54. Arancio O, Zhang HP, Chen X, Lin C, Trinchese F, Puzzo D et al. RAGE potentiates Abeta-induced perturbation of neuronal function in transgenic mice. EMBO J 2004; 23: 4096–4105.

55. Fang F, Lue LF, Yan S, Xu H, Luddy JS, Chen D et al. RAGE potentiates Abeta-induced perturbation of neuronal function in transgenic mice. EMBO J 2004; 23: 4096–4105.

56. Fang F, Lue LF, Yan S, Xu H, Luddy JS, Chen D et al. RAGE-dependent signaling in microglia contributes to neuroinflammation, Abeta accumulation, and impaired learning/memory in a mouse model of Alzheimer’s disease. FASEB J 2010; 24: 1043–1055.

57. Takuma K, Fang F, Zhang W, Yan S, Fukuzaki E, Du H et al. RAGE-mediated signaling contributes to intraneuronal transport of amyloid-beta and neuronal dysfunction. Proc Natl Acad Sci USA 2009; 106: 20021–20026.

58. Chang CT, Wu MS, Tian YC, Chen KH, Yu CC, Liao CH et al. Enhancement of epithelial sodium channel expression in renal cortical collecting ducts cells by advanced glycation end products. Nephrol Dial Transplant 2007; 22: 722–731.

59. Du H, Guo L, Fang F, Chen D, Sosunov AA, McKhann GM et al. Cyclophilin D deficiency attenuates mitochondrial and neuronal perturbation and ameliorates learning and memory in Alzheimer’s disease. Nat Med 2008; 14: 1097–1105.