RAGE SIGNALING MEDIATES DEFICITS IN HIPPOCAMPAL FUNCTION IN MODELS OF DIABETES

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ABSTRACT

Diabetes is a prevalent metabolic disorder that affects various body functions and systems. Effects of diabetes on cognitive function have been reported in animal models of diabetes, particularly learning and memory impairments and changes in hippocampal synaptic plasticity such as long-term potentiation (LTP), which depends primarily on NMDA and AMPA subtypes of glutamate receptors. The Receptor for Advanced Glycation End-products (RAGE) has been particularly implicated in vascular and peripheral nervous system complications of diabetes. These observations led us to hypothesize that RAGE signaling in models of diabetes can alter the function of NMDA and AMPA subtypes of glutamate receptors, leading to dysfunction in synaptic transmission and subsequent impairment in learning and memory.

Our findings showed that although recognition memory was unaffected in streptozotocin (STZ)-induced diabetes in both genotypes, hippocampal-dependent spatial memory was impaired in STZ-induced diabetic mice in wild-type (WT) but not in the RAGE knockout (RAGE-KO) group. This impairment in spatial memory was consistent with deficits in synaptic plasticity, i.e. LTP and paired pulse facilitation (PPF), and reduction in the expression and phosphorylation of the GluA1 subunit of the AMPA receptor in WT STZ-induced diabetic mice. These changes were associated with the activation of the mitogen activated protein kinase (MAPK) pathway, leading to increased total p38, phospho-p38, and nuclear factor-kappa beta (NF-κB) and decreased phospho c-Jun N-terminal kinase (pJNK) and its kinase, mitogen activated protein kinase kinase 7 (pMEK7). In WT hippocampal cultures, high glucose caused a reduction of AMPA-evoked currents, as well as a reduction in cell excitability, and an increase in cytosolic ROS.

This is the first study, to the best of our knowledge, that shows the contribution of RAGE signaling in abnormal hippocampal synaptic transmission and cognitive function in diabetes, which could help identify potential targets for therapeutic interventions.

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LIST OF ABBREVIATIONS

Aβ	Amyloid beta
AChE	Acetylcholinesterase
ACSF	Artificial cerebrospinal fluid
AD	Alzheimer's disease
ADAM10	A disintegrin and metalloprotease 10
AGEs	Advanced glycosylation end-products
AHP_{AMPL}	After-hyperpolarization amplitude
AKT	A serine/threonine protein kinase (Protein kinase B)
AMPA	Alpha-amino-3-hydroxy-5-methylisoxazole propionate
ANOVA	Analysis of variance
AP-5	2-amino-5-phosphonovaleric acid
APs	Action potentials
AP_{AMPL}	Action potential peak amplitude
AP_{HW}	Action potential half-width
AP_{ISI}	Action potential inter-spike interval
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BM	Barnes maze
Ca^{+}	Calcium ion
CA	Cornu Ammonis
CaMK	Calcium/calmodulin-dependent protein kinase
ChAT	Choline acetyltransferase
C_M	Membrane capacitance
CNS	Central nervous system
CNT	Control
COX	Cyclooxygenase
cRAGE	Cleaved RAGE
CREB	cAMP-response element binding protein
DG	Dentate gyrus

DM	Diabetes mellitus
DMEM	Dulbecco's modified eagle medium
DMV	Dorsal motor nucleus of the vagus nerve
DN RAGE	Dominant-negative RAGE
DPN	Diabetic peripheral neuropathy
DRG	Dorsal root ganglion
EC	Entorhinal cortex
ECF	Extracellular fluid
ELISA	Enzyme-linked immunosorbent assay
EPM	Elevated plus maze
ERK	Extracellular-regulated kinase
esRAGE	Endogenous secretory RAGE
fEPSPs	Field excitatory postsynaptic potentials
FITC	Fluorescein isothiocyanate
fRAGE	Full-length RAGE
GABA	Gamma-aminobutyric acid
GADD45β	Growth arrest and DNA damage-inducing protein β
GCRs	Glucocorticoid receptors
GFAP	Glial fibrillary acidic protein
GSK3β	Glycogen synthase kinase 3β
H_2O_2	Hydrogen peroxide
HG	High-glucose
HMGB1	High-mobility group box 1 protein
HPA	Hypothalamic-pituitary-adrenal
HRP	Horse radish peroxidase
IGFs	Insulin-like growth factors
JAK	Janus kinase
JNK	C-Jun N-terminal kinase
K^+	Potassium ion
LPP	Lateral perforant pathway
LPS	Lipopolysaccharides

LTD	Long-term depression
LTP	Long-term potentiation
Mac-1	Macrophage-1 antigen
MAPK	Mitogen activated protein kinase
MAPK2K	Mitogen-activated protein kinase kinase 2
mAPP	Mutant amyloid precursor protein
MCI	Mild cognitive impairment
MEK7	Mitogen activated protein kinase kinase 7
mEPSCs	Miniature excitatory postsynaptic currents
Mg^{2+}	Magnesium ion
MMP9	Matrix metalloproteinase 9
MPP	Medial perforant pathway
mRNA	Messenger ribonucleic acid
MTL	Medial temporal lobe
MWM	Morris water maze
Na^{+}	Sodium ion
nAChRs	Nicotinic acetylcholine receptors
NADPH	Nicotinamide adenine dinucleotide phosphate
Nav1.3	Voltage-gated sodium channel type 3
NF-κB	Nuclear factor-kappa beta
NMDA	N-methyl-D-aspartate
NOD	Non-obese diabetic
NOR	Novel object recognition
NOS	Nitric oxide synthase
NP-40	Nonidet P-40
OF	Open field
PFC	Prefrontal cortex
PI	Propidium iodide
pJNK	Phospho c-Jun N-terminal kinase
PKA	Protein kinase A
PKC	Protein kinase C

PPF	Paired pulse facilitation
PVN	Paraventricular nucleus
RAGE	Receptor for Advanced Glycation End-products
RAGE-KO	RAGE knockout
Rho	Ras homologous
R_M	Membrane resistance
ROS	Reactive oxygen species
S100B	S100 calcium-binding protein B
SAPK	Stress-activated protein kinases
SCG	Superior cervical ganglion
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Ser831	Amino acid residue serine 831
Ser845	Amino acid residue serine 845
SOD	Superoxide dismutase
sRAGE	Soluble RAGE
STAT	Signal transducers and activators of transcription
STZ	Streptozotocin
TBS	Theta burst stimulation
tDRG	Thoracic dorsal root ganglia
V_{TH}	Threshold voltage
V_M	Resting membrane potential
WT	Wild type

CHAPTER 1

GENERAL INTRODUCTION

1.1 Diabetes

Diabetes is a complex metabolic disorder characterized by elevated blood glucose level (hyperglycemia) due to deficits in insulin secretion or resistance to insulin action, or both (Kharroubi and Darwish 2015). The vast majority of diabetic cases are classified into one of two broad categories: type 1 diabetes, which is caused by the autoimmune destruction of insulin-secreting pancreatic beta cells, or type 2 diabetes, which is caused by a combination of resistance to insulin action and an inadequate insulin secretion (Kharroubi and Darwish 2015). In addition, a transient form of diabetes developed during the course of pregnancy is classified as gestational diabetes (Alfadhli 2015). Hyperglycemia is the hallmark characteristic of all types of diabetes and it has the potential to cause long-lasting complications due to its chronic nature (Papatheodorou et al., 2015). These complications are divided into microvascular and macrovascular, with the former having much higher prevalence than the latter (Deshpande et al., 2008; Papatheodorou et al., 2015). Microvascular complications include damage to the kidneys (nephropathy) leading to renal failure, to the eyes (retinopathy) with potential loss of vision, and to the nerves (neuropathy) leading to sensory loss, and damage to the limbs as well as to the gastrointestinal and genitourinary systems (American Diabetes Association 2010). Macrovascular complications, on the other hand, can lead to atherosclerosis, cardiovascular, peripheral arterial, and cerebrovascular diseases and stroke (American Diabetes Association 2010). These complications represent the leading causes of diabetes-related morbidity and mortality worldwide (Cusick et al., 2005). In addition to micro- and macrovascular complications, hyperglycemia can also affect cells directly, in particular neurons, independently of the vasculature, in a phenomenon known as glucose neurotoxicity (Tomlinson and Gardiner, 2008), which is linked to oxidative stress and activation of a number of signaling pathways.

1.2 Diabetes and the central nervous system (CNS)

It has been traditionally accepted that the CNS was spared from diabetic complications since it was considered an insulin-independent organ with constant glucose levels (Ahmadpour 2012; Blázquez et al. 2014). However, in recent decades, studies have provided strong pieces of evidence that not only show important functions of insulin in the brain (Blázquez et al. 2014; Gray et al., 2014), but also confirm changes in brain glucose concentrations following diabetic/hyperglycemic states (Jacob et al., 2002; Elizabeth et al., 2012). The effects of diabetes on the CNS, known as diabetic encephalopathy (Sima, 2010), is linked to structural and functional changes (Stiles and Seaquist 2010; Seaquist 2015; Moheet et al., 2015). Alterations in neurotransmission, electrophysiological abnormalities, and neurobehavioural changes are widely reported in the diabetic brain (Trudeau et al., 2004; Li and Sima 2004; Malone 2016). In particular, demyelination and delayed conduction velocity (Manschot et al., 2003; Huang et al., 2012), degenerative changes and neuronal loss (Sadeghi et al., 2016), cerebral atrophy (Šerbedžija et al., 2012), impaired neurogenesis (Stranahan et al., 2008; Dorsemans et al., 2017), increased gliosis (Wanrooy et al., 2018), enlarged lateral ventricles (Mazaika et al., 2018), white matter hyperintensities (Weinger et al., 2008), increased blood-brain barrier (BBB) permeability (Hawkins et al., 2007), impaired synaptic plasticity (Gispen and Biessels 2000), changes in neurotransmitter synthesis and release (Trudeau et al., 2004), cerebral ischemia (Shukla et al., 2017), and increased risks of cognitive dysfunction (Kodl and Seaquist 2008) such as dementia (Gudala et al., 2013) and depression (Bădescu et al., 2016a) have been observed in clinical and experimental diabetes (Li and Sima 2004; Biessels and Reijmer 2014).

1.2.1 Diabetes and cognitive function

Diabetes is accompanied by an erosion of cognitive function, as shown by longitudinal and cross-sectional studies in diabetic patients (Trudeau et al. 2004). Perhaps, the most recognizable negative impact of diabetes in cognitive function is its connection to dementia. Type 2 diabetes has long been considered a risk factor for Alzheimer's disease (AD), vascular dementia, and other forms of dementia (Lee et al., 2018). Reports concentrating on the prevalence of diabetes and AD, and hyperglycemia as a risk factor for AD, strongly support a link between these two diseases (González-Reyes et al., 2016; Kim et al., 2016; Pruzin et al., 2018). Particularly, hyperglycemia

increases amyloid β accumulation on brain lesions, as well as increase oxidative stress, markers of neuroinflammation, mitochondrial dysfunction, and neurodegeneration (Macauley et al., 2015; Rom et al., 2018; Silzer et al., 2018).

Cognitive decline has been widely reported in type 2 diabetic patients. Among the most commonly cognitive functions affected are psychomotor speed, executive function, verbal memory, processing speed, complex motor functioning, working memory, immediate and delayed recall, verbal fluency, visual retention, and attention (Gregg et al., 2000; Grodstein et al., 2001; Fontbonne et al., 2001; Munshi et al., 2006; Kanaya et al., 2004; Messier, 2005; Kodl and Seaquist, 2008; Moheet et al., 2015). Furthermore, the incidence of cognitive decline in type 2 diabetic patients correlates with the duration of disease (Kodl and Seaquist, 2008). Although diabetes might also increase the risk of young-onset dementia (that is, before the age of 65), the vast majority of individuals with diabetes who develop dementia are over the age of 65 (Biessels and Despa, 2018).

Similarly, type 1 diabetic patients show negative impact on cognitive functions such as information processing speed, psychomotor efficiency, attention, memory, learning, problem solving, motor speed, general intelligence, visuoconstruction, visual perception, somatosensory examination, motor strength, mental flexibility and executive function (Kodl and Seaquist, 2008; Brands et al., 2005; Wessels et al., 2007; Perantie 2008; Northam 2009; Ohmann 2010; Moheet et al., 2015).

Although hallmark complications of diabetes affecting peripheral tissues, such as retinopathy and nephropathy, usually develop later in the course of the disease, the onset of cognitive dysfunction has been found to develop early in patients with type 1 diabetes (Kodl and Seaquist, 2008). Cognitive functions, including general intelligence, vocabulary, learning, and speed of information processing, for example, have been affected as early as 2 years after diagnosis in children with type 1 diabetes (Northam et al., 1998).

Cognitive dysfunction, especially learning and memory impairments, have also been widely reported in animal models of diabetes (Kodl and Seaquist 2008; Wrighten et al., 2009; Rostami et al., 2013, Moheet et al., 2015; Saedi et al., 2016).

Table 1.1 provides a summary of cognitive domains that negatively affected by diabetes in type 1 and type 2 diabetic patients.

Table 1.1 Summary of cognitive domains that are negatively affected by diabetes in type 1 and type 2 diabetic patients.

Cognitive Domain	Type of Diabetes	Citation
Information processing speed	Type 1 and type 2	Brands et al., 2006; Kodl and Seaquist, 2008
Psychomotor speed	Type 1 and type 2	Ryan et al., 2003; Gregg et al., 2000
Attention	Type 1 and type 2	Wessels et al., 2007; Fontbonne et al., 2001
Learning and memory	Type 1 and type 2	Weinger et al., 2008; Zilliox et al., 2016
Executive function	Type 1 and type 2	Munshi et al., 2006; Weinger et al., 2008
Problem solving	Type 1	Kodl and Seaquist, 2008
Motor speed and strength	Type 1	Ryan et al., 2003
Vocabulary	Type 1	Weinger et al., 2008
General intelligence	Type 1	Northam et al., 1998
Visuoconstruction	Type 1	Wessels et al., 2007
Mental flexibility	Type 1	Kodl and Seaquist, 2008
Verbal memory	Type 2	Messier, 2005
Delayed and immediate recall	Type 2	Grodstein et al., 2001
Complex motor function	Type 2	Kumar et al., 2009
Verbal fluency	Type 2	Kanaya et al., 2006
Depression	Type 2	Bruce et al., 2003; Holt et al., 2014

1.2.2 Pathogenesis of CNS complications of diabetes

Among the several factors involved in the pathogenesis of CNS complications in diabetes, hyperglycemia-induced oxidative stress and insulin/C-peptide deficiency are considered critically important (Li and Sima 2004; Muriach et al., 2014). Hyperglycemia-induced oxidative stress is one of the well-investigated theories regarding diabetes-induced CNS complications (Li and Sima 2004; Muriach et al., 2014). It is well known that hyperglycemia induces oxidative stress through the polyol pathway, enhanced production of mitochondrial reactive oxygen species (ROS), increased nonenzymatic glycation of proteins, glucose autoxidation, enhanced lipid peroxidation by-products, and eventually imbalances in the generation of ROS and their scavengers (Mercuri et al., 2000; Lipinski 2001; Opara 2002; Li and Sima 2004).

High lipid content and oxygen consumption rate and relative paucity of antioxidant enzymes make the brain especially vulnerable to such oxidative stress (Muriach et al., 2014; Barcia et al., 2015). One of the main consequences of oxidative stress is the activation of stress-sensitive signaling pathways such as c-Jun N-terminal kinase/stress-activated protein kinases (JNK/SAPK), p38 mitogen-activated protein kinase (MAPK), advanced glycosylation end-products (AGEs)/receptor for AGEs (RAGE), and subsequent activation of nuclear factor-κB (NF-κB) and the production of gene products which cause cellular and tissue damage and chronic complications of diabetes (Evans et al., 2002).

In addition to oxidative stress-mediated complications, deficiency in the insulin/C-peptide, a peptide cleaved from proinsulin during the insulin biosynthesis, is also proposed as another mechanism underlying diabetes-induced CNS complications (Li and Sima 2004; Sima et al., 2004). Insulin in the brain contributes to numerous distinct roles such as regulating energy expenditure, glucose uptake and homeostasis, feeding behaviour, reproduction, cell proliferation and differentiation, cognition, and memory, in addition to its neuromodulatory, neurotrophic and neuroprotective effects (Blázquez et al. 2014; Gray et al., 2014). Insulin deficiency is shown to play an important role in diabetes-induced neuronal loss/apoptosis in the CNS (Li and Sima 2004; Sima et al., 2004). Treatment with insulin mediated an anti-apoptotic effect and partially corrected diabetes-induced cognitive deficits (Li et al., 2003; Li and Sima 2004; Sima et al., 2004). Similarly, treatment with C-peptide was able to prevent neuronal apoptosis in the hippocampus of diabetic rats (Li et al., 2002b).

The role of diabetes-induced oxidative stress and neuronal loss in CNS complications is most extensively studied in the hippocampus (Li et al., 2002a; Yang et al., 2013; Foghi and Ahmadpour 2013; Muriach et al., 2014; Sadeghi et al., 2016; Ahmad et al., 2017).

1.2.3 Diabetes and the hippocampus

1.2.3.1. Hippocampus structure and function

Hippocampus is a part of the limbic system that is located alongside the medial to inferior horn of the lateral ventricle in the temporal lobe of the cerebral cortex (Campbell and MacQueen 2004). The hippocampus has a highly distinctive morphology, composed of two regions, the Cornu Ammonis (CA) and the dentate gyrus (DG) which principally contain the pyramidal cells and the granule cells respectively (Taupin 2007). The CA region itself can be divided into CA1, CA2, CA3, and CA4 subregions based on pyramidal neuron morphology (Campbell and MacQueen 2004). The hippocampus forms a unidirectional network, with input from the entorhinal cortex that makes connections with the DG and CA3 pyramidal neurons through the perforant path. In addition to input from entorhinal cortex, CA3 pyramidal neurons also receive input from the DG via the mossy fibre pathway and send axons to CA1 pyramidal neurons via the Schaffer collateral pathway, as well as to the contralateral hippocampus through the associational/commissural pathway. CA1 pyramidal neurons receive inputs from the perforant path as well and send axons to the subiculum, which in turn send the main hippocampal output back to the entorhinal cortex and form a loop (Kesner 2013) (Figure 1.1).

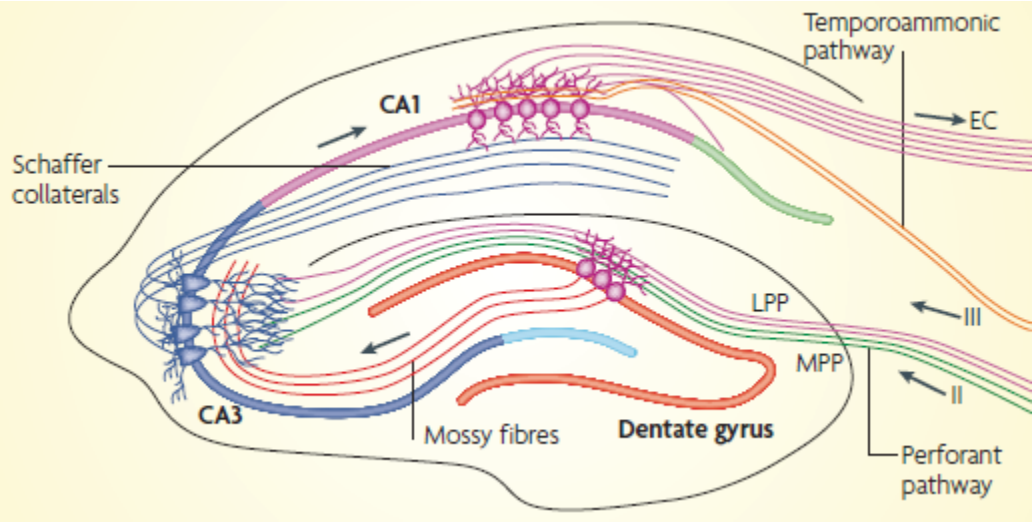


Figure 1.1. The neuronal circuitry in the hippocampus. Layer II neurons in the entorhinal cortex (EC) send axons to the dentate gyrus via the lateral perforant pathway (LPP) and the medial perforant pathway (MPP). The dentate gyrus then projects to CA3 pyramidal neurons through the mossy fibre pathway. CA3 pyramidal neurons send the information to pyramidal neurons in CA1 via the Schaffer collateral pathway, which in turn send back-projections into deep-layer neurons of the EC (Reproduced with permission from Deng et al., 2010).

The hippocampus is also a part of the brain that shows a high degree of synaptic plasticity (Taupin 2007). It is also one of the unique regions of the brain where neurogenesis continues throughout adulthood (Bonfanti and Peretto 2011; Anand and Dhikav 2012).

The hippocampus is involved in several important physiological functions such as learning and memory, spatial navigation, emotional behaviours, and regulation of hypothalamic functions (Anand and Dhikav 2012). Many of these physiological hippocampal functions are mediated, at least in part, by the two major subtypes of ionotropic glutamate receptors in the CNS, N-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methylisoxazole propionate (AMPA) receptors (Voglis and Tavernarakis 2006). NMDA receptors are tetrameric assemblies of different subunits (GluN1, GluN2A-D and GluN3A-B) and act as non-selective cation channels (Hansen et al., 2018). To open the cation pore, they require the binding of glutamate and glycine, coupled with depolarization of the postsynaptic membrane to relieve the magnesium (Mg^{2+}) blockade from the channel's pore (Voglis and Tavernarakis 2006). AMPA receptors, on the other hand, are tetrameric ion channels composed of different subunits (GluA1-GluA4) that conduct sodium (Na^{+}) and potassium (K^{+}) ions primarily, although they can be permeable to calcium as well depending on their subunit composition (Gouaux, 2004). The effect of glutamate on the hippocampal excitatory synapses has a dual effect mediated by both types of ionotropic excitatory receptors, a fast component mediated by AMPA receptors and a slow component mediated by NMDA receptors (Huganir and Nicoll 2013; Strong et al., 2014).

In the CA1 region of the hippocampus, it is now well known that the induction of long-term potentiation (LTP) is primarily mediated by AMPA and NMDA subtypes of glutamate receptors (Lynch, 2004; Citri and Malenka, 2008). LTP is a form of synaptic plasticity that serves as an important electrophysiological tool for the study of processes involved in learning and memory (Baudry and Lynch 2001; Lynch 2004). AMPA-mediated rapid responses to synaptically released glutamate can shift the membrane potential from resting to a depolarized potential and relieve Mg^{2+} blockade of NMDA receptors (Fleming and England, 2010). Once open, NMDA receptors allow calcium (Ca^{+}) influx into the postsynaptic neuron, triggering various signaling cascades involved in the regulation of the expression, trafficking and function of glutamate receptors, especially AMPA receptors, which are critical for the generation of synaptic potentiation (Baudry and Lynch 2001; Fleming and England, 2010).

The regulation of AMPA receptors is primarily mediated by two mechanisms: 1) phosphorylation of the receptor subunits by kinases that become activated following NMDA-mediated Ca^{+} influx, and the subsequent increase in the open probability of the receptor, and 2) changes in the surface expression of the receptor at the postsynaptic membrane, such as insertion of new AMPA receptors following Ca^{2+}-mediated cascades (Voglis and Tavernarakis 2006) (Figure 1.2).

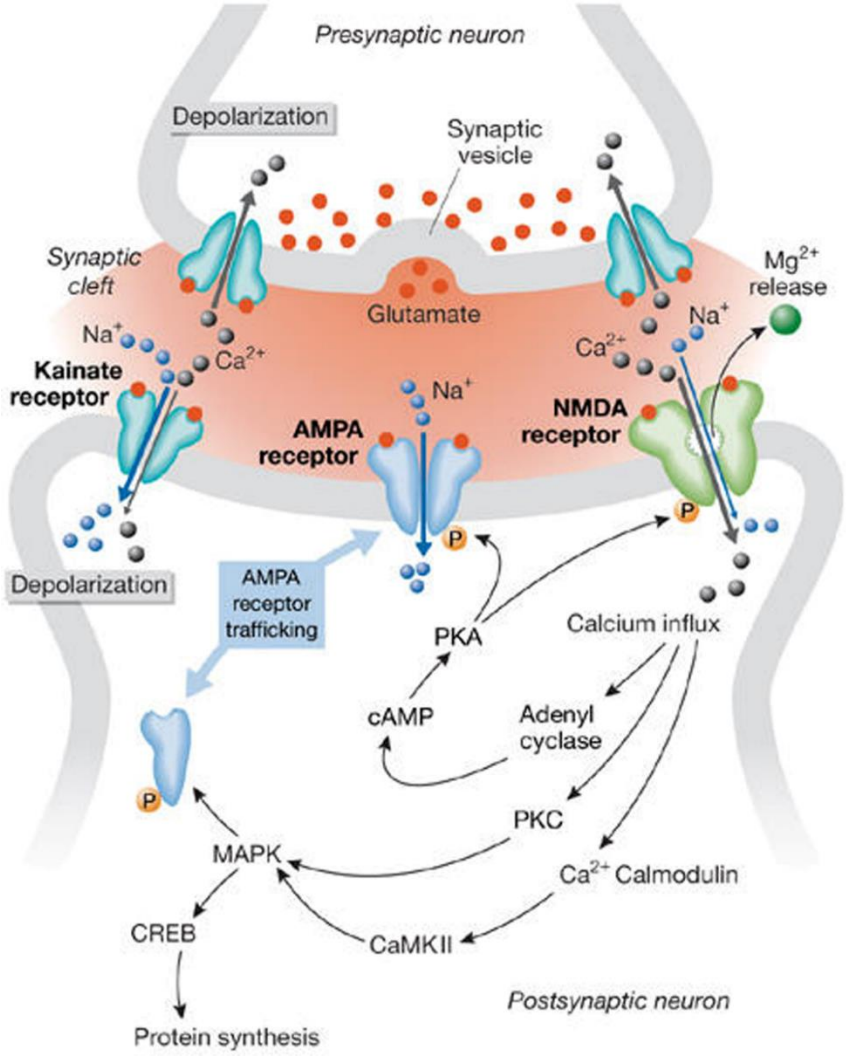


Figure 1.2. Hippocampal synaptic plasticity and glutamate receptors. Glutamate released from the presynaptic terminal binds to the glutamate receptors at the postsynaptic terminal, leading to the passage of Na^+ from AMPA and kainate receptors and subsequent postsynaptic depolarization. This shift in the membrane potential triggers the release of Mg^{2+} from the NMDA receptor. Ca^+ influx through the NMDA receptor initiates various signaling cascades, such as further regulating the expression, trafficking and phosphorylation of AMPA receptors, which is critical for the generation of synaptic potentiation (Reproduced with permission from Voglis and Tavernarakis 2006).

The hippocampus is shown to be very vulnerable to a variety of insults including stress, hypoxia, ischemia, aging, and metabolic disturbances such as those taking place in diabetes (Di Paola et al., 2008; Stranahan et al., 2008; Murray et al., 2014; Bartsch and Wulff 2015; Bartsch et al., 2015; Gasparova et al., 2018). Diabetes results in a variety of functional and structural changes in the hippocampus, and some of the most common will be discussed below.

1.2.3.2. Increased neuronal loss and decreased neurogenesis

Increased neuronal cell loss/apoptosis is widely reported in the hippocampus of spontaneous and induced animal models of diabetes (Li et al., 2002a; Sima and Li 2005; Lebed et al., 2008; Rostami et al., 2013; Sadeghi et al., 2016). These kinds of neurodegenerative alterations are suggested to underlie the volumetric changes observed in the hippocampus in diabetes, such as a reduction of grey matter density in diabetic humans, especially in those with poorer glycemic control (Musen et al., 2006; Ates et al., 2007; foghi et al., 2013). In addition to increased neuronal loss, an ample body of literature shows significant decrease in hippocampal neurogenesis in animal models of diabetes (Jackson-Guilford et al., 2000; Saravia et al., 2004; Beauquis et al., 2006; Stranahan et al., 2008; Zhang et al., 2008; Ho et al., 2013). Diabetes-induced inflammation is suggested as one of the mechanisms involved in reduced hippocampal neurogenesis (Chesnokova et al., 2016). Pro-inflammatory cytokines are shown to suppress hippocampal neural progenitor cell proliferation (Chesnokova et al., 2016). Proinflammatory cytokines can induce the hypothalamic-pituitary-adrenal (HPA) axis to become hyperactive and cause an elevation in glucocorticoid levels, which in turn disrupt hippocampal neurogenesis due to the high density of glucocorticoid receptors (GCRs) in the hippocampus (Stranahan et al., 2008; Chesnokova et al., 2016; Odaka et al., 2017). High levels of cytokines, on the other hand, can induce apoptosis of newborn neurons and trigger oxidative stress that damages the developing neurons (Chesnokova et al., 2016).

1.2.3.3 Astroglial alterations

Astrocytes, the most abundant cell type in the CNS, play an important role in brain energy metabolism and are therefore a major potential target during abnormal glucose homeostasis, such

as in diabetes (Prebil et al., 2011; Jha and Morrison 2018). Hyperglycemia is shown to affect primary astrocytes in culture by increasing inflammatory cytokine expression, ROS production, and cell death, as well as by inhibiting proliferation and migration (Wang et al., 2012; Li et al., 2018). In addition, high glucose is reported to increase glycolytic metabolism and enhance adenosine triphosphate (ATP) and glycogen content in cultured astrocytes, leading to changes in energy metabolism (Li et al., 2018). Hyperglycemia also caused deficits in gap junctional communication between astrocytes in cultures as well as in brain slices of STZ-treated rats (Gandhi et al., 2010). Moreover, increases in glial fibrillary acidic protein (GFAP) and S100 calcium-binding protein B (S100B) expression have been observed in the hippocampus of rodent models of diabetes, indicating increases in astrocyte reactivity in response to diabetes (Saravia et al., 2002; Baydas et al., 2003a; Coleman et al., 2004). Remarkably, these effects were attenuated by antioxidant treatment, which further supports the central role of oxidative stress in mediating diabetes-induced astroglial alterations (Baydas et al., 2003a; Okuyama et al., 2018).

1.2.3.4 Impairment in synaptic plasticity

Synaptic plasticity – the ability of synapses to change their synaptic strength in response to specific patterns of activity – is modulated by morphological and biochemical mechanisms that are thought to be the cellular events underling learning and memory (Massaad and Klann 2011). Morphological studies show a decreased length of apical dendrites as well as a reduced number of apical branch points of CA3 pyramidal neurons in STZ-induced diabetic rats (Magarinos and McEwen 2000), which may be linked to electrophysiological dysfunctions in the CA1 region of the hippocampus (Trudeau et al., 2004). In fact, deficit in the expression of LTP at hippocampal CA3–CA1 synapses is considered by many as one of the main underlying mechanisms of diabetes-induced cognitive impairments (Biessels et al., 2002; Trudeau et al., 2004; Kumar 2011). Impairment in LTP is well documented in animal models of diabetes (Izumi et al., 2003; Trudeau et al., 2004 ; Artola et al., 2005; Kumar 2011; Sasaki-Hamada et al., 2012; Grillo et al., 2015).

Both pre- and postsynaptic components are suggested to contribute to LTP alterations in the hippocampus in diabetes (Trudeau et al., 2004). At the presynaptic level, changes in LTP might be caused by alterations in neurotransmitter synthesis and release (Trudeau et al., 2004). In this regard, measurements of paired pulse facilitation (PPF) – a form of presynaptic plasticity – is

considered as a useful electrophysiological tool for detection of changes in presynaptic function, such as probability of neurotransmitter release (Jackman and Regehr 2017). Changes in neurotransmitter synthesis or release in diabetes are reported in several brain regions including the hippocampus (Trudeau et al., 2004). In contrast, at the postsynaptic level, changes in LTP can be linked to modifications in expression and function of glutamate receptors.

1.2.3.5 Changes in expression and function of glutamate receptors

Glutamate receptors, the major excitatory receptors in the central nervous system, play important roles in controlling synaptic plasticity during learning and memory processes (Voglis and Tavernarakis, 2006). Abnormal regulation of glutamate receptors thus appears to mediate diabetes-induced impairment in synaptic plasticity and the development of cognitive deficits (Trudeau et al., 2004).

Findings in the diabetic hippocampus describe changes in AMPA and NMDA receptors expression and function, which may help explain the impaired synaptic plasticity found in diabetic models. STZ induced-diabetic animals showed changes in electrophysiological properties (Gardoni et al, 2002; Marshad et al., 2018) as well as in the expression level of different subunits of NMDA receptors in the hippocampus (Valastro et al, 2002; Gardoni et al, 2002; Nardin et al, 2016; Wang et al., 2019). Similarly, changes in the electrophysiological properties of AMPA receptors as well as in the expression and post-translational modifications of AMPA receptor subunits are reported in animal models of diabetes (Valastro et al., 2002; Trudeau et al., 2004; Castilho et al., 2012; Wang et al., 2019). These changes in glutamate receptors, which will be discussed thoroughly in Chapter 4, are linked to impairment in synaptic plasticity and cognitive function in diabetic models (Valastro et al., 2002; Trudeau et al., 2004; Sasaki-Hamada et al., 2012; Wang et al., 2019).

1.3 AGEs and their receptor

In recent years, a large number of studies have focused on the factors contributing to the pathogenesis of diabetic complications. Hyperglycemia is still considered the primary cause of diabetic complications (Peppa et al., 2003). Its deleterious effects are attributable, at least in part,

to the formation of AGEs (Peppa et al., 2003; Singh et al., 2014). AGEs are a complex and heterogeneous group of molecules formed from the nonenzymatic reaction of glucose with free amino groups of lipids, proteins, and nucleic acids, called the Millard reaction (Ulrich and Cerami 2001; Cho et al., 2007). This reaction occurs increasingly in the body as we age and is accelerated under conditions of elevated glucose availability, i.e. hyperglycemia, (Nass et al., 2007). AGEs are also formed through the polyol pathway, which becomes active when intracellular glucose concentrations are elevated (Lorenzi 2007). The fructose produced by the polyol pathway can become phosphorylated and break down into compounds that are powerful glycosylating agents and enter into the formation of AGEs (Lorenzi 2007). In addition to the abovementioned pathways, AGEs can also arise from products of autoxidation of glucose, which yields highly reactive oxidative compounds (Daroux et al., 2012). AGEs bind to their receptor, RAGE, which is a member of the immunoglobulin superfamily of cell surface molecules (Singh et al., 2014) and is expressed in a variety of cell types such as immune cells, neurons, astrocytes, skeletal cells, endothelial cells, smooth muscle cells, myocardial cells, and alveolar epithelial cells (Nedić et al., 2013). RAGE is composed of five domains including three extracellular domains (a V-type domain with ligand binding properties and two C-type domains), a single transmembrane domain that anchors RAGE to the membrane and a short C-terminal cytosolic tail that mediates interaction with cytosolic transduction molecules (Stern et al. 2002; Singh et al., 2014).

Several isoforms of RAGE are reported, including the full-length RAGE (fRAGE), the membrane-bound RAGE lacking the extracellular ligand binding domain (N-truncated form, ΔN RAGE), the dominant-negative RAGE (DN RAGE) lacking the C-terminal cytosolic tail, and the soluble RAGE lacking the transmembrane domain but conserving the binding domain. Soluble RAGE, which acts as a decoy receptor to sequester circulating ligands (Han et al., 2011), is divided into two subcategories, i.e. endogenous secretary RAGE (esRAGE) and cleaved RAGE (cRAGE), with the former generated from the splice variants and the latter via the cleavage of full-length RAGE by a membrane α-secretase, a disintegrin and metalloproteinase 10 (ADAM 10) and matrix metalloproteinase 9 (MMP9) (Han et al., 2011) (Figure 1.3).

Although initially identified as a receptor for AGEs, RAGE is a multiligand receptor that binds to a variety of ligands such as S-100 calcium-binding protein, high-mobility group box 1 protein (HMGB1), Amyloid-β (Aβ), macrophage-1 antigen (Mac-1), and phosphatidylserine, to mention

a few (Fritz 2011). Most RAGE ligands are involved in acute and chronic inflammatory events, and many signaling cascades triggered by RAGE lead to the production and activation of pro-inflammatory and inflammatory mediators (Riuzzi et al., 2018). It is not thus surprising that RAGE is implicated in a number of pathological conditions such as atherosclerosis, arthritis, stroke, neurodegeneration, cancer and diabetes (Riehl et al., 2009; Riuzzi et al., 2018).

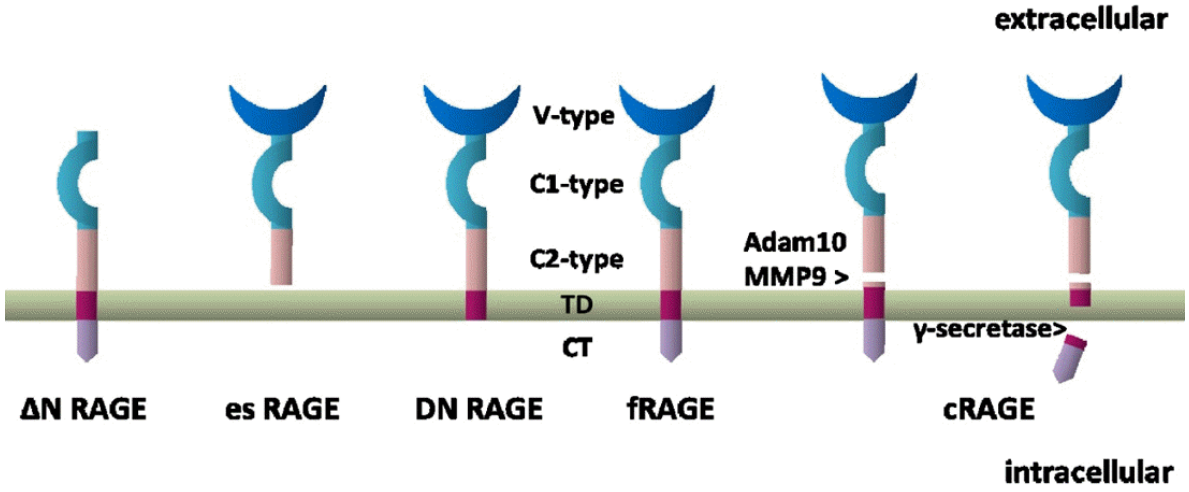


Figure 1.3. RAGE isoforms. Different isoforms of RAGE are generated from alternative mRNA splicing and/or proteolytic cleavage by ADAM10 and MMP9. Full-length RAGE (fRAGE), membrane-bound RAGE (N-truncated form, ΔN RAGE), dominant-negative RAGE (DN RAGE), endogenous secretary RAGE (esRAGE), and cleaved RAGE (cRAGE) (Reproduced with permission from Han et al., 2011).

1.3.1 RAGE and diabetic complications

RAGE plays an important role in the development of diabetic complications such as nephropathy, retinopathy, neuropathy, and macrovascular diseases (Ramasamy et al., 2005; Yamaguchi et al., 2009; Chen et al., 2012; Kanasaki et al., 2013; Manigrasso et al., 2014).

In the kidneys, for example, RAGE is expressed in a number of cell types, such as podocytes and endothelial cells (Manigrasso et al., 2014). Diabetes-induced AGEs accumulation is more prominent in the kidney since it is a major site of AGEs clearance (Manigrasso et al., 2014). In addition, STZ-induced hyperglycemia upregulates renal expression of HMGB1 in glomerular and tubular epithelial cells in parallel with increased RAGE expression (Kim et al., 2011). The appearance of another RAGE ligand, S100, in the podocyte was associated with more severe clinical and pathological indices of diabetic nephropathy (Yamaguchi et al., 2009). Engagement of RAGE with its ligands leads to the generation of ROS and amplifies inflammation (Sanajou et al., 2018). In addition to reducing antioxidant enzymes and cellular glutathione levels, RAGE activation results in the upregulation of nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase), nitric oxide synthase (NOS), and cyclooxygenase (COX), leading to exacerbation of inflammatory responses in the kidneys (Kim et al., 2010; Sanajou et al., 2018). Such chronic inflammatory states are mediated largely by RAGE-induced NF-κB activation that enhances the expression and production of various inflammatory cytokines, chemokines, and adhesion molecules in the diabetic kidneys (Kanasaki et al., 2013). RAGE deletion was beneficial in delaying the progression of diabetic renal disease (Manigrasso et al., 2014).

In concert with the abovementioned findings, RAGE and its ligands AGEs, HMGB1, and S100 showed significant upregulation in the diabetic eye, thereby implicating RAGE as a major player in the development of diabetic retinopathy (Manigrasso et al., 2014). Interaction of RAGE with its ligands in the diabetic retina induces inflammatory responses through the MAPKs-NF-κB pathway, thereby leading to the disruption of the retinal vascular barrier (Mohammad et al., 2013). Inhibition of RAGE was able to block the development of certain lesions of diabetic retinopathy (Chen et al., 2012).

RAGE expression is also increased in peripheral neurons in diabetic neuropathy (Juranek et al., 2013), and lack of its expression prevented cellular changes in autonomic (Chandna et al, 2015a)

and sensory (Lam et al., 2018) neurons. RAGE-induced oxidative stress and activation of the polyol pathway was associated with dysregulation of PKC and (Na^+, K^+)-ATPase activity and subsequent impairment in nerve function (Wada and Yagihashi 2005). Similarly, diabetes-induced endothelial injury in the peripheral nervous tissues was mediated through the AGE-RAGE axis and the involvement of MAPKs, Ras homologous (Rho) GTPases, phosphoinositol-3 kinase (PI3K), and Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathways, finally resulting in NF-κB activation and subsequent expression of numerous pro-inflammatory molecules; in addition, many of these pathways lead to the generation of ROS by NADPH oxidase, thereby exacerbating the development of micro- and macrovascular complications of diabetes (Wautier et al., 2001; Wada and Yagihashi 2005; Daroux et al., 2012). The activation of precise RAGE-mediated signaling pathways thus appears to be dependent on the cell type as well as the degree of the cellular stress (Daffu et al, 2013).

1.3.1.1 RAGE-induced oxidative stress in diabetic complications

In almost all of the complications mentioned above, RAGE effects were largely mediated through oxidative stress-induced inflammation via stress signaling-NF-κB pathways. AGE formation via the Millard reaction, polyol pathway, and glucose autoxidation produce a large amount of oxidants (Yagihashi et al., 2011). ROS themselves may fuel further generation of AGEs, thereby initiating a vicious cycle of oxidative stress (Ramasamy et al., 2005). In addition, RAGE-mediated activation of membrane-associated NADPH oxidase generates ROS, which along with the ROS continuously generated as by-product of ATP biosynthesis, exacerbate oxidative stress production and consumption of the antioxidant defenses (Daffu et al, 2013). Consistent with this, AGEs mediate decreased glutathione stores and reduced activity of superoxide dismutase (SOD) and catalase (Obrosova 2002; Jiang et al., 2004; Ramasamy et al., 2005). AGEs also stimulate the recruitment of RAGE-expressing inflammatory cells and the release of other RAGE ligands, such as HMGB1 and S100. Therefore, other ligands can amplify RAGE activation and contribute to further cellular stress (Daffu et al, 2013). One of the most common downstream consequences of almost all these pathways is the activation of NF-κB (Ramasamy et al., 2005). In addition to mediating the inflammatory responses, NF-κB leads to the upregulation of RAGE, as the RAGE promoter

contains two NF-κB responsive elements, thereby forming a positive feedback loop that exacerbates inflammation and tissue injury (Li et al., 2012).

1.3.1.1.1 RAGE and the MAPKs pathway

As mentioned in the previous sections, there are several stress-activated signaling pathways downstream from the RAGE-ligand interaction. The MAPKs signaling cascade is one of those pathways. The MAPK family, which consists of three major members, i.e. extracellular-regulated kinase (ERK), p38 MAPK, and JNK, is a group of serine/threonine protein kinases that mediate proliferation, differentiation, and cell survival in mammalian cells by responding to extracellular signals such as growth factors, mitogens, and cellular stress (Morrison 2012). MAPKs are not only a well-established downstream signaling molecules of RAGE activation (Yeh et al., 2001), but are also involved in cognitive function, synaptic plasticity and glutamate receptor trafficking (Thomas and Huganir 2004; Gu and Stornetta 2007; Morel et al., 2018). The MAPK signaling cascade plays an important role in NMDA receptor-dependent and independent LTP as well as in the metabotropic glutamate receptors (mGluRs)- and NMDA receptor-induced LTD in the hippocampus (Li et al., 2007; Izumi et al., 2008; Corrêa and Eales 2012). In addition, MAPK signaling cascades are strongly implicated in the control of AMPA receptor trafficking during synaptic plasticity (Zhu et al., 2002 and 2005; Huang et al., 2004; Krapivinsky et al., 2004; Boudreau et al., 2007). Changes in ERK, p38 and JNK expression and phosphorylation/activity are widely reported in models of diabetes (Purves et al., 2001; Chen et al., 2005; Jing et al., 2013; Liu et al. 2016; Dalli et al., 2018), which could contribute to the changes in glutamate receptor expression and function, synaptic plasticity, and cognitive function in diabetes. Oxidative stress is one of the important upstream mediators of MAPKs signaling (Son et al., 2013). Increase in oxidative stress, exacerbated by RAGE-ligands binding, is shown to phosphorylate and activate various protein kinases in the MAPK signaling pathway (Sharma et al., 2010), which is required for the NF-κB activation and the subsequent inflammatory responses and tissue injury (Yeh et al., 2001) that happens in the diabetic condition.

1.3.1.2 RAGE and CNS complications of diabetes

The physiological functions of RAGE in the CNS remain largely unknown, with few reports indicating RAGE involvement in neurite outgrowth and neuronal differentiation during development (Wang et al., 2008), although its expression decreases significantly upon cellular differentiation (Kim et al., 2012). RAGE is normally expressed at low levels in adult tissues, but it is significantly upregulated and activated in a wide variety of cell types under pathological conditions, especially at sites where its ligands accumulate (Sorci et al., 2013; Lee and Park 2013). The degree of RAGE activation under pathological conditions and its subsequent adverse effects seem to be highly dependent on the type of ligand, downstream signaling pathways, and the cell type (Sorci et al., 2013).

RAGE activation within the adult nervous system is shown to mediate neuronal damage and dysfunction due to the overproduction of ROS, cytokines and pro-inflammatory molecules, as discussed earlier, and therefore it is involved in the pathogenesis of a number of CNS disorders such as Parkinson's, Huntington's and Alzheimer's diseases (Piras et al., 2016).

Since Aβ is a RAGE ligand, significant research has concentrated on the role of RAGE in synaptic dysfunction and cognitive impairments in AD-like models, but much less in known about the role of RAGE in the CNS complications of diabetes. In the context of AD, for example, AGE-induced AD models showed impairments in LTP and memory tasks that were significantly attenuated by RAGE blockade (Zhou 2011; Tan et al., 2015). Similarly, hippocampal neurons and brain slices treated with AGEs showed decreased synaptic density and impaired hippocampal LTP; effects that were largely reduced by genetic RAGE depletion (Zhang et al, 2014). In the context of diabetes, long-term diabetes (4 to 8 months) increased expression of RAGE in neurons and glial cells and impaired cognitive function (Toth et al., 2006). However, whether/how RAGE led to such cognitive impairment remained to be investigated.

1.4 Rationale and hypothesis

RAGE is strongly implicated in the pathogenesis of diabetes. Although the role of RAGE is extensively investigated in micro- and macrovascular complication of diabetes, especially nephropathy, retinopathy and neuropathy, less is known about the role of RAGE in glucose neurotoxicity, as well as in CNS complications of diabetes. In particular, the role of RAGE on hippocampal dysfunction, such as impairment in synaptic plasticity and cognitive function, which are widely reported in diabetes, remained to be investigated.

We, therefore, hypothesize the following:

1) RAGE signaling underlies cognitive dysfunction in a mouse model of STZ-induced diabetes (Chapter 2).

2) RAGE signaling alters expression and function of glutamate receptors in high glucose or STZ-induced diabetic conditions, leading to subsequent impairment in hippocampal synaptic transmission (Chapter 3).

The main body of the thesis is written in a “manuscript” style, comprising of two papers (Chapter 2 and Chapter 3) that have been submitted for publication to peer-reviewed journals.

CHAPTER 2

Diabetes induces RAGE-dependent hippocampal spatial memory impairments

2.1 Abstract

Diabetes is a prevalent metabolic disorder that has long been associated with changes in different regions of the brain including the hippocampus. Changes in hippocampal synaptic plasticity and subsequent impairment in cognitive functions, such as learning and memory, are well documented in animal models of type 1 and type 2 diabetes. It is known that the receptor for advanced glycation end products (RAGE) contributes to micro- and macrovascular complications of diabetes. However, it is still unknown if RAGE plays a similar role in the development of central nervous system (CNS) complications of diabetes. Therefore, we hypothesize that RAGE signaling under diabetic condition underlies cognitive dysfunction such as learning and memory impairments in a mouse model of STZ-induced diabetes. Control and streptozotocin (STZ)-induced diabetic mice from wild-type (WT) and RAGE-KO groups were used for the behavioural experiments. While STZ-induced diabetes decreased locomotor activity in the open field (OF) test, it did not affect the recognition memory in the novel object recognition (NOR) test in either genotype. Spatial memory, however, was impaired in STZ-induced diabetic mice in WT but not in RAGE-KO group in both the Barnes maze (BM) and the Morris water maze (MWM) tests. Our findings indicate that the parameters associated with locomotor activity and recognition memory were independent of RAGE expression in STZ-induced diabetic mice. In contrast, the parameters associated with hippocampal-dependent spatial memory were dependent on RAGE expression/signaling.

Momeni Z, Kiir TAB, Yamamoto Y, Bekar LK, Campanucci VA. Diabetes induces RAGE-dependent hippocampal spatial memory impairments. The work in this chapter has been submitted to Physiology and Behavior.

Contribution: Momeni Z was responsible for performing all experiments, data analysis, and preparation of the manuscript. Kiir helped in the collecting behaviour data; Yamamoto provided the RAGE-KO mice and edited the manuscript; Bekar provided access to behavior equipment/software and helped with data analysis.

2.2 Introduction

Diabetes is a common metabolic disorder characterized by hyperglycemia due to deficient insulin production (type 1) or resistance to insulin action (type 2) or both (Kharroubi and Darwish, 2015). Both types of diabetes are associated with chronic complications such as retinopathy, nephropathy, angiopathy, and neuropathy (Muriach et al., 2014). Central neuropathy – the effect of diabetes on the CNS – manifests as structural and functional alterations in the brain and spinal cord (Selvarajah 2006; Muriach et al., 2014). Some of these changes are reported in different regions of the brain including the hippocampus (Wrighten et al., 2009; Hernández-Fonseca et al., 2009; Seaquist 2010; Moheet et al., 2015). A large body of evidence collected from rodent models of diabetes show electrophysiological abnormalities in the hippocampus, as well as abnormal hippocampal-dependent behaviours, especially tasks associated with learning and memory and cognitive function (Wrighten et al., 2009; Rostami et al., 2013, Saedi et al., 2016). Studies performed on rodent models of type 1 diabetes showed dysfunctional synaptic plasticity, such as impaired long-term potentiation (LTP) and long-term depression (LTD) (Artola et al., 2005; Kamal et al., 2006) and subsequent impairments in spatial memory tasks (Biessels et al., 1998). In addition, type 2 diabetes is considered as a strong risk factor for the development of Alzheimer's disease (AD), which is characterized by progressive decline of cognitive functions and learning and memory impairments (Jia et al., 2017).

In the search of mechanisms underlying such diabetes-induced cognitive decline, a great attention has been paid to RAGE, a member of the immunoglobulin protein family of cell surface molecules (Schmidt et al., 2001; Kodl and Seaquist, 2008). Hyperglycemia is linked to the formation of advanced glycation end products (AGEs) and the generation of reactive oxygen species (ROS), which are both considered as major contributors to the development of nervous system complications of diabetes (Yamagishi and Matsui, 2010). Interaction of AGEs with their receptor RAGE and the subsequent generation of oxidative stress trigger a cascade of events that induce sustained activation of transcription factor nuclear factor kappa beta (NF-κB), a proinflammatory gene marker, and further upregulation of RAGE and ROS production, a positive feedback loop that intensifies diabetic complications and inflammation-induced tissue injury (Yamagishi and Matsui, 2010).

The involvement of RAGE in cognitive impairments has been well studied in the context of AD (Zhou 2011; Tan et al., 2015; Tan et al., 2015; Lubitz et al., 2016). Exogenous AGEs were shown to induce tau hyperphosphorylation at multiple AD-related brain sites both *in vitro* and *in viv*o and to increase the level of RAGE and impair spatial memory (Zhou 2011; Tan et al., 2015). Similarly, poorer memory and higher hippocampal levels of amyloid beta (Aβ) and RAGE were observed in AD mice on a high-AGE diet (Lubitz et al., 2016). These effects were attenuated by RAGE blockade (Zhou, 2011; Tan et al., 2015; Hong et al., 2016).

It remains unclear, however, if RAGE plays a similar role in the development of CNS complications of diabetes, particularly cognitive impairments. In the only published study, long-term diabetic mice (18 to 33 weeks diabetic) demonstrated increased expression of RAGE in neurons and glial cells and displayed cognitive dysfunction (Toth et al., 2006). However, whether RAGE led to this cognitive deficit was not investigated.

In addition, although AGEs infusion to the brain, a model used in AD studies, can mimic the diabetic state to some extent, it should be taken into consideration that the pathogenesis of hyperglycemia includes but is not limited to AGEs. Therefore, there is a lack of information regarding RAGE-mediated CNS complications, especially cognitive dysfunction, during a real hyperglycemic state in animal models.

Therefore, in this study, we hypothesize that RAGE signaling during diabetes contributes to cognitive dysfunction in STZ-induced diabetic mice. To test this hypothesis, we took advantage of a constitutive RAGE-KO mouse model along with the WT mice in the behavioural tests. We performed the open field (OF) test to measure locomotor activity and anxiety-like behaviour (Seibenhener and Wooten, 2015), followed by the novel object recognition (NOR) test to evaluate recognition memory (Antunes and Biala, 2012). Despite the widespread use of NOR test in rodents, a strong consensus has not yet developed regarding the brain structures necessary for the task performance. For example, although there is agreement about the role of perirhinal cortex in NOR performance, there is less agreement on the role of hippocampus (Winters et al., 2008; Broadbent et al., 2010; Squire et al., 2010). While some studies show impaired recognition memory after hippocampal lesions (Clark et al., 2000; Zola et al., 2000; Broadbent et al., 2010), others report spared recognition memory following hippocampal damage (Winters et al. 2004; Forwood et al. 2005; Mumby et al. 2005). It seems that the degree and type of lesion

(permanent vs. temporary/reversible), and retention intervals may play roles in such controversies (Broadbent et al., 2004; Albasser et al., 2010; Broadbent et al., 2010).

In addition to the NOR test, Barnes maze and Morris water maze tests are also used to evaluate cognitive functions, especially those related to hippocampal-dependent spatial learning and memory (Vorhees and Williams, 2006; Gawel et al., 2019). However, due to undue stress associated with water in the MWM test (Harrison et al., 2009), we performed both tests to ensure that the results can reflect a more robust and reliable understanding of spatial learning and memory performance in the experimental groups.

Our findings reveal that STZ-induced diabetes reduced locomotor activity in both WT and RAGE-KO groups. Although STZ-induced diabetes did not lead to significant changes in object recognition memory in either genotype, it led to impairment in hippocampal-dependent spatial memory in WT but not in RAGE-KO group. These findings constitute novel evidence for the contribution of RAGE signaling in hippocampal spatial memory impairments during diabetes.

2.3 Experimental Procedures

2.3.1 Mice and Treatments. To maintain a colony of RAGE-KO mice on a C57BL/6 background, heterozygous mice were generated by back-crossing RAGE-KO (homozygous) mice (Myint et al., 2006) with C57BL/6 WT mice, as previously described (Lam et al., 2018). Genomic DNA extraction and polymerase chain reaction were used for genotyping (Myint et al., 2006).
For type 1 diabetes, male mice (4-6 weeks old) received daily intraperitoneal (i.p.) injection of 50 mg STZ/kg body weight for three consecutive days, while age-matched controls received citrate buffer injections. Blood glucose measurements were obtained one week after the injections using a CONTOUR Glucose Meter (Bayer Inc., Toronto, ON, Canada), and animals with blood glucose levels >15 mM glucose were considered diabetic. Diabetic animals were kept for one month prior to the experiments and blood glucose was measured once more at the end of the one-month period.

2.3.2 Behavioural Experiments. Separate groups of mice were used for each behavioural experiment. Mice were brought to the experimental room in their home cages at least half an hour before the behavioural experiments to allow them to acclimate to the test environment. The experimental room was kept at a controlled temperature (22 ± 2°C). Mice were tested in the OF, NOR, BM and MWM. All trials were video-recorded, and the behavioural analysis was carried out with the help of the Noldus Ethovision XT software (Leesburg, VA, USA).

2.3.2.1 Open Field Test. The OF apparatus consisted of a solid gray square shaped (40 × 40 cm) box. The animals were individually put in the center of the OF apparatus, placed in a standard lit room, and were allowed free and uninterrupted movement for 25 minutes. Total time the animals spent in the center, total distance traveled, and total movement time were measured (Seibenhener and Wooten, 2015).

2.3.2.2 Novel Object Recognition Test. In this test, all groups of mice were subjected to three phases*:* habituation, acquisition and testing, as described previously (Lueptow, 2017). For habituation, the mouse was placed into the box and allowed to explore freely for 15 minutes. No objects were placed in the box during the habituation phase. On the following day, the acquisition

trial was conducted in which two copies of the same object were placed in the box arena. Mice were allowed to explore the two objects for 15 minutes and the time spent exploring each object as well as the total exploration time were recorded. After 24 hours, mice were exposed to a different copy of the same object used in the acquisition phase and a novel object (not used in the acquisition phase) and allowed to explore for 15 minutes. The time spent exploring each object and the total exploration time were recorded. The novel object discrimination index was calculated as the time spent exploring the novel object divided by the total time spent exploring both objects.

The choice for novel or familiar object was counterbalanced and the position of each object was alternated between trials.

2.3.2.3 Barnes Maze Test. The BM consisted of a white circular platform, 100 cm in diameter, with 20 equally-spaced holes around the circumference and an escape box fitted under one of the holes, placed in a standard lit room with visual cues in the periphery. After habituation, during which animals were placed on the maze for a 2-minute undisturbed exploration, mice underwent training trials. The sound of a buzzer was used during both acquisition and testing sessions as an aversive stimulus to increase the animal's motivation to escape from the platform during the 2-minute exploration and was turned off as soon as the animal entered the escape hole. If the animal did not find the escape box during the 2-minute exploration, it would be gently guided to the escape box, followed by cessation of the buzzer, and allowed to stay there for 1 minute. Mice underwent two training trials per day with approximately 15-minue intertrial interval for four consecutive days. The amount of time spent to find the escape box (latency) as well as the number of incorrect holes explored (errors), escape velocity, and total distance traveled were measured during the training sessions. In the probe trial, in which the escape box was not present, the time spent in the target quadrant (where the escape box was previously located) was measured. The probe trial was conducted 48 hours after the last training day (day 4). Animals were allowed to explore the maze for 2 minutes during both training and probe sessions (Gawel et al., 2019).

2.3.2.4 Morris Water Maze Test. A circular maze made of white plastic (120 cm diameter, 80 cm height) was two thirds filled with water (22 to 23°C) and made opaque by the addition of a

white non-toxic food coloring. Extra-maze landmark cues that were visible from the maze were used. In this task, as previously described (Bromley-Brits et al., 2011), mice were first subjected to a visible platform phase in which they explored the pool to locate a visible platform. This phase can help to exclude mice with visual or locomotor impairments, as well as to habituate them to the testing conditions. The following day, mice were tested with a hidden platform task. In this phase, a fixed platform was hidden 1 cm below the water surface and was not moved throughout the experiments. The entrance point into the maze was changed every trial (we used 4 release points every day, one for each trial). Mice received four training trials per day, with approximately 15-minue intertrial interval, and were allowed to swim for 60 seconds during each trial. If the animal did not find the platform during the 60-second period, it would be gently guided to the platform and allowed to stay on it for 20 seconds. The training sessions were performed for four consecutive days and latency to reach the platform, swimming distance and swimming velocity were measured. The probe trial was conducted 48 hours after the last training day (day 4). In this phase, the platform was removed and mice were allowed to swim for 60 seconds and the time spent in the target quadrant (where the platform was previously located) was measured to evaluate memory retention.

2.3.3 Statistical Analysis. All values are reported as mean ± SEM and the level of significance was set at 0.05 for all statistical tests performed. For statistical comparisons, we used two-way analysis of variance (ANOVA), three-way repeated measures ANOVA, and one-sample t-test as indicated in the figure legends. The F values, p values and t values are reported in the figure legends. Statistical analyses were carried out with InStat 3.0 or Prism 8.0 (GraphPad Software Inc., La Jolla, CA, USA).

This work was approved by the University of Saskatchewan's Animal Research Ethics Board (Campanucci: protocol 20090082) and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

2.4 Results

2.4.1 Induction of diabetes by STZ injection in mice. No significant difference in body weight was found between the groups before citrate buffer/STZ treatment, nor in the blood glucose level (Figure 2.1). Although both control and STZ-treated mice showed increases in body weight over time, STZ-treated mice displayed significant decreases in body weight gain as compared with aged-matched controls (Figure 2.1 A). After STZ injection, mice were severely diabetic as indicated by elevated blood glucose levels compared with age-matched controls (Figure 2.1 B).

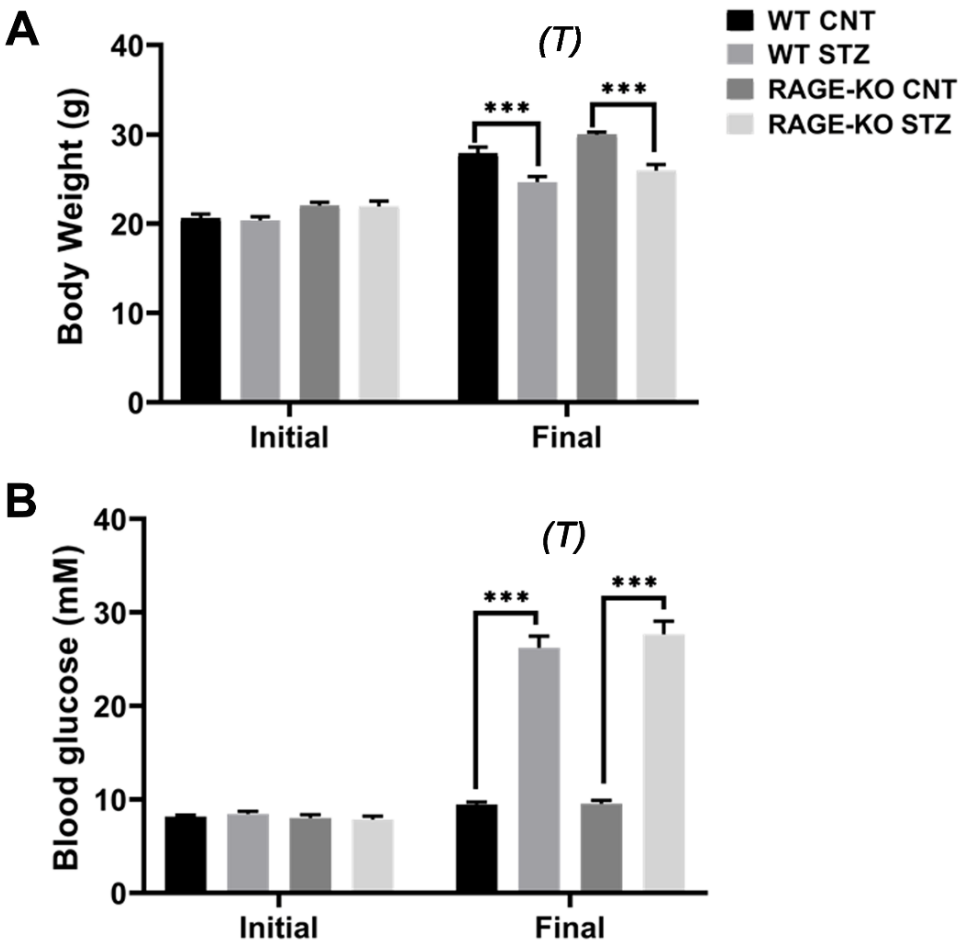


Figure 2.1. Body weights and glycemic values of control and STZ-induced diabetic mice. Bar graphs summarize the mean ± SEM values of initial and final **(A)** body weights and **(B)** blood glucose concentrations in control and STZ-induced diabetic mice from WT and RAGE-KO groups. "Initial" refers to before STZ or citrate buffer injection and "final" refers to 5 weeks after the injections. Values are expressed as the mean ± SEM from WT CNT (n=23), WT STZ (n=28), RAGE-KO CNT (n=20), and RAGE-KO STZ (n=25). Means were statistically compared by three-way repeated measures ANOVA, followed by Tukey's multiple comparison test; ***, $p<0.001$. All groups showed significant differences between their final and their initial body weight values (A, $p<0.001$). There was no significant difference between initial and final blood glucose values in control mice from both genotypes (B). *(T)*: significant main effect of STZ treatment ($F_{(1, 92)}$=18.44, $p<0.001$ in A and $F_{(1, 92)}$=240, $p<0.001$ in B). There was also a significant main effect of time ($F_{(1, 92)}$=321.9, $p<0.001$ in A and $F_{(1, 92)}$=378.8, $p<0.001$ in B).

2.4.2 STZ-induced diabetic mice showed lower locomotor activity as compared with controls. The OF test was used to measure locomotor activity and anxiety-like behaviours in STZ-induced diabetic mice and age-matched controls based on the conflict between a rodent's natural aversion to open areas and its willingness to explore new areas (Schmitt and Hiemke, 1998).

Our findings show a significant main effect of STZ treatment on the time spent in the center (Figure 2.2 A), distance traveled (Figure 2.2 B) and total movement time (Figure 2.2 C) in the OF test. STZ-induced diabetic mice in both genotypes showed significant reduction in total distance traveled and total movement time as compared to their age-matched controls (Figure 2.2 B-C). There was a significant genotype difference between WT and RAGE-KO mice in the total time spent in the center of the OF (Figure 2.2 A).

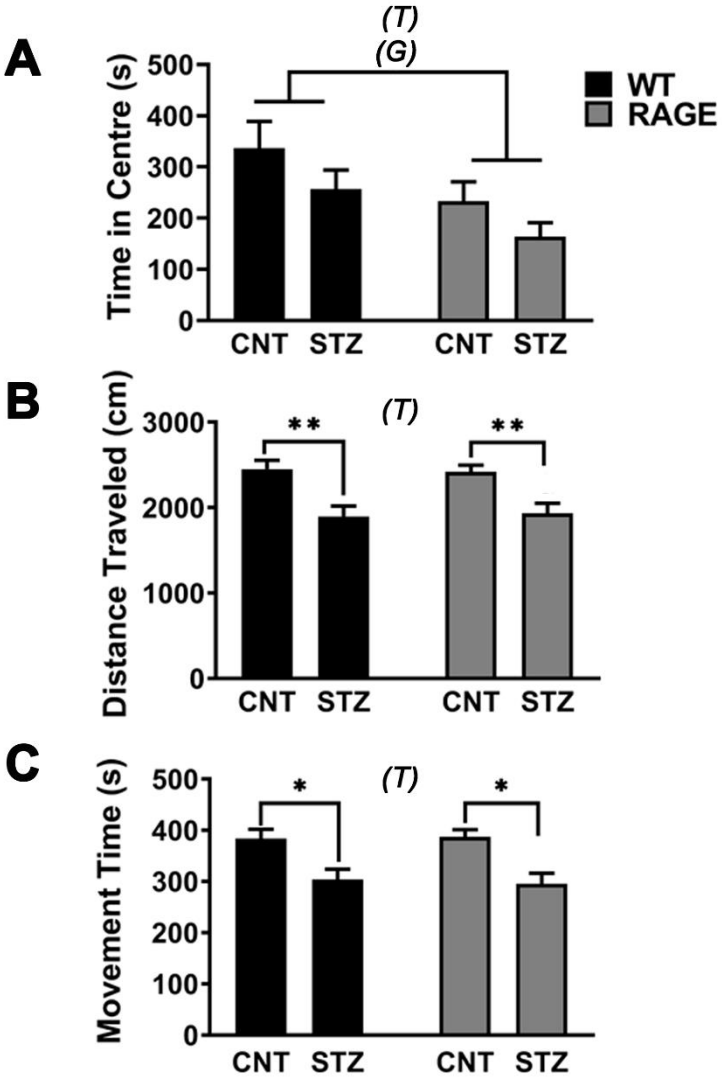


Figure 2.2. Behavioural parameters in the open field test. (A) Total time the animal spent in the center, **(B)** total distance traveled in the maze and **(C)** total movement time were measured to determine locomotor activity and anxiety-like behaviour in control and STZ-induced diabetic mice. Values are expressed as the mean ± SEM from WT CNT (n=21), WT STZ (n=25), RAGE-KO CNT (n=25), and RAGE-KO STZ (n=30). Means were statistically compared by two-way ANOVA, followed by Tukey's multiple comparison test; *, $p<0.05$; **, $p<0.01$. *(G)*: significant genotype differences ($F_{(1, 97)}=6.01$, $p<0.05$ in A); *(T)*: significant main effect of STZ treatment ($F_{(1, 97)}=4.19$, $p<0.05$ in A; $F_{(1, 97)}=23.32$, $p<0.001$ in B; $F_{(1, 97)}=20.36$, $p<0.001$ in C).

2.4.3 Recognition memory was not affected by STZ-induced diabetes. The NOR test was used to assess recognition memory (Broadbent et al., 2010). Our findings show no significant differences in the time spent exploring the two objects during the acquisition phase (Figure 2.3 A). There was, however, a significant genotype difference in total exploration time in the acquisition phase (Figure 2.3 A). On the testing phase, although all groups explored the novel object for a longer period (Figure 2.3 B), the novel object discrimination index was not significantly different between control and STZ-induced diabetic mice in either WT or RAGE-KO group or between genotypes (Figure 2.3 C). All groups, however, performed above chance (50%) during the testing phase (Figure 2.3 C).

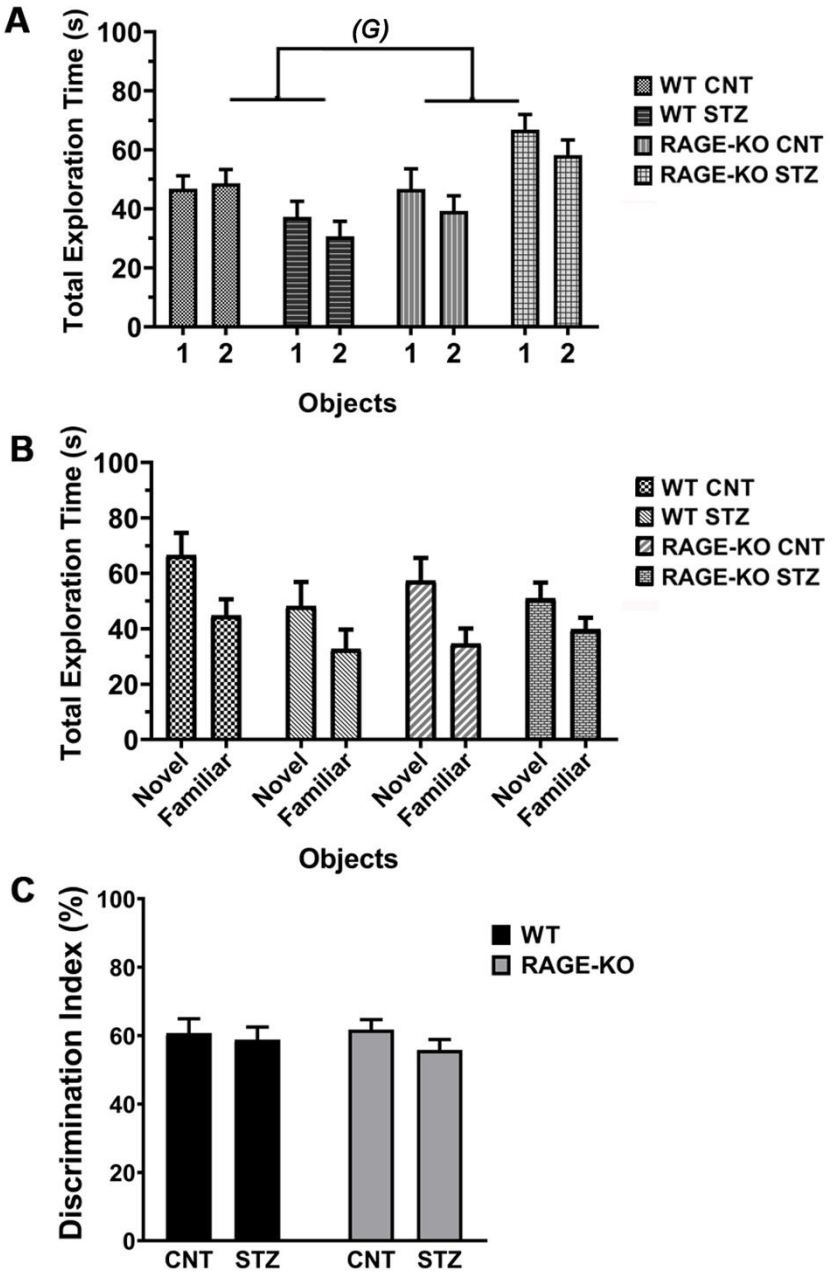


Figure 2.3. The novel object recognition test. Objects exploration during the **(A)** acquisition and **(B)** testing day**.** 1 and 2 in A show the two copies of the same object used during the acquisition phase. **(C)** The discrimination index was calculated as the time spent exploring the novel object divided by the total time spent exploring both objects. Values are expressed as the mean ± SEM from WT CNT (n=20), WT STZ (n=18), RAGE-KO CNT (n=23), and RAGE-KO STZ (n=28). Means were statistically compared by two-way ANOVA, followed by Tukey's multiple comparison test. *(G)*: significant genotype differences ($F_{(3, 170)}$=9.9, *p<0.001* in A). There was a significant difference from the chance in WT CNT ($t_{(19)}$= 2.66; p<0.05), WT STZ ($t_{(17)}$= 2.37; p<0.05), RAGE-KO CNT ($t_{(22)}$= 4.03; p<0.01) and RAGE-KO STZ ($t_{(27)}$=2.05; p<0.05) by one sample t-test in C.

2.4.4 WT STZ-induced diabetic mice showed impairment in hippocampal-dependent spatial memory. The BM test was used to evaluate hippocampal-dependent spatial learning and memory (Gawel et al., 2019). During the acquisition phase of BM (Figure 2.4), there was a significant main effect of STZ treatment on escape latency and velocity, as well as a significant difference between the two genotypes in escape latency, number of errors, and velocity (Figure 2.4 A, C-D).

On the probe day (Figure 2.5), there was a significant interaction effect between genotype and STZ treatment in the percent of time spent in the target quadrant, meaning that the effect of STZ on the time spent in the target quadrant differed significantly between the two genotypes (Figure 2.5 A). Consistent with this, a significant decrease in the percent of time spent in the target quadrant was found in STZ-induced diabetic mice as compared with control in WT, but not in RAGE-KO group (Figure 2.5 A). The distance traveled and velocity showed significant decreases in STZ-induced diabetic mice from both genotypes (Figure 2.5 B-C).

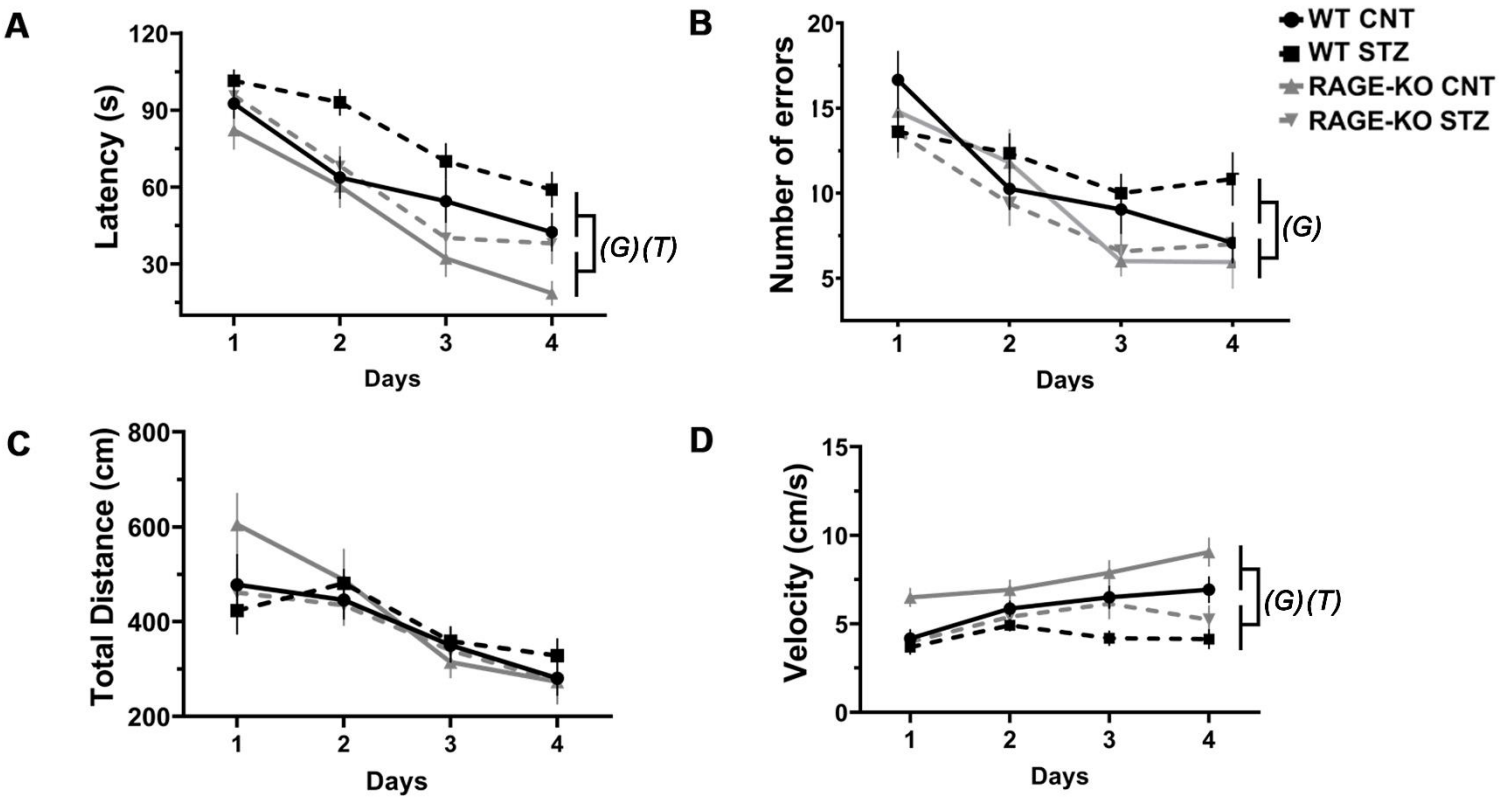


Figure 2.4. Behavioural parameters during the acquisition phase of Barnes maze test. (A) Latency to enter the escape hole, (**B**) number of errors committed before entering the escape hole, **(C)** total distance traveled and **(D)** escape velocity in control and STZ-induced diabetic mice from WT and RAGE-KO groups during the four days of training. Mice received two trials per day. Values are expressed as the mean ± SEM from WT CNT (n=13), WT STZ (n=14), RAGE-KO CNT (n=11), and RAGE-KO STZ (n=11). Means were statistically compared by repeated measures of three-way ANOVA. *(G)*: significant genotype differences ($F_{(1, 94)}$=18.87, *p<0.001* in A; $F_{(1, 94)}$=6.16, *p<0.05* in B; $F_{(1, 94)}$=14.4, *p<0.001* in D); *(T)*: significant main effect of STZ treatment ($F_{(1, 94)}$=14.21, *p<0.001* in A; $F_{(1, 94)}$=31.96, *p<0.001* in D). There was also a significant main effect of time ($F_{(2.957, 278.0)}$=49.33, *p<0.001* in A; $F_{(2.889, 271.5)}$=21.83 *p<0.001* in B; $F_{(2.507, 235.6)}$=18.15, *p<0.001* in C; $F_{(2.671, 251.1)}$=7.77, *p<0.001* in D).

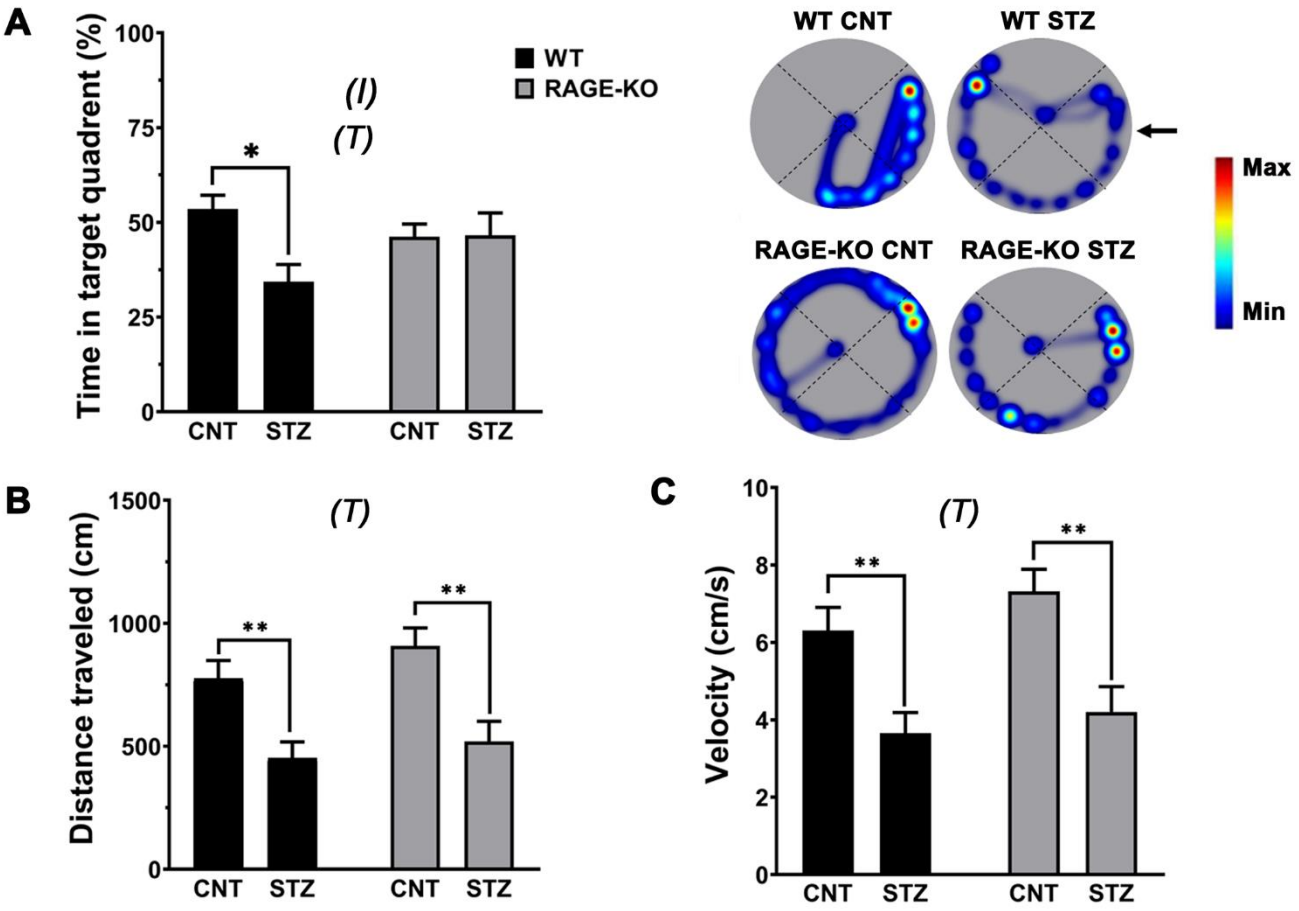


Figure 2.5. Behavioural parameters during the probe trial of Barnes maze test. (A) Percent of total time spent in the target quadrant and representative heat maps of the path traveled by CTN and STZ-induced diabetic mice from WT and RAGE-KO groups during the probe trial. The arrow shows the target quadrant (where the drop box was previously located) and the color scale represents the minimum and maximum time spent in a given area. **(B)** Total distance traveled and **(C)** velocity during the 2 min exploration period. Probe trial was conducted 48 hr after the last training day. Values are expressed as the mean ± SEM from WT CNT (n=13), WT STZ (n=14), RAGE-KO CNT (n=11), and RAGE-KO STZ (n=11). Means were statistically compared by two-way ANOVA, followed by Tukey's multiple comparison test; *, $p<0.05$; **, $p<0.01$. *(T)*: significant main effect of STZ treatment ($F_{(1, 45)}=4.52$, $p<0.05$ in A; $F_{(1, 45)}=24$, $p<0.001$ in B; $F_{(1, 45)}=23.44$, $p<0.001$ in C); *(I)*: significant interaction between genotype and STZ treatment ($F_{(1, 45)}=4.81$, $p<0.05$ in A).

2.4.5 WT STZ-induced diabetic mice showed spatial memory impairment in MWM. Similar to the BM, MWM was used to assess hippocampal-dependant spatial learning and memory (Vorhees and Williams, 2006). Our data showed no significant difference in latency, distance traveled and velocity between control and STZ-induced diabetic mice in either genotype during the habituation (visible platform) phase of MWM (Figure 2.6). There was, however, a significant main effect of STZ treatment on swimming velocity (Figure 2.6 C).

During the acquisition (hidden platform) phase of MWM (Figure 2.7), there was a significant main effect of STZ treatment on latency (Figure 2.7 A) and a significant difference between the two genotypes in latency and swimming velocity (Figure 2.7 A and C).

On the probe trial (Figure 2.8), there was a significant interaction effect between genotype and STZ treatment in the percent of time spent in the target quadrant, indicating that the effect of STZ on the time spent in the target quadrant differed significantly between the two genotypes (Figure 2.8 A). Distance traveled and swimming velocity showed significant differences between the two genotypes (Figure 2.8 B-C).

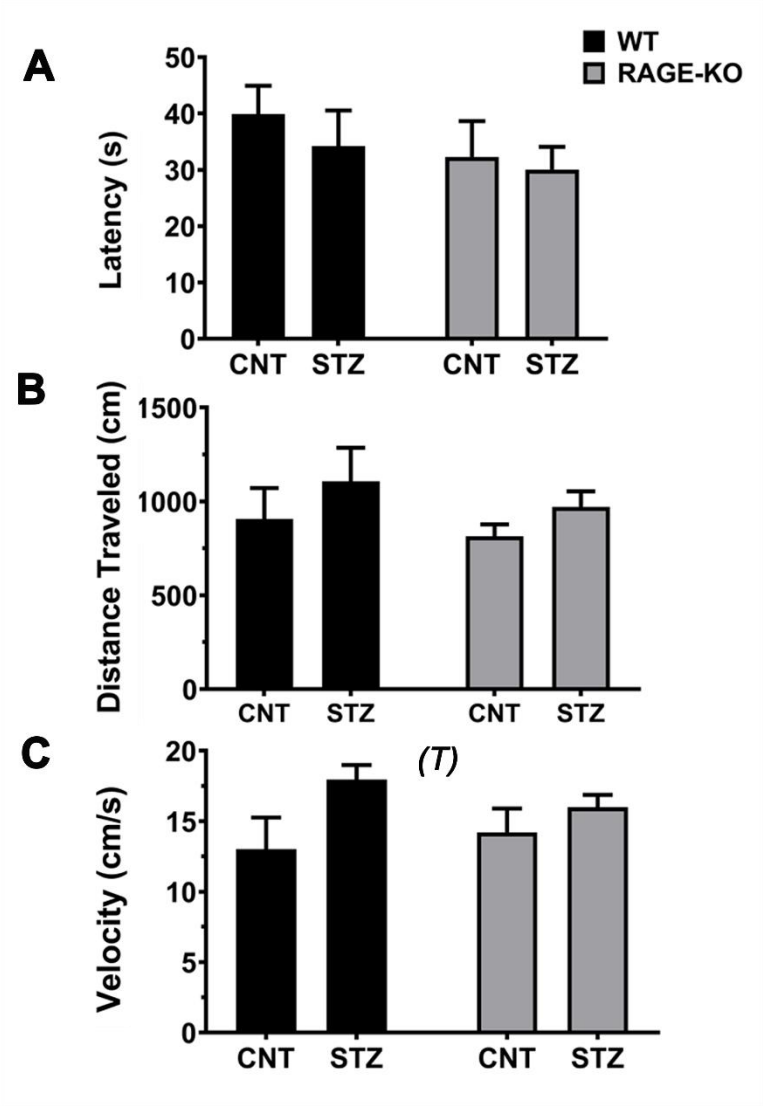


Figure 2.6. Behavioural parameters during the visible platform phase of Morris water maze. (A) Latency to find the platform, **(B)** total distance traveled and **(C)** swimming velocity in control and STZ-induced diabetic mice from WT and RAGE-KO groups. Values are expressed as the mean ± SEM from WT CNT (n=13), WT STZ (n=10), RAGE-KO CNT (n=10), and RAGE-KO STZ (n=10). Means were statistically compared by two-way ANOVA, followed by Tukey's multiple comparison test. *(T)*: significant main effect of STZ treatment ($F_{(1, 39)}$=4.14, $p<0.05$ in C).

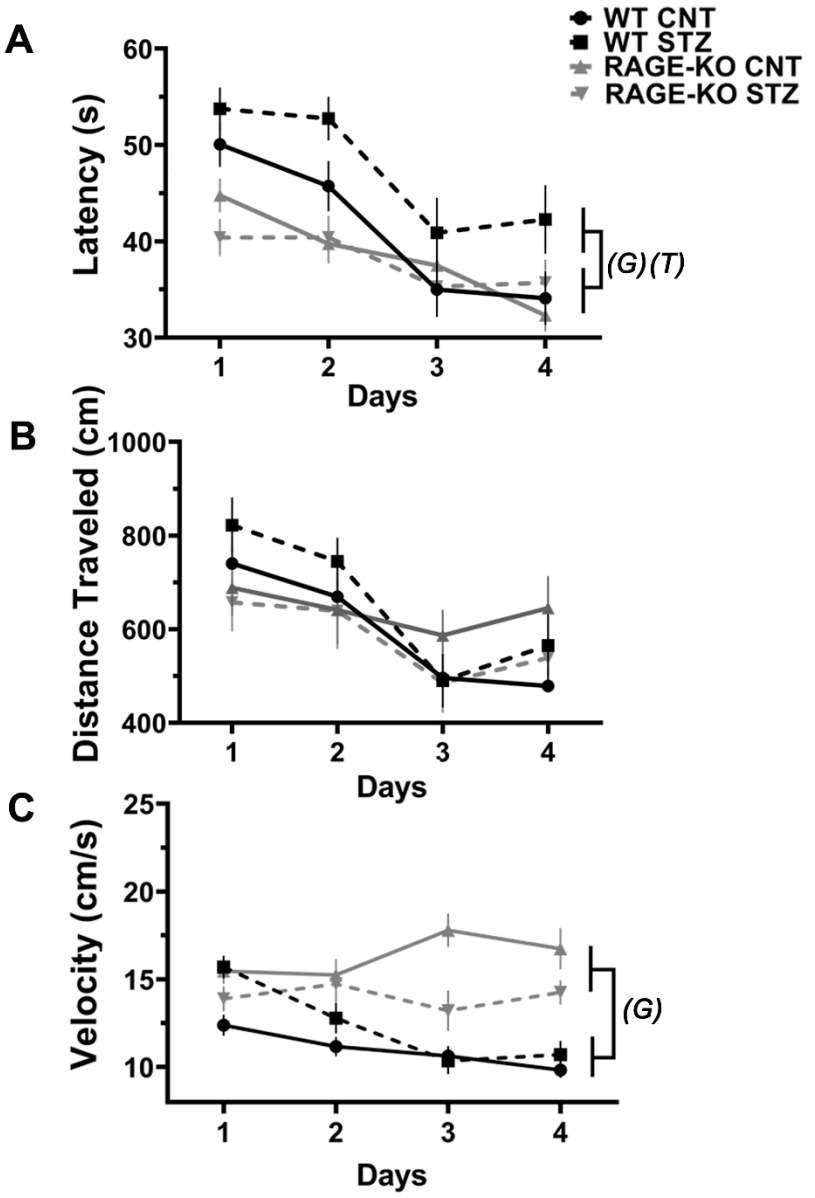


Figure 2.7. Behavioural parameters during the acquisition phase of Morris water maze. **(A)** Latency to find the platform, **(B)** total distance traveled, and **(C)** swimming velocity in control and STZ-induced diabetic mice from WT and RAGE-KO groups during the four days of training. Mice received four trials per day. Values are expressed as the mean ± SEM from WT CNT (n=13), WT STZ (n=10), RAGE-KO CNT (n=10), and RAGE-KO STZ (n=10). Means were statistically compared by three-way repeated measures ANOVA. *(G)*: significant genotype differences ($F_{(1, 168)}$=19.8, *p<0.001* in A; $F_{(1, 168)}$=76.43, *p<0.001* in C); *(T)*: significant main effect of STZ treatment ($F_{(1, 168)}$=4.46, *p<0.05* in A). There was also a significant main effect of time ($F_{(2.918, 490.3)}$=20.74, *p<0.001* in A; $F_{(2.861, 480.7)}$=13.46, *p<0.001* in B).

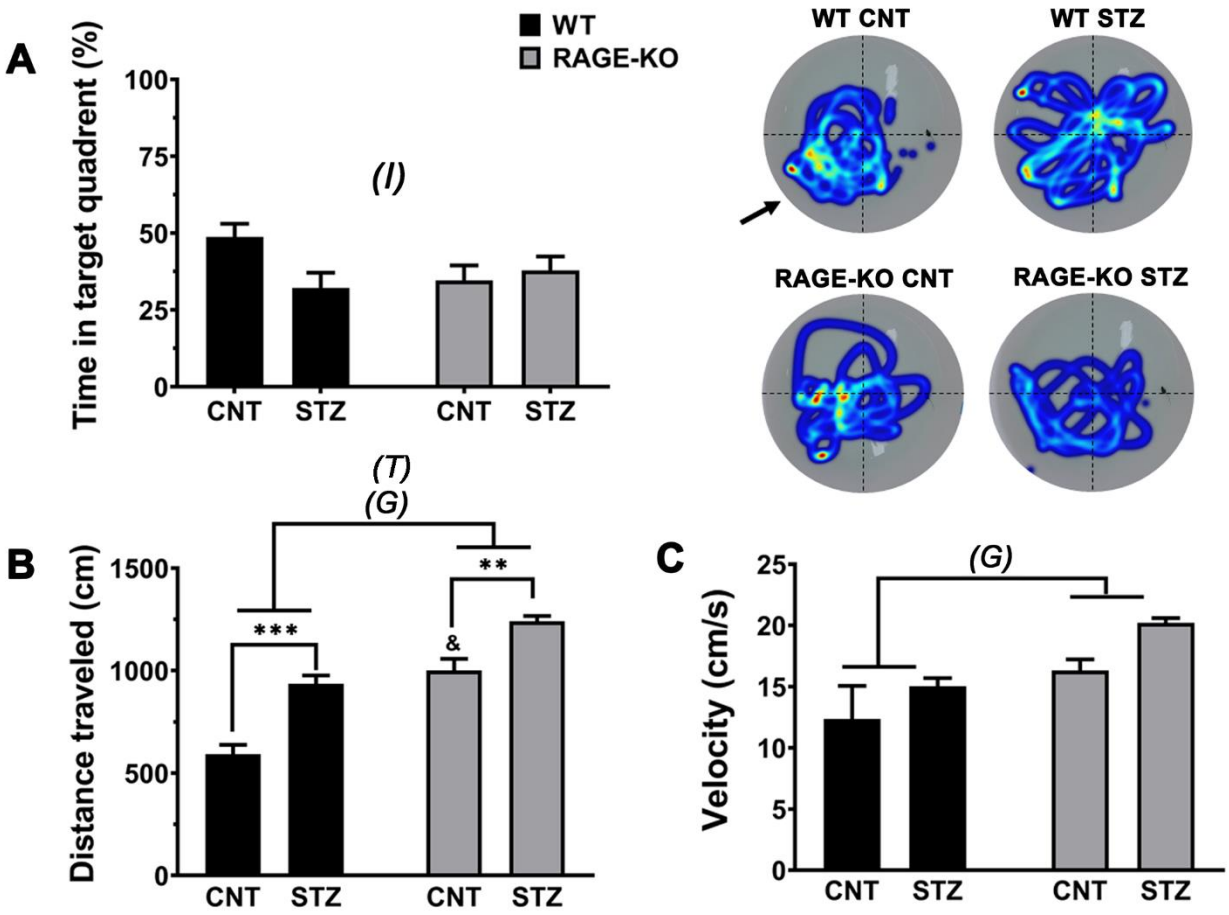


Figure 2.8. Behavioural parameters during the probe trial of Morris water maze. (A) Percent of total time spent in the target quadrant and representative heat maps of the path traveled by CTN and STZ-induced diabetic mice from WT and RAGE-KO groups during the probe trial of the MWM. The arrow shows the target quadrant (where the platform was previously located). **(B)** Total distance traveled and **(C)** velocity during the 2 min exploration period. The probe trial was conducted 48 hr after the last training day. Values are expressed as the mean ± SEM from WT CNT (n=13), WT STZ (n=10), RAGE-KO CNT (n=10), and RAGE-KO STZ (n=10). Means were statistically compared with two-way ANOVA, followed by Tukey's multiple comparison test; **, *p<0.01;* ***, *p<0.001; (T)*: significant main effect of STZ treatment ($F_{(1, 39)}$=38.31, *p<0.05* in B); *(I)*: significant interaction between genotype and STZ treatment ($F_{(1, 39)}$=4.52, *p<0.05* in A); *(G)*: significant genotype differences ($F_{(1, 39)}$=57.63, *p<0.001* in B; $F_{(1, 39)}$=6.79, *p<0.05* in C).

2.5 Discussion

In this study, we provide novel evidence for the involvement of RAGE in hippocampal spatial memory impairments under STZ-induced diabetic conditions.

2.5.1 Anxiety-like behaviour and locomotor activity in STZ-induced diabetic mice

We used the OF test for anxiety-like behaviour, by quantifying the animals' preference to avoid the center of the field in an unknown environment (Choleris et al., 2001). In this regard, STZ is shown to induce anxiogenic activity. For instance, STZ-induced diabetic rats showed more anxiogenic activity in comparison to non-diabetic rats (Ramanathan et al., 1998), by spending less time in central arena of the OF and more time in the closed arms in the elevated plus maze (EPM) (Aksu et al., 2012). Anxiety-like behaviours are also shown in STZ-induced diabetic rodents subjected to social interaction and zero maze tests (Gupta et al., 2014; Damian et al., 2014; Chu et al., 2017). Changes in neurotransmitters, such as gamma-aminobutyric acid (GABA) and monoamines in different brain regions and increases in the hypothalamic–pituitary–adrenal axis (HPA) activity and glucocorticoid levels are shown to contribute to diabetes-associated anxiety in many studies (Ramanathan et al., 1998; Duarte et al., 2000; Gomez et al., 2003; Antony et al., 2010; Kumar et al., 2010; Shpakov et al., 2011; Torres et al., 2013; Gupta et al., 2014). In our study, STZ treatment caused a significantly decreased time spent in the center of the OF, which may indicate an increase in anxiety-like behaviour. However, a significant decrease in locomotor activity, and not necessarily a change in anxiety-like behaviour, might have contributed to the decrease in the time spent in the centre of the OF in our study.

Our findings also showed a significant genotype difference in the time spent in the centre of the OF. Although a previous study showed no significant difference between WT and RAGE-KO mice in anxiety-like behaviour in the OF test (Sakatani et al., 2009), the two by two factorial design, i.e. intervention of an additional factor (STZ) in our experimental design, might explain discrepancy between the two studies.

Our results also demonstrated a significant effect of STZ treatment on decreased distance traveled and movement time in STZ-induced diabetic mice in both genotypes in the OF test, which is consistent with other studies that reported a significant reduction in locomotor activity, exploratory

behaviour, and distance traveled in the OF, passive avoidance, NOR, and the activity cage tests in response to STZ-induced diabetes (Mayer et al., 1990; Haider et al., 2013; Bădescu et al., 2016b; Bensaoula et al., 2016; Patel et al., 2016; El-Marasy et al., 2017). Hyperglycemia-induced decreases in striatal cholinergic receptor expression and function as well as glutamate toxicity and cellular damage in the cerebellum were associated with impaired locomotor activity in STZ-induced diabetes (Sherin et al., 2012; Nagayach et al., 2014).

Since our findings show similar trends in the parameters associated with locomotor activity in both WT and RAGE-KO groups, as reported previously (Sakatani et al., 2009), we suggest that these parameters are independent of RAGE expression/signaling.

2.5.2 Novel object recognition memory

In rodents, the NOR test has been widely used for assessing recognition memory (Broadbent et al., 2009) based on the tendency of the animal to spend more time exploring a novel object than a familiar one (Lueptow, 2017). STZ-induced diabetes from 8 to 10 weeks showed a recognition memory deficit in the NOR test (King et al., 2013; Jabbarpour et al., 2014; Patel et al., 2016). The results of our 5-week STZ-induction of diabetes in mice performing the NOR test, however, showed no significant difference between control and STZ-induced diabetic mice in either WT or RAGE-KO group in the novel object discrimination index during either 5 min or 15 min exploration.

As mentioned earlier, reports are controversial regarding the role of the hippocampus in recognition memory. Although hippocampus is suggested to play an important role in recognition memory encoding and consolidation (Cohen et al., 2013), other brain regions, including the perirhinal regions, are reported to play more important and/or more direct roles (Winters et al., 2008). It is suggested that the hippocampus is involved in object recognition when spatial or contextual components are involved in the task (Winters et al. 2004; Forwood et al. 2005). It is also suggested that the hippocampus may play a time-limited role in object recognition memory (Broadbent et al., 2010). Although the role of the hippocampus in recognition memory remains controversial, our result showing a spared recognition memory during potential hippocampal damage by STZ-induced diabetes (Pamidi et al., 2012) suggest minimal or lack of hippocampal

involvement in the task and/or larger involvement of other brain regions, such as perirhinal regions, or the possibility of compensatory mechanisms by other structures within the medial temporal lobe (MTL) (Cohen et al., 2013).

Regardless of the brain areas involved, the role of RAGE in recognition memory has not been studied so far. Since our data show a similar trend in the parameters associated with recognition memory in both WT and RAGE-KO groups, we can infer that recognition memory is independent of RAGE expression/signaling, at least during the period our experiments lasted.

2.5.3 Hippocampal-dependent spatial learning and memory

The central role of the hippocampus in spatial learning and memory is well established (Moser et al., 2008; Pilly and Grossberg, 2012), including spatial navigation in animals (Eichenbaum, 2017). Hippocampal lesions in animals were associated with impaired performance on spatial tasks (Broadbent et al., 2006; Clark et al., 2007).

In animal models of diabetes, hippocampal-dependent spatial learning and memory showed impairments following 1, 2, 4, and 12 weeks of STZ-induced diabetes in the MWM (Baydas et al., 2003b; Babri et al., 2013; Ghasemi et al., 2016), as well as in the BM test (Jolivalt et al., 2010; Enhamre-Brolin et al., 2013; King et al., 2013; Anderson et al., 2014; Tender and Razdan 2017). Furthermore, STZ-induced diabetic rats showed learning deficits in the hippocampal-dependent version of the MWM, while this effect was absent in a non-hippocampal dependent version of the maze (Biessels et al., 1998; Stranahan et al., 2008). Studies performed on STZ-induced diabetic animals suggest that cognitive impairments elicited by diabetes might result from electrophysiological dysfunction in the CA1 region of the hippocampus, particularly defects in the expression of LTP (Biessels et al., 2002), changes in the expression and function of glutamate receptors (Gispen and Biessels, 2000; Trudeau et al., 2004), alteration in neurotransmitter synthesis or release (Trudeau et al., 2004), morphological changes such as decreased length of apical dendrites as well as reduced number of apical branch points of CA3 pyramidal neurons (Magarinos and McEwen, 2000), and decreased hippocampal neurogenesis (Stranahan et al., 2008).

Consistent with the behavioural findings of the above-mentioned studies, our results show a significant effect of STZ on spatial cognitive performance. The results of our spatial behavioural tasks show spatial memory impairment in WT STZ-induced diabetic mice in both BM and MWM tests. The significant interaction between STZ treatment and the genotype in the time spent in the target quadrant in both of these tests indicates that the effect of STZ on spatial memory differed significantly between the two genotypes, suggesting that hyperglycemia does not affect the spatial memory performance in the RAGE-KO group, further supporting the involvement of RAGE expression and/or signaling in cognitive dysfunction under diabetic conditions.

Unfortunately, the role of RAGE in initiating and/or exacerbating cognitive abnormalities in diabetes has not been well explored yet. In one study, D-ribosylation-derived AGEs formation caused spatial learning and memory impairment in the MWM test through RAGE-dependent astrocytic inflammation (Han et al., 2014). An elevated serum AGE level was associated with a higher risk of mild cognitive impairment (MCI) in type 2 diabetic patients (Wang et al., 2016). Similarly, serum AGEs and RAGE showed an increase in the circulation of MCI elderly diabetic patients compared to controls (Gorska-Ciebiada et al., 2015), suggesting the AGE-RAGE system as a potential contributor to the development of cognitive decline in diabetes. Involvement of RAGE in cognitive dysfunction, however, has been more investigated in the context of AD. Double transgenic mice, with mutant amyloid precursor protein (mAPP) and targeted neuronal overexpression of RAGE, displayed early abnormalities in spatial learning and memory before such changes were observed in mAPP mice (Arancio et al., 2004). In contrast, transgenic mice with a dominant-negative form of RAGE targeted to neurons crossed with mAPP mice showed preservation of spatial learning and memory (Arancio et al., 2004). Exogenous AGEs were shown to induce tau hyperphosphorylation and spatial memory deficit through RAGE-mediated pathways (Zhou, 2011), and RAGE blockade was able to significantly attenuate memory impairments in AGE-induced AD models (Tan et al., 2015). Direct interaction of neuronal RAGE with its ligands can provoke oxidative stress and activation of mitogen-activated protein kinase (MAPK) signaling pathways, which subsequently mediate synaptic deficits and cognitive dysfunction (Yan et al., 2012). In addition to neuronal RAGE, RAGE-dependent signaling in microglia also contributed to neuroinflammation, accumulation of Aβ, and impaired learning and memory in a mouse model of AD (Fang et al., 2010). Although more research is still required regarding the role of RAGE in the

context of CNS complications of diabetes, our constitutive RAGE-KO model seems to be spared from STZ-induced diabetic effect on spatial memory function.

Our spatial behavioural tasks also showed changes in the velocity and distance traveled in response to STZ treatment. Although STZ-induced diabetic mice showed lower velocity and distance traveled during the probe trial of the BM test, the opposite trend was observed in the MWM test. The lower velocity and distance traveled in STZ-induced diabetic mice in the BM test is compatible with the lower locomotor activity observed in the open field test. However, the higher velocity and distance traveled in STZ-induced diabetic mice in the MWM can be partly due to the higher anxiety level of this group as compared with age-matched controls, probably due to the higher tendency of anxious animals to avoid water (Bondarenko, 2017). This finding also supports the results of the OF test, suggesting that less time spent in the centre of the OF in STZ-induced diabetic mice can be due to anxiety-like behaviour and not merely impairment in locomotor activity. Interestingly, RAGE-KO mice, which showed significantly higher anxiety-like behaviour in the OF test as compared to WT mice, displayed significantly more distance traveled and velocity during the probe trial in the MWM when compared to the age-matched WT mice. The latter is consistent with a report showing that RAGE deletion causes hyperactivity and increased home cage activity in mice (Sakatani et al., 2009).

It also needs to be mentioned that the NOR test showed a significant genotype difference in the total exploration time during the acquisition phase of the task. As there is no study available regarding the role of RAGE in exploratory behaviours, especially those in the NOR test, we cannot explain at this point the effect of genotype we observed in the acquisition phase and not the testing phase of the task. If it is due to, again, anxiety-like phenotype, as observed in the OF and the MWM tests, or other factors such as hyperactivity (Sakatani et al., 2009) of the RAGE-KO mice needs further investigations.

In conclusion, although the effect of STZ on cognitive function, such as learning and memory impairments, is widely reported in diabetes, the role of RAGE in these effects has never been investigated and, to the best of our knowledge, this is the first study that shows the involvement of RAGE signaling in hippocampal-dependent spatial memory dysfunction under STZ-induced diabetic condition.

CHAPTER 3

RAGE signaling is required for AMPA receptor dysfunction in the hippocampus of diabetic mice

3.1 Abstract

Diabetes in humans has been associated for a long time with cognitive dysfunction. In rodent animal models, cognitive dysfunction can manifest as impaired hippocampal synaptic plasticity. Particular attention has been concentrated on the receptor for advanced glycation end products (RAGE), which is implicated in multiple diabetic complications involving the development of vascular and peripheral nerve abnormalities. In this study, we hypothesize that RAGE signaling alters glutamate receptor function and expression, impairing synaptic transmission in the hippocampus. Using preparations of hippocampal slices from male mice, we show a RAGE-dependent decrease in long-term potentiation (LTP) and an increase in paired-pulse facilitation (PPF) following STZ-induced diabetes. Consistently, in hippocampal cultures from male and female neonatal mice, high glucose caused a RAGE-dependent reduction of AMPA- but not NMDA-evoked currents, and an increase in cytosolic reactive oxygen species (ROS). Hippocampi from diabetic WT mice showed increased RAGE expression concomitant with a decrease of both expression and phosphorylation (Ser 831 and 845) of the AMPA GluA1 subunit. We found these changes correlated to activation of the MAPK pathway, consistent with increased detection of total p38, pp38, nuclear factor-kappaB (NF-κB), and decreased pJNK and its kinase, pMEK7. As no changes in expression or phosphorylation of regulatory proteins were observed in hippocampi from diabetic RAGE-KO mice, we report a RAGE-dependent impairment in the hippocampi of diabetic WT mice, with reduced AMPA receptor expression/function and LTP deficits.

Momeni Z, Urban R, Yamamoto Y, Campanucci VA. RAGE signaling is required for AMPA receptor dysfunction in the hippocampus of diabetic mice. The work in this chapter is under review by Physiology and Behavior.

Contribution: Momeni was responsible for preforming all experiments, data analysis, and preparation of the manuscript. Urban helped in collecting electrophysiology data; Yamamoto provided the RAGE KO mice and edited the manuscript.

3.2 Introduction

Diabetes is a metabolic disorder characterized by chronic hyperglycemia due to the impaired production, secretion (type 1), or action of insulin (type 2; Ozougwu et al., 2013), affecting several organ systems, including the brain (Watkins and Thomas, 1998). Studies have indicated that both types of diabetes, type 1 and type 2, induce deleterious changes in the hippocampus leading to cognitive dysfunction, including learning and memory impairments (Saedi et al., 2016). streptozotocin (STZ)-induced type 1 diabetic rats, as well as other rodent models of type 1 (e.g. alloxan-induced) and type 2 (e.g. *db/db* mice and Zucker rat) diabetes, have shown dysfunctional hippocampal plasticity and impairments in cognitive performance (Wrighten et al., 2009; Rostami et al., 2013).

Hyperglycemia-induced oxidative stress is proposed as one of the main mechanisms underlying such diabetes-related complications (Giacco and Brownlee, 2010). Oxidative stress is caused by the accumulation of ROS, which are usually generated by the mitochondria as by-products of ATP biosynthesis, as well as by cytoplasmic enzymes and non-enzymatic glucose oxidation (Russell et al., 2002). One of the non-enzymatic glucose metabolic pathways with critical importance in diabetes is the formation of advanced glycation end products (AGEs) (Nass and Simm, 2009). AGEs are formed by the non-enzymatic reaction of sugar with amino groups on macromolecules (Nass and Simm, 2009) and this reaction, which occurs in our bodies increasingly as we age, is exacerbated under diabetic conditions due to the increased availability of glucose (Nass and Simm, 2009). AGEs bind to their receptor, RAGE, which is a member of the immunoglobulin protein family of cell surface molecules (Fang et al., 2010). Findings that RAGE interacts with multiple ligands and that some of these ligands accumulate under diabetic conditions (Yao et al., 2010) support the possibility of RAGE involvement in the development of diabetic complications (Ramasamy et al., 2011). RAGE activation induces oxidative stress and increases expression of inflammatory mediators (Granic et al., 2009). Thus, while hyperglycemia ignites the generation of AGEs and their subsequent interaction with RAGE, the interplay of RAGE with other ligands further amplifies oxidative stress and promotes RAGE up-regulation, a cycle that intensifies diabetic complications and inflammation-induced tissue injury (Bartsch et al., 2015).

Electrophysiological studies in STZ-induced diabetic rodents have revealed deficits in the generation of LTP, as a measure of impaired learning and memory processes (Biessels et al., 1998;

Lynch, 2004; Artola et al., 2005). Furthermore, STZ-induced and spontaneously diabetic animals have shown changes in the expression and/or function of NMDA and AMPA subtypes of glutamate receptors (Trudeau et al., 2004; Sasaki-Hamada et al., 2012), which play a critical role in induction and maintenance of LTP (Riedel et al., 2003). The strength of individual excitatory synapses is determined in part by the number of AMPA receptors present at synapses. Changes in the regulation and targeting of synaptic AMPA receptors reduce capacity of synaptic transmission and plasticity (McCormack et al., 2006), which may underlie cognitive impairment (Thomas and Huganir, 2004; Stornetta and Zhu, 2011). In diabetes it remains unclear, however, if changes in synaptic AMPA receptors and RAGE signaling are linked as part of the CNS pathology associated with the disease.

In this study, we hypothesize that RAGE signaling in diabetic conditions (*in vitro* high glucose or *in vivo* STZ-induced diabetes) interferes with the expression and function of synaptic glutamate receptors in the hippocampus, leading to deficits in synaptic transmission. Our findings show that hyperglycemia decreases the expression of the AMPA GluA1 subunit and its phosphorylation in WT but not in RAKE-KO mice. We also show that RAGE expression is required for LTP impairment in hippocampal slices from STZ-induced diabetic mice and for changes in expression and/or phosphorylation of signaling proteins including NF-κB, p38, and JNK, which are all downstream from RAGE. In particular, we found reduced phospho-JNK (pJNK), accompanied by a reduction in its kinase phospho-MEK7 (pMEK7) and an increase in phospho-p38 (pp38), which correlated with reduction in hippocampal neuronal excitability. Taken together, these findings constitute novel evidence for the contribution of RAGE signaling in abnormal hippocampal synaptic transmission in diabetes.

3.3 Materials and Methods

3.3.1 Animals

Heterozygous mice generated by back-crossing RAGE-KO (homozygous) mice (Myint et al., 2006) with C57BL/6 wild-type (WT) mice were used to maintain a colony of RAGE-KO mice on a C57BL/6 background, as previously described (Lam et al., 2018). Genomic DNA extraction and polymerase chain reaction were used for genotyping (Myint et al., 2006).

Male and female neonate mice [postnatal day 0 (P0)–P2] were used for *in vitro* experiments, and 4-6 weeks old male mice were used for STZ induction of diabetes. For STZ treatment, mice received daily i.p. injections of 50 mg STZ/kg body weight for three consecutive days, while age-matched controls received citrate buffer injections. Blood glucose measurements were obtained 10 days after injections using a CONTOUR Glucose Meter (Bayer Inc., Toronto, ON, Canada), and animals with blood glucose levels >15 mM were considered diabetic. Diabetic animals were kept for one month prior to the experiments, and blood glucose was measured once more when the animals were sacrificed. Determination of serum insulin level was performed using a mouse insulin enzyme-linked immunosorbent assay (ELISA) kit (Mercodia, Uppsala, Sweden) according to the manufacturer's instructions. Insulin levels were measured at the end of the one-month period when the animals were sacrificed.

3.3.2 Primary hippocampal culture. Hippocampal neurons were cultured from neonatal (P0-P2) mice as previously described (Falzone and Stokin, 2012). Briefly, hippocampal regions were dissected from WT and RAGE-KO mice and incubated in a mixture of papain (45 units) in phosphate-buffered saline (PBS), enriched with 0.05% of DNase, for 20 minutes at 37˚C, followed by gentle trituration in 10% fetal bovine serum (FBS) in Dulbecco's modified Eagle's medium. Cells were grown on poly-D-lysine-coated coverslips at 37˚C under 5% CO_2 in 500 mM L-glutamine and custom neurobasal medium (5 mM glucose) supplemented with B27. Cultured neurons were maintained in medium containing either 5 mM glucose (control) or 25 mM glucose (high glucose) for 1-2 weeks.

To monitor neuronal loss, cultured hippocampal neurons were labelled with annexin V conjugated with fluorescein isothiocyanate (FITC) and propidium iodide (PI), using annexin V-FITC

apoptosis detection kit (Sigma-Aldrich). As a positive control, some cells were exposed to 1 mM hydrogen peroxide (H_2O_2) for 2 hours to induce cell death. Images were collected with an AxioObserver inverted microscope and Zen software (Carl Zeiss, Oberkochen, Germany), and results are presented as percentage of positive cells to total cells counted.

3.3.3 Intracellular ROS levels. ROS level changes were evaluated in experimental groups (Lam et al., 2018) using the ROS-sensitive dye CM-H_2DCFDA (Molecular Probes, Eugene, OR, USA), and H_2O_2 (100 μM for 24 hr.) was used as a positive control (Xu et al., 2012). Cultures were incubated for one hour at 37°C with medium containing CM-H_2DCFDA (10 μM) and subsequently washed three times with control extracellular solution (see below). The cultures were then placed on the stage of an inverted microscope (AxioObserver, Carl Zeiss, Germany) and viewed through a 40x (1.3 numerical aperture) Plan Neofluor oil-immersion objective lens (Zeiss) at 37ºC. To obtain fluorescent images, we excited the cultures with 470 nm wavelength light using a Colibri 2.0 LED illumination system (Zeiss) and collected 510-550 nm wavelength emissions with an AxioCam camera (Zeiss) controlled by AxioVision v4.8 software (Zeiss). For each neuron recorded, the background fluorescence was subtracted from its mean fluorescence intensity.

3.3.4 Whole-cell patch-clamp electrophysiology. Cultured hippocampal neurons maintained for 1–2 weeks in control and high glucose were used for whole-cell patch-clamp recording. An Axopatch 200B amplifier (Molecular Devices, Palo Alto, CA) equipped with a 1 GΩ cooled head-stage feedback resistor and a Digidata 1400A analog-to-digital converter (Molecular Devices) were used for current and voltage clamp protocols. pClamp 10 (Molecular Devices) and Origin 9.0 software (OriginLab Corporation, Northampton, MA, USA) were used for data acquisition and analysis. Patch pipettes were made from thin-wall borosilicate glass capillaries (World Precision Instruments, FL, USA) and were pulled using a vertical puller (PC 10; Narishige Scientific Instrument Lab., Tokyo, Japan). Pipette tips were polished with a microforge (Narishige) to a final resistance of 3–8 MΩ when filled with intracellular recording solution containing (in mM): 65 KF, 55 KAc, 5 NaCl, 0.2 $CaCl_2$, 1 $MgCl_2$, 10 EGTA, 2 MgATP, and 10 HEPES (all from Sigma-Aldrich) at pH=7.2. Cultured neurons were perfused continuously at 1 mL/min with control extracellular solution consisting of (in mM): 140 NaCl, 5.4 KCl, 25 HEPES, 5 glucose, and 5

μg/mL phenol red (all from Sigma-Aldrich) at pH = 7.4. NMDA (50 μM)/glycine (1 μM)- or AMPA (50 μM)-containing extracellular solution was delivered at a perfusion rate of 1 mL/min using a fast-step pressurized perfusion system.

3.3.5 Hippocampal slice electrophysiology. Acute hippocampal slices were prepared from control and STZ-induced diabetic mice (Pitcher et al., 2011). Briefly, parasagittal hippocampal slices (300 μm) were prepared from the hippocampi of WT and RAGE-KO mice, using a vibrating tissue slicer (VTS1200S, Vibram Instruments, Germany) and were placed in a holding chamber containing artificial cerebrospinal fluid (aCSF) at 32°C for 30 min and then at room temperature for at least 1 h before recordings. A single slice was then transferred to the recording chamber and superfused with aCSF composed of 124 mM NaCl, 2.5 mM KCl, 1 mM NaH_2PO_4, 1.3 mM $MgCl_2$, 11 mM d-glucose, 26 mM $NaHCO_3$, and 2 mM $CaCl_2$ (all from Sigma-Aldrich), saturated with 95% O_2 (balance 5% CO_2) at 2 mL/min at room temperature, pH 7.4, 315–325 mOsm. Synaptic responses were evoked by stimulating Schaffer collateral afferents using bipolar tungsten electrodes located ~50 μm from the pyramidal cell body layer in CA1. Extracellular field excitatory postsynaptic potentials (fEPSPs) were recorded using aCSF-filled glass micropipettes placed in the stratum radiatum 60–80 μm from the cell body layer. Stimulus-response curves were used to establish a stimulation voltage that would induce a 60–70% maximal fEPSP amplitude. LTP was induced by theta burst stimulation (TBS) consisting of 15 bursts of four pulses at 100 Hz, delivered to Schaffer collateral afferents at an interstimulus interval of 200 ms. In LTP experiments, the value of fEPSP amplitude from the 10 min period before TBS was defined as baseline (100%) although the recording was continued for 20 min until a stable baseline was reached. For PPF, we used a range of interstimulus intervals (25 ms to 800 ms), and the PPF ratio was calculated by dividing the amplitude of the second fEPSP by that of the first fEPSP. We averaged data from five paired-pulse stimulations for each slice. Raw data were amplified using a MultiClamp 700B amplifier and a Digidata 1440A acquisition system and were analyzed using pClamp 10 (Molecular Devices) and Origin 9.0 software (OriginLab Corporation, Northampton, MA, USA).

3.3.6 Western blotting. We used whole extracts from both cultured neurons and hippocampal tissues collected from adult mice. For the whole-cell extracts from cultured neurons, cultures maintained for 1–2 weeks in control and high-glucose conditions were lysed using a 1% Nonidet

P-40 (NP-40) lysis buffer containing a protease and phosphatase inhibitor cocktail. At least 3 replicas were used per condition (control and high glucose) for both WT and RAGE-KO mice. Each replica was generated from 5 pups. For hippocampal tissues, whole hippocampi of control and STZ-induced diabetic mice were homogenized in ice-cold CelLytic™ MT Cell Lysis Reagent (Sigma-Aldrich) containing a protease and phosphatase inhibitor cocktail. At least 3 replicas were used per condition (CNT or STZ) for both WT and RAGE-KO mice. Equal amounts of protein were loaded per group, separated on 12% SDS polyacrylamide gels and then electrotransferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were incubated overnight at 4°C with the following primary antibodies: rabbit anti-GluN1, anti-GluN2A, anti-GluN2B, anti-GluN2C, anti-GluN2D, anti-GluN3A, and anti-GluN3B (1:1000, NMDA Receptor Antibody Explorer Kit, Alomone Labs); rabbit anti-GluA1, anti-GluA2, anti-GluA3 and anti-GluA4 (1:1000, AMPA Receptor Antibody Explorer Kit, Alomone Labs); and rabbit anti-phospho-GluA1 (Ser831 and Ser845) (1:1000, Abcam), rabbit anti-RAGE (1:1000; Abcam), rabbit anti-NF-κB p65 (1:1000, Abcam), rabbit anti-ADAM10 (1:1000, Abcam), rabbit anti-Erk1/2 and anti-phospho Erk1/2 (Thr202/Thr204) (1:1000, Cell Signaling), rabbit anti-p38 MAPK and anti-phospho-p38 MAPK (Thr180/Thr182) (1:1000, Cell Signaling), rabbit anti-JNK and anti-phospho JNK (Thr183/Thr185) (1:1000, Cell Signaling), rabbit anti-phospho MKP1/2 (Ser296 and Ser318) (1:500, Thermofisher Scientific), rabbit anti-phospho-MEK7 (Ser277 and Thr275) (1:1000; Abcam) and mouse anti-α-tubulin (1:2000; Sigma); followed by horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (1:20000; Bio-Rad Laboratories). Protein signals were visualized using enhanced chemiluminescence reagents (Bio-Rad) and quantified by densitometry using ImageJ software (NIH, Bethesda, MD, USA).

3.3.7 Experimental design and statistical analysis. All values are reported as mean ± SEM and the level of significance was set at 0.05 for all statistical tests performed. To compare two means, we used parametric Student's t-tests or non-parametric Mann-Whitney U tests as indicated in the figure legends. To compare multiple means, we used two-way ANOVA and three-way repeated measures ANOVA as indicated in the table and figure legends. Statistical analyses were carried out with InStat 3.0 or Prism 8.0 (GraphPad Software Inc., La Jolla, CA, USA). Details for statistical tests used are provided within figure legends. Each *n* number is indicated in the figure

legends. All sample sizes and experimental designs were based on previously published data from our lab and similar experiments in the field.

This work was approved by the University of Saskatchewan's Animal Research Ethics Board (Campanucci: protocol 20090082) and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

3.4 Results

3.4.1 Induction of diabetes by STZ injection in mice

After STZ injection, mice were severely diabetic as indicated by elevated blood glucose and low circulating insulin levels (below detectable range for ELISA, i.e., <0.2 μg/L) compared with age-matched controls in both genotypes (Table 3.1). Body weights indicate that, even though all mice gained weight between initial and final measurements, STZ-treated mice from both genotypes displayed significantly less body weight gain as compared with their aged-matched controls (Table 3.1). The lack of significant differences in blood glucose and insulin levels between genotypes, confirms that WT and RAGE-KO mice had similar basal levels and that STZ induced a similar diabetic state in both genotypes, as previously shown (Shoji et al., 2006; Soro-Paavonen et al., 2008; Hamada et al., 2010).

Table 3.1. Glycemic and body weight values.

Groups	Blood glucose (mM)		Bodyweight (g)		Insulin (µg/L)
	Initial	Final	Initial	Final	Final
WT CNT	9.5 ± 0.26 (n=18)	10.29 ± 0.28 (n=18)	16.6 ± 0.57 (n=18)	26.95 ± 0.4 (n=18)	2.41 ± 0.63 (n=11)
WT STZ	9.4 ± 0.24 (n=14)	30.51 ± 1.29 (n=14)	15.37 ± 0.77 (n=14)	20.83 ± 0.91 (n=14)	< 0.2 (n=7)
RAGE-KO CNT	8.96 ± 0.32 (n=15)	10.56 ± 0.42 (n=15)	18.18 ± 0.46 (n=15)	25.9 ± 0.57 (n=15)	2.77 ± 0.87 (n=9)
RAGE-KO STZ	9.21 ± 0.17 (n=13)	29.95 ± 1.48 (n=13)	17.23 ± 1.01 (n=13)	21.16 ± 0.88 (n=13)	< 0.2 (n=7)
Blood glucose Initial WT STZ vs. Final WT STZ: *p<0.001* Initial RAGE-KO STZ vs. Final RAGE-KO STZ: *p<0.001* Final WT CNT vs. Final WT STZ: *p<0.001* Final RAGE-KO CNT vs. Final RAGE-KO STZ: *p<0.001*			**Bodyweight** Initial WT CNT vs. Final WT CNT: *p<0.001* Initial WT STZ vs. Final WT STZ: *p<0.001* Initial RAGE-KO CNT vs. Final RAGE-KO CNT: *p<0.001* Initial RAGE-KO STZ vs. Final RAGE-KO STZ: *p<0.01* Final WT CNT vs. Final WT STZ: *p<0.001* Final RAGE-KO CNT vs. Final RAGE-KO STZ: *p<0.001*		

Blood glucose concentrations, body weights, and insulin levels in nondiabetic control (CNT) and STZ-induced diabetic mice from WT and RAGE-KO groups. Measurements were taken before STZ or citrate buffer injections (Initial) and/or at the end of the experiment (Final). Means were statistically compared by three-way repeated measures ANOVA, followed by Tukey's multiple comparisons test. Significant main effect of STZ treatment on blood glucose: $F_{(1, 56)}$=424.3, *p<0.001*; and on body weight: $F_{(1, 56)}$=37.59, *p<0.001*. Significant main effect of time on blood glucose: $F_{(1, 56)}$=520.4, *p<0.001*; and on body weight: $F_{(1, 56)}$=227.7, *p<0.001*.

3.4.2 STZ-induced diabetes caused impairment in hippocampal synaptic plasticity in WT, but not in RAGE-KO, mice

To study the effect of diabetes on synaptic strength in the presence and absence of RAGE expression, we examined hippocampal synaptic plasticity in brain slices from STZ-induced diabetic WT and RAGE-KO mice. We recorded fEPSPs from hippocampal slices of control and STZ-induced diabetic mice from both genotypes. We observed a significant main effect of STZ treatment on TBS-induced LTP as well as a significant difference between the two genotypes (Figure 3.1). There was also a significant interaction between STZ treatment and the genotype, indicating that the effect of STZ on LTP differed significantly between the two genotypes. Consistent with this, the TBS-induced LTP was significantly lower in STZ-induced diabetic WT mice, but not in STZ-induced diabetic RAGE-KO mice (Figure 3.1). To evaluate whether diabetes also induces presynaptic changes, we quantified PPF. The PPF ratio showed a significant increase at the 25 ms interval in STZ-induced diabetic WT mice, but, again, not in RAGE-KO mice (Figure 3.2). These findings demonstrate that under diabetic conditions, changes in hippocampal synaptic plasticity require RAGE expression and involve both pre- and postsynaptic components.

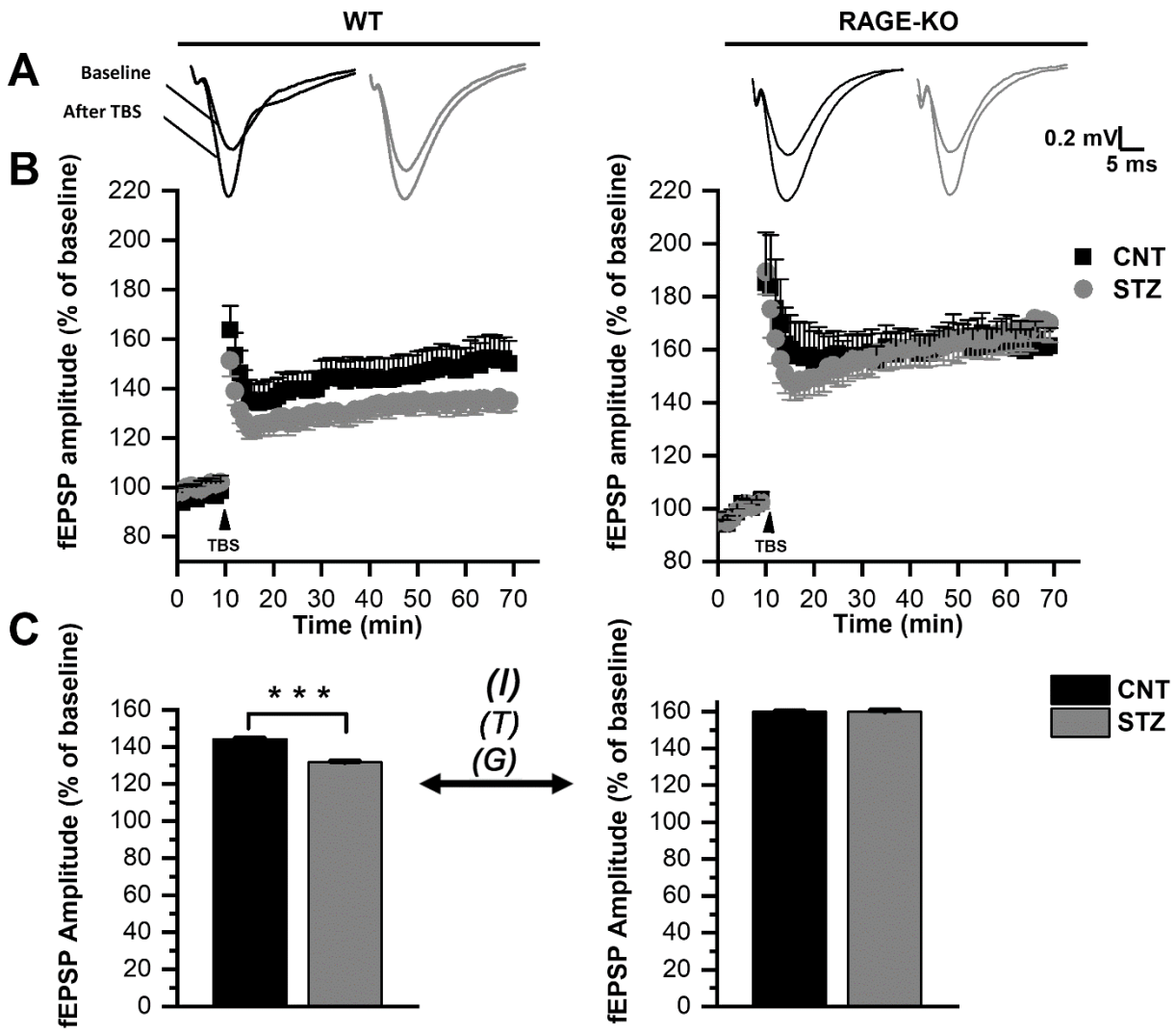


Figure 3.1. RAGE-dependent synaptic plasticity impairment in STZ-induced diabetic mice. (A) Representative traces and **(B)** time-course of field EPSP amplitude in slices from control (CNT) and STZ-induced diabetic mice in WT and RAGE-KO groups expressed as the percentage of baseline. **(C)** Bar graphs summarizing LTP during the last 50-min period after TBS delivery in control (CNT) and STZ-induced diabetic groups. Values expressed as the mean ± SEM from WT CNT (n=9 slices from 5 mice), WT STZ (n=10 slices from 5 mice), RAGE-KO CNT (n=8 slices from 4 mice) and RAGE-KO STZ (n=9 slices from 5 mice). Means were statistically compared by two-way ANOVA followed by Sidak's multiple comparison test; *** $p<0.001$. *(G)*: significant genotype differences ($F_{(1, 194)}$=1475, $p<0.001$); *(T)*: significant main effect of STZ treatment ($F_{(1, 194)}$=105.2, $p<0.001$); *(I)*: significant interaction between genotype and STZ treatment ($F_{(1, 194)}$=145.6, $p<0.001$).

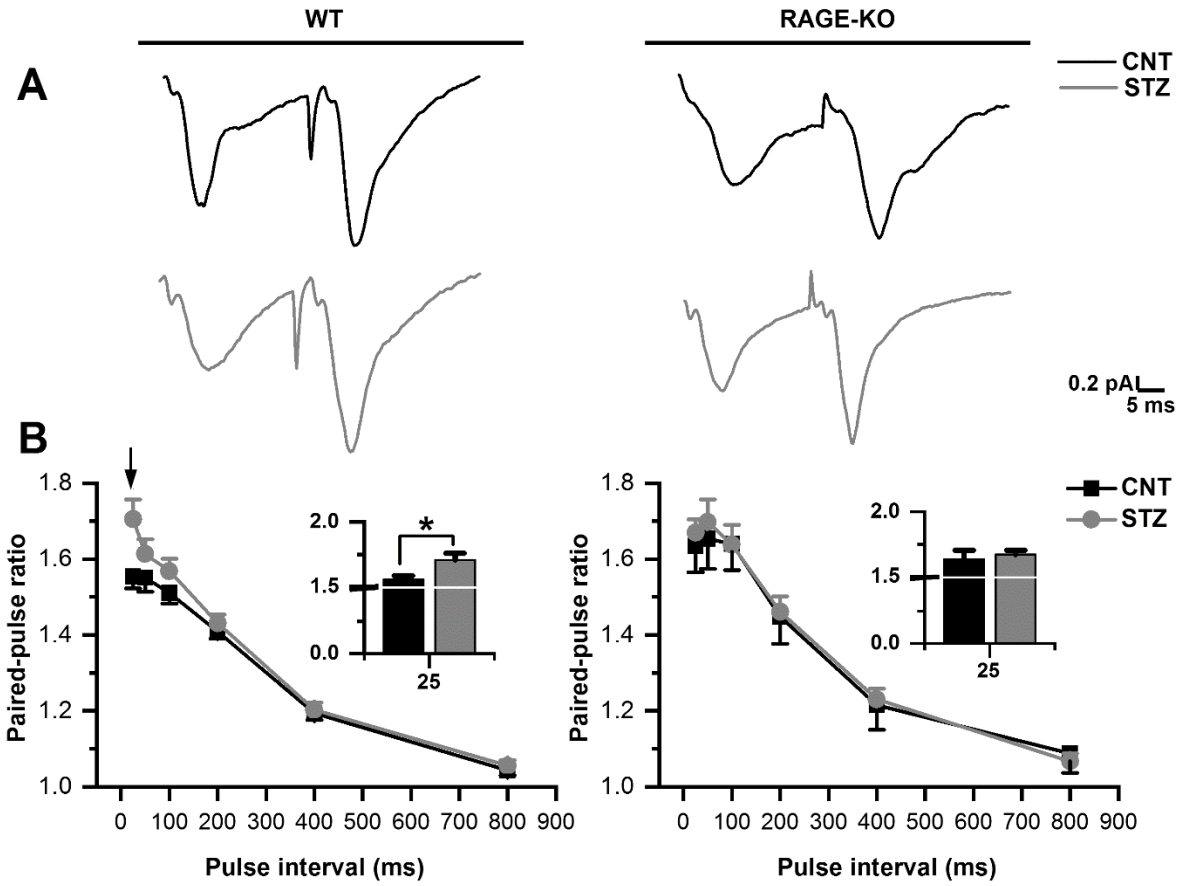


Figure 3.2. RAGE-dependent PPF impairment in STZ-induced diabetic mice. (A) Sample traces showing the average of five paired pulses at 25 ms intervals in slices from control (CNT) and STZ-induced diabetic mice in WT and RAGE-KO groups. **(B)** Graphs summarizing paired-pulse ratio as the average amplitude of the second fEPSP divided by the average amplitude of the first fEPSP. Inset, shows a comparison of the paired-pulse ratio solely at the 25 ms interval, at which the significant difference was observed. In each hippocampal slice, facilitation was tested using pairs of stimuli at intervals ranging from 25 ms to 800 ms. Values expressed as the mean ± SEM from WT CNT (n=13 slices from 6 mice), WT STZ (n=15 slices from 7 mice), RAGE-KO CNT (n=9 slices from 5 mice), and RAGE-KO STZ (n=10 slices from 5 mice). Means were statistically compared by two-way ANOVA at specific intervals followed by Sidak's multiple comparison test; * $p<0.05$.

3.4.3 STZ-induced diabetes decreased the expression of the AMPA GluA1 subunit in the hippocampus of WT, but not RAGE KO, mice

Since induction and maintenance of LTP requires AMPA and NMDA receptor function, we next concentrated on the expression levels of AMPA and NMDA receptor subunits in hippocampi of control and STZ-induced diabetic mice, from both WT and RAGE-KO genotypes. Western blot analysis showed a significant reduction in expression of the AMPA receptor GluA1 subunit in the hippocampal tissues from WT diabetic mice, but not from RAGE-KO mice (Figure 3.3).

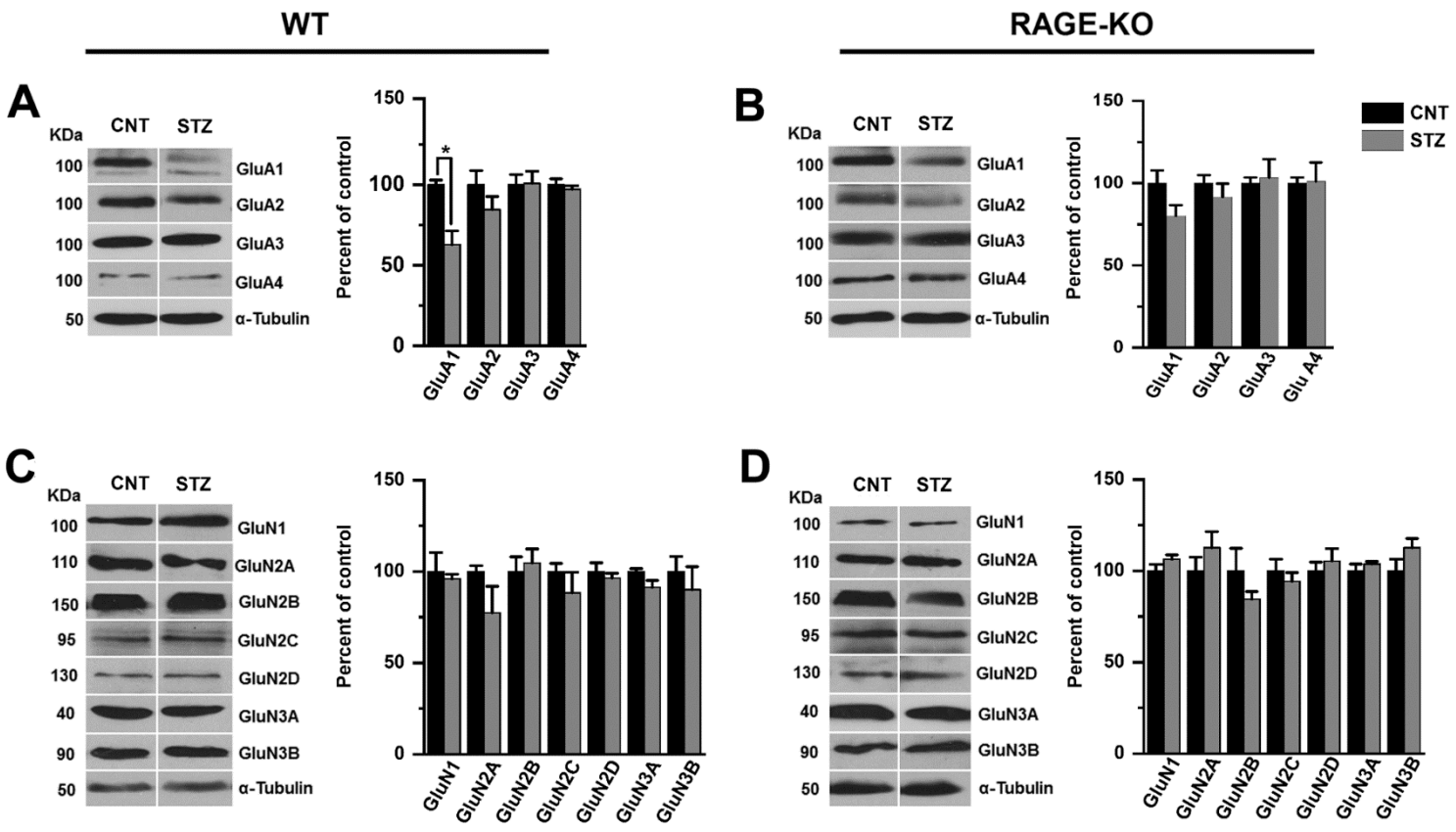


Figure 3.3. RAGE-dependent changes in AMPA receptor expression, but not NMDA receptors, in STZ-induced diabetic mice. Representative immunoblots showing levels of AMPA receptor subunits in the hippocampi of control (CNT) and STZ-induced diabetic mice in **(A)** WT and **(B)** RAGE-KO groups. Levels of NMDA receptor subunits in the hippocampi of control (CNT) and STZ-induced diabetic mice are shown in **(C)** for WT and **(D)** for RAGE-KO groups. Bar graphs show the mean ± SEM levels of each protein after normalization to tubulin expressed as a percentage of control (n=4 in each group). Means were statistically compared by the Mann-Whitney U test; * $p<0.05$. Protein samples for Western blotting were obtained from the bilateral hippocampi of four mice.

3.4.4 STZ-induced diabetes activated the MAPK signaling pathway in diabetic WT, but not RAGE KO, mice

To better understand the link between RAGE downstream signaling and reduced AMPA receptor function, we next investigated regulatory proteins in the MAPK pathway. Hippocampi from STZ-induced diabetic mice had, as expected (Chandna et al 2015a and 2015b), a significant increase in RAGE and NF-κB expression, accompanied by no change in the α-secretase ADAM10, which cleaves membrane-bound RAGE to generate a secretory form (Raucci et al., 2008). The increase in NF-κB detected in WT mice was absent in hippocampi from RAGE-KO mice, which lack functional RAGE expression (Figure 3.4) (Chandna et al., 2015a; Myint et al., 2006; Harashima et al., 2006).

Next, we concentrated on the kinases downstream from RAGE, which are linked to NF-κB expression (Figure 3.5). We observed significant increases in total p38 and pp38, whereas there were no changes in total ERK1/2 and pERK1/2 in the hippocampi of WT STZ-induced diabetic mice. Levels of p38 and pp38 kinases, however, were unchanged in the hippocampi of RAGE KO STZ-induced diabetic mice (Figure 3.5 A-B).

Lastly, we concentrated on the c-Jun N-terminal kinase (JNK), which is required for GluA1 turnover at the plasma membrane (Myers et al., 2012). In hippocampal tissues from WT diabetic mice we observed an increase in total JNK accompanied by a decrease in pJNK. We also observed a decrease in pMEK7, which phosphorylates JNK (Tournier et al., 2001), and no change in its phosphatase, pMKP1/2. Consistent with a decrease in pJNK, we also observed a decrease in GluA1-pSer831 and GluA1-pSer845; these phosphorylated version of GluA1 are both required for surface expression of GluA1 subunits. None of these changes were observed in hippocampal tissues from RAGE-KO diabetic mice (Figure 3.5 C-D).

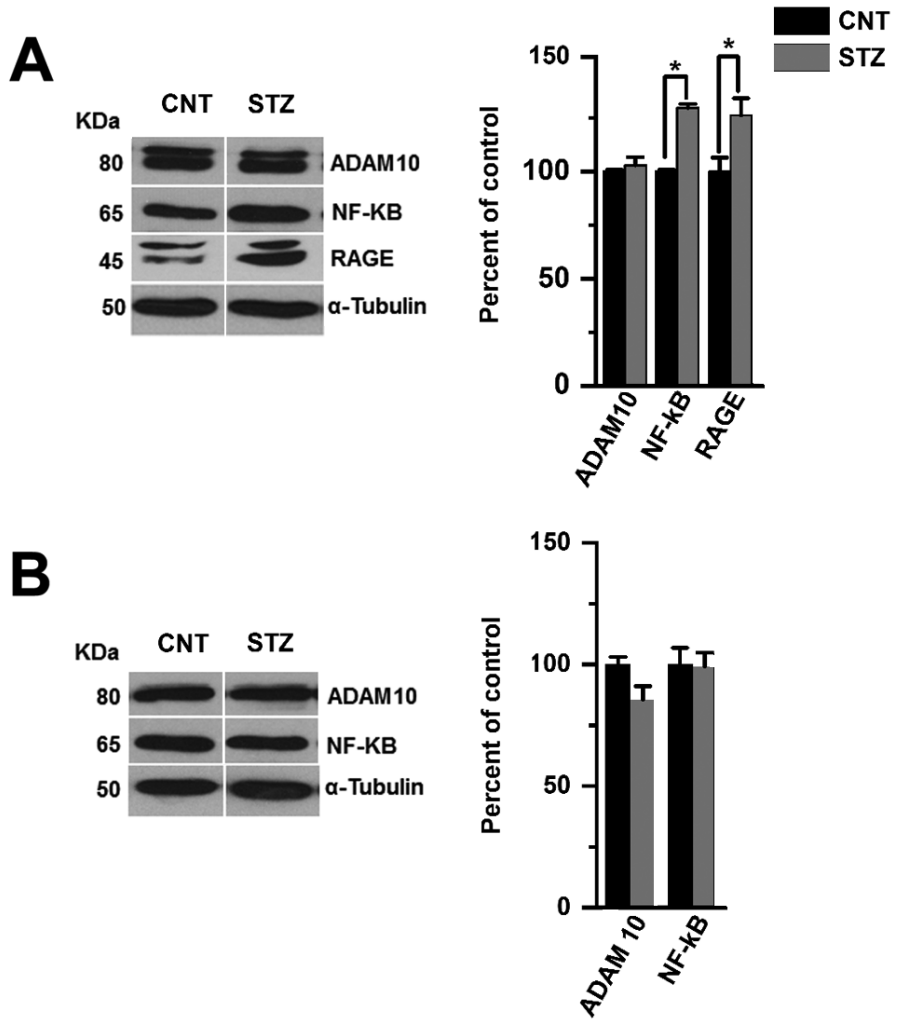


Figure 3.4. STZ-induced diabetic mice in WT showed changes in the expression levels of RAGE and NF-κB. Representative immunoblots showing levels of RAGE and NF-κB in the hippocampi of control (CNT) and STZ-induced diabetic mice in **(A)** WT and **(B)** RAGE-KO groups. Bar graphs show the mean ± SEM levels of each protein after normalization to tubulin expressed as a percentage of control (n=4 in each group). Means were statistically compared by the Mann-Whitney U test; * $p<0.05$. Protein samples for Western blotting were obtained from the bilateral hippocampi of four mice.

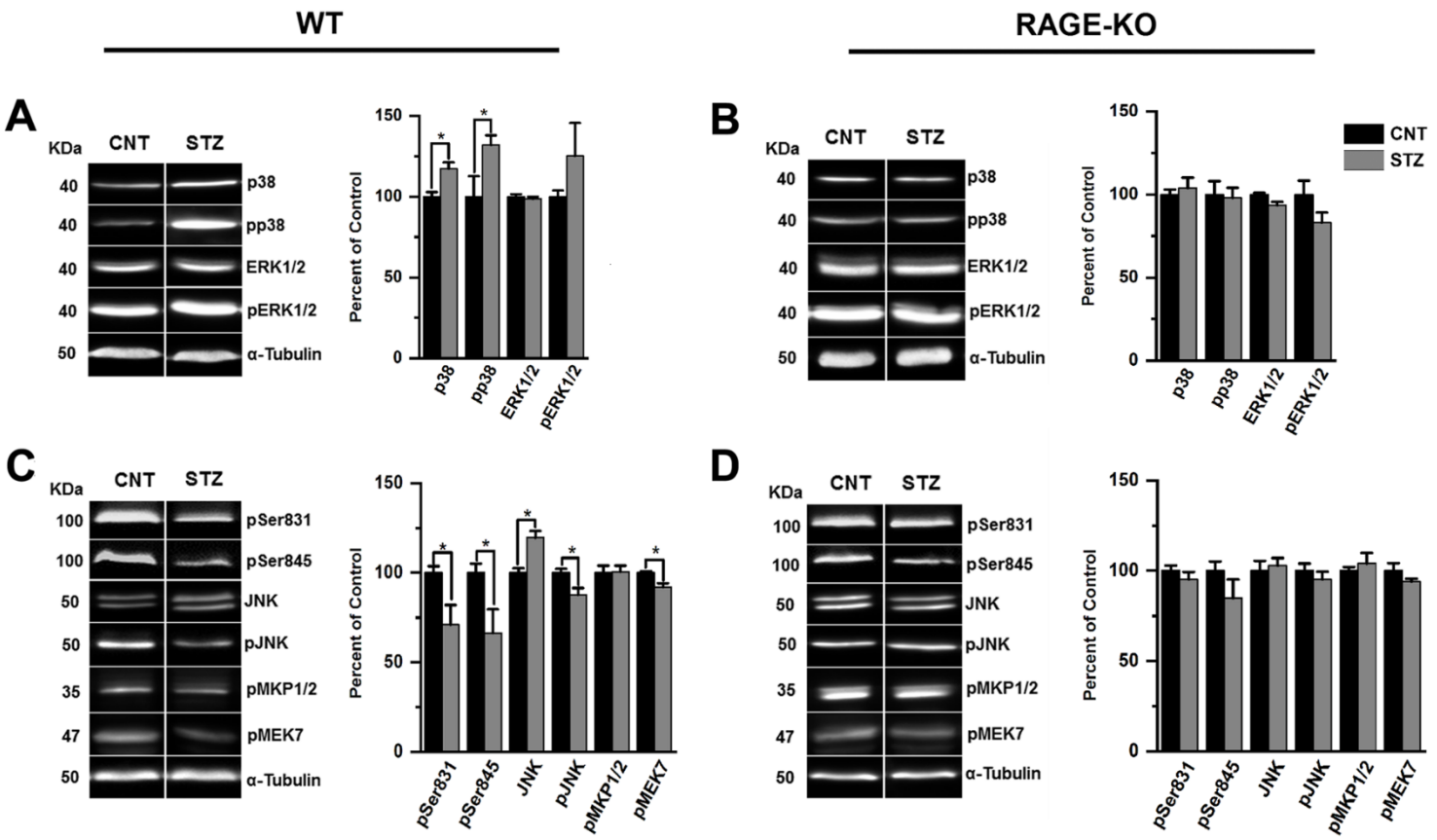


Figure 3.5. STZ-induced diabetic mice in WT showed changes in the phosphorylation state of the AMPA GluA1 subunit and regulatory proteins in the MAPK pathway downstream from RAGE. Representative immunoblots showing levels of phospho-Ser831 and phospho-Ser845 GluA1, total p38 and pp38, total ERK1/2 and pERK1/2, total JKN and pJNK as well as JNK kinase pMEK and JNK phosphatase pMKP1/2 in the hippocampi of control (CNT) and STZ-induced diabetic mice in **(A and C)** WT and **(B and D)** RAGE-KO groups. Bar graphs show the mean ± SEM levels of each protein after normalization to tubulin expressed as a percentage of control (n=4 in each group). Means were statistically compared by the Mann-Whitney U test; * $p<0.05$. Protein samples for Western blotting were obtained from the bilateral hippocampi of four mice.

3.4.5 High glucose decreased AMPA-mediated currents in hippocampal neurons from WT, but not from RAGE KO, mice

To better understand the effect of hyperglycemia at the cellular level, we next examined the function of the glutamate receptors responsible for TBS-induced LTP. Thus, we study the effect of high glucose on AMPA and NMDA receptor function in the presence and absence of RAGE expression.

Currents were evoked using AMPA (50 μM) or NMDA (50 μM) in voltage-clamp mode, at a holding potential of -60 mV, and were expressed as current densities. To better quantify the magnitude of fast-inactivating currents, we also calculated the ionic charge (area under the curve) carried by the evoked currents.

In the WT cultures, high glucose caused a significant reduction in AMPA-evoked ionic charge, while NMDA-evoked ionic charge was unaffected (Figure 3.6 D-E). No significant changes were observed in AMPA- or NMDA-evoked current density or ionic charge in either the control or high-glucose RAGE-KO cultures (Figure 3.6 A-F). These data are consistent with our observations of reduced expression and phosphorylation of AMPA GluA1 subunits and suggest that activation of the RAGE signaling pathway in high glucose modulates AMPA receptor function, but not NMDA, in hippocampal neurons.

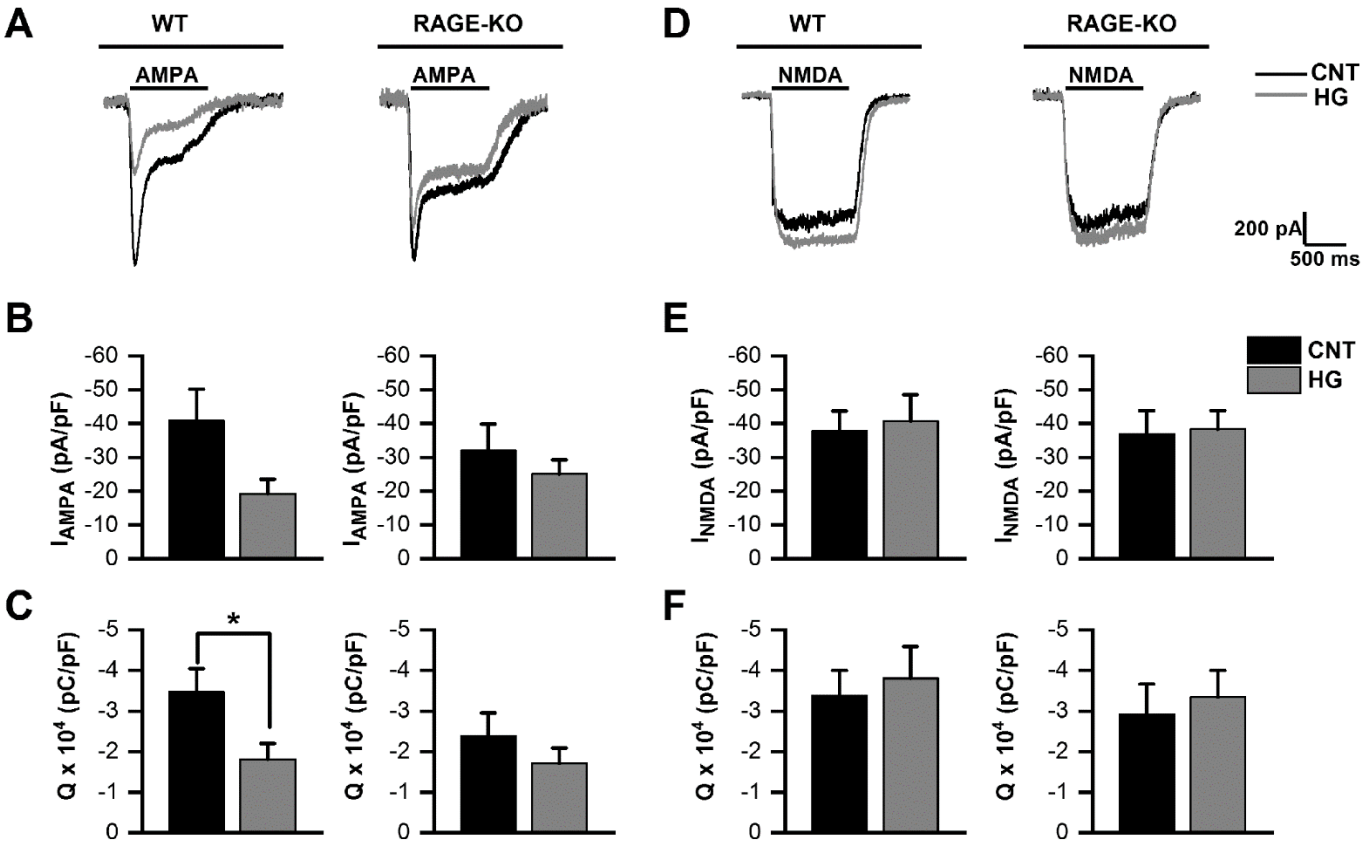
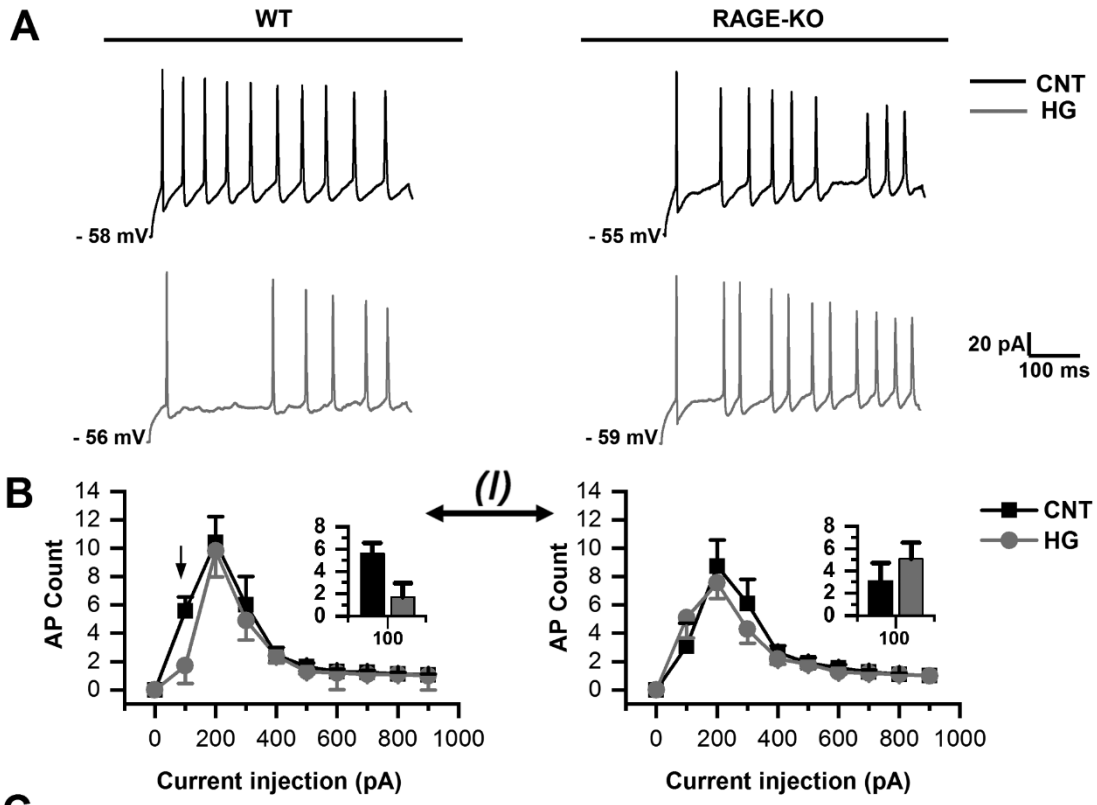


Figure 3.6. Effect of high glucose on whole-cell currents evoked by AMPA and NMDA receptors in cultured hippocampal neurons. Representative example traces of AMPA (50 μM)- **(A)** and NMDA (50 μM)-evoked **(D)** currents in cultured hippocampal neurons from WT and RAGE-KO mice, maintained in either control (CNT) or high-glucose (HG) conditions. Bar graphs summarize **(B)** AMPA- and **(E)** NMDA-evoked current density (I_{AMPA} or I_{NMDA}) and **(C, F)** ionic charge (Q x 10^4) values expressed as the mean ± SEM. Means were statistically compared by two-way ANOVA, followed by Sidak's multiple comparison test; * *p<0.05.* For AMPA experiments: WT CNT (n=11), WT HG (n=10), RAGE-KO CNT (n=12), and RAGE-KO HG (n=10). For NMDA experiments: WT CNT (n=16), WT HG (n=11), RAGE-KO CNT (n=10), and RAGE-KO HG (n=10).

3.4.6 High glucose caused a decrease in neuronal excitability in WT, but not in RAGE-KO, hippocampal neurons

To explore whether high glucose affects the ability of hippocampal neurons to participate in synaptic transmission, we concentrated on parameters of cell excitability. We evoked action potentials in cultured hippocampal neurons from WT and RAGE-KO mice maintained in either control or high-glucose conditions. Action potentials were generated in current clamp mode by injecting a series of depolarizing current steps (0-900 pA, at 100-pA increments) for 500 ms. To mitigate the effects of differences in resting membrane potentials among cells, we held the cells at approximately -60 mV before applying the current step protocol. We observed a significant interaction between genotype and high glucose treatment in action potential counts at the 100 pA depolarizing current step, indicating that the effect of high glucose on neuronal excitability differed significantly between the two genotypes (Figure 3.7 A-B). The reduced excitability at the 100 pA step was not accompanied by changes in other parameters of action potentials, such as threshold voltage (V_{th}), peak amplitude (AP_{ampl}), half-width (AP_{hw}), inter-spike interval (APisi), or after-hyperpolarization amplitude (AHP_{ampl}) (Figure 3.7 C). Furthermore, no significant changes were observed in passive membrane properties between cultured neurons from either WT or RAGE-KO mice exposed to control and high-glucose conditions, including resting potential (V_m), membrane resistance (R_{in}), and membrane capacitance (C_m) (Table 3.2).



C

Groups	Vth (mV)	AP ampl (mV)	AP hw (ms)	AP isi (ms)	APH ampl (mV)
WT CNT (n=13)	-42.23 ± 1.53	76.7 ± 5.91	2.37 ± 0.3	60.06 ± 6.36	-12.35 ± 1.01
WT HG (n=7)	-41.6 ± 1.74	73.53 ± 5.87	2.42 ± 0.26	52.54 ± 6.81	-11.96 ± 1.47
RAGE-KO CNT (n=7)	-42 ± 4.16	61.67 ± 13.26	3.56 ± 0.9	44.19 ± 1.92	-8.05 ± 0.22
RAGE-KO HG (n=10)	-41.42 ± 1.42	70.72 ± 5.2	2.83 ± 0.38	45.83 ± 7.43	-10.98 ± 1.61

Figure 3.7. High glucose decreases cell excitability in cultured hippocampal neurons from WT mice. (A) Representative action potential traces from WT and RAGE-KO cultured hippocampal neurons (P0-P2) maintained in either control (CNT) or high-glucose (HG) conditions. Action potentials were generated by a series of depolarizing current steps at 100-pA increments for 500 ms. Example traces are from a 100-pA depolarizing current step. **(B)** Mean action potential counts (AP count) expressed as the mean ± SEM from WT CNT (n=17), WT HG (n=10), RAGE-KO CNT (n=11), and RAGE-KO HG (n=11). The inset, shows a comparison of the AP count solely at the 100-pA depolarizing current step. **(C)** Table summarizes the action potential parameters, such as threshold voltage (*Vth*), peak amplitude (*APampl)*, half width (*APhw*), inter-spike interval (*APisi*), and after-hyperpolarization amplitude (*AHPampl*). Means were statistically compared by two-way ANOVA, followed by Sidak's multiple comparison test. *(I):* significant interaction between HG treatment and the genotype ($F_{(1, 45)}$=4.913, $p<0.05$).

Table 3.2. Passive membrane properties.

Groups	Cm (pF)	Rm (MΩ)	Vm (mV)
WT CNT (n=23)	32.08 ± 2.19	406.27 ± 29.94	-53.98 ± 1.2
WT HG (n=20)	30.23 ± 2.13	435.39 ± 36.87	-56.32 ± 2.88
RAGE-KO CNT (n=18)	36.66 ± 2.67	501.43 ± 61.57	-52.79 ± 1.56
RAGE-KO HG (n=19)	37.16 ± 3.58	481.38 ± 57.85	-54.02 ± 1.9

Passive membrane properties of cultured hippocampal neurons from WT and RAGE-KO mice maintained in either control (CNT) or high-glucose (HG) conditions. Table summarizes cell capacitance (*Cm*), membrane resistance (*Rm*) and membrane potential (*Vm*) during whole-cell recordings. Means were statistically compared by two-way ANOVA, followed by Sidak's multiple comparison test.

3.4.7 High glucose caused an increase in oxidative stress in WT, but not in RAGE-KO, hippocampal neurons

To investigate whether the redox state of hippocampal neurons may contribute to hippocampal abnormalities during high glucose condition, as observed in other neurons (Vincent et al., 2005; Chandna et al., 2015a), we next monitored both cytosolic ROS levels and cell viability. For cytosolic ROS we used the ROS sensitive dye CM-H_2DCFDA. We observed a significant main effect of high glucose treatment on cytosolic ROS levels as well as a significant difference between the two genotypes (Figure 3.8). There was also a significant interaction between high glucose treatment and the genotype, indicating that the effect of high glucose on cellular redox state differed significantly between the two genotypes. Consistent with this, we observed a significant increase in ROS levels in hippocampal neurons cultured from WT but not from RAGE-KO mice (Figure 3.8). The latter further supports the link between RAGE signaling and activation of the MAPK pathway that eventually leads to further expression of RAGE and decreased surface expression of the AMPA GluA1 subunit.

Next, we evaluated cell viability in hippocampal cultures under control and high glucose conditions by annexin V and PI fluorescence (Lam et al., 2018). The evaluation of cell viability served two purposes: first, to confirm that contrary to the historical assumption that cultured neurons could not survive in normal glucose levels, our cells were healthy in 5 mM glucose and second, to evaluate whether the high glucose treatment had any impact on cell viability of hippocampal neurons, as it has been previously reported for hippocampal and peripheral neurons (Russell et al., 2002; Vincent et al., 2005; Kahya et al., 2016). We observed no significant difference in neuronal viability between control (5 mM glucose) and high glucose (25 mM glucose) conditions in either genotype (Figure 3.9). In contrast, hippocampal neurons exposed to 1 mM H_2O_2 as a positive control showed a significant increase in the percent of cells positive for annexin V and PI (indicative of cell death). These data thus confirm the survival of primary hippocampal culture in control glucose levels (5 mM) and also the lack of significant cell loss under high glucose (25 mM) condition.

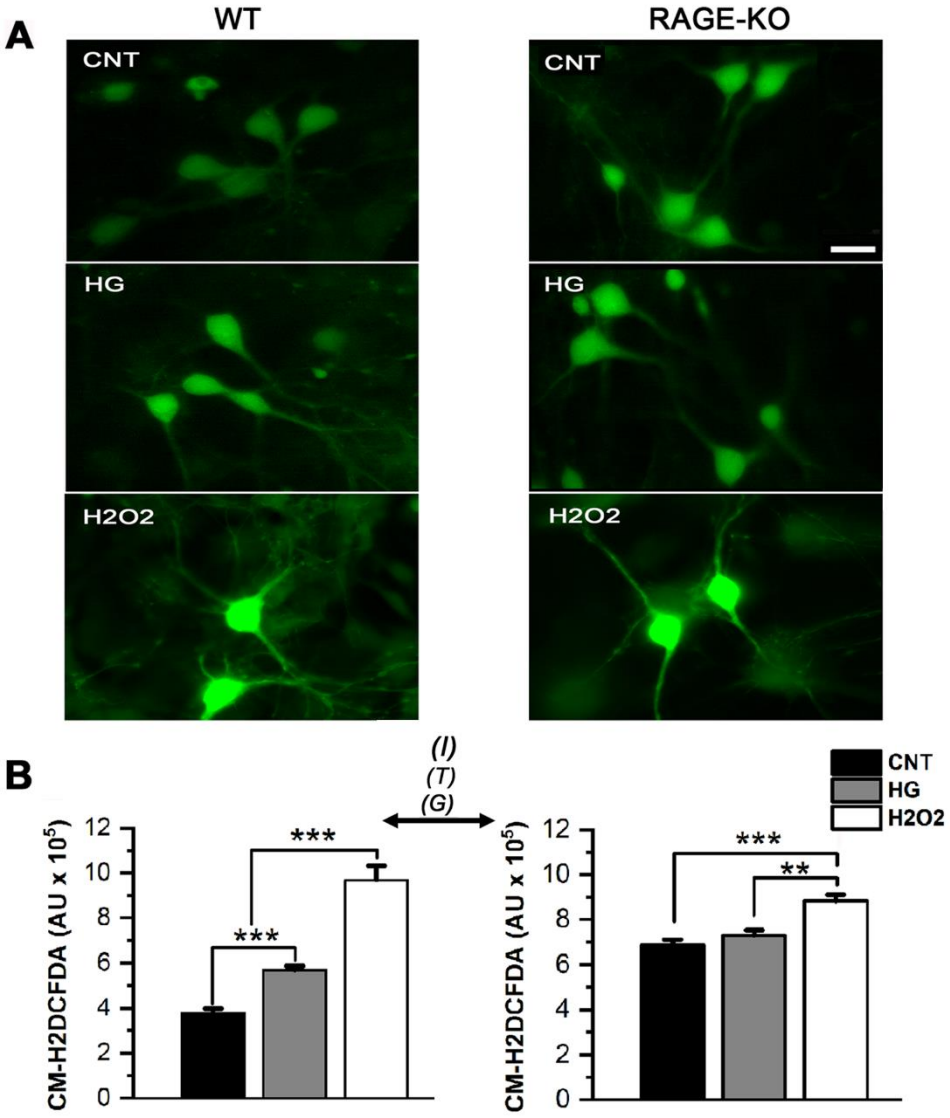


Figure 3.8. High glucose induces intracellular ROS accumulation in cultured hippocampal neurons from WT mice. (A) Representative images of ROS detection by CM-H_2DCFDA fluorescence from WT and RAGE-KO cultured hippocampal neurons (P0-P2) in control (CNT), high-glucose (HG), and 100 µM H_2O_2 (as positive control). **(B)** Bar graphs summarize the mean ± SEM pixel intensity for neurons from WT CNT (n=140), WT HG (n=175), WT H_2O_2 (n=148), and RAGE-KO CNT (n=178), RAGE-KO HG (n=168), and RAGE-KO H_2O_2 (n=151) mice respectively. Means were statistically compared by two-way ANOVA followed by Sidak's multiple comparison test; ** *p<0.01;* *** *p<0.001*. *(T)*: significant main effect of HG treatment ($F_{(1, 657)}$ = 28.42, *p<0.001*); *(G)*: significant genotype differences ($F_{(1, 657)}$=115.6, *p<0.001); (I)*: significant interaction between genotype and HG treatment ($F_{(1, 657)}$=11.27, *p<0.001).* Scale bars represent 30 µm.

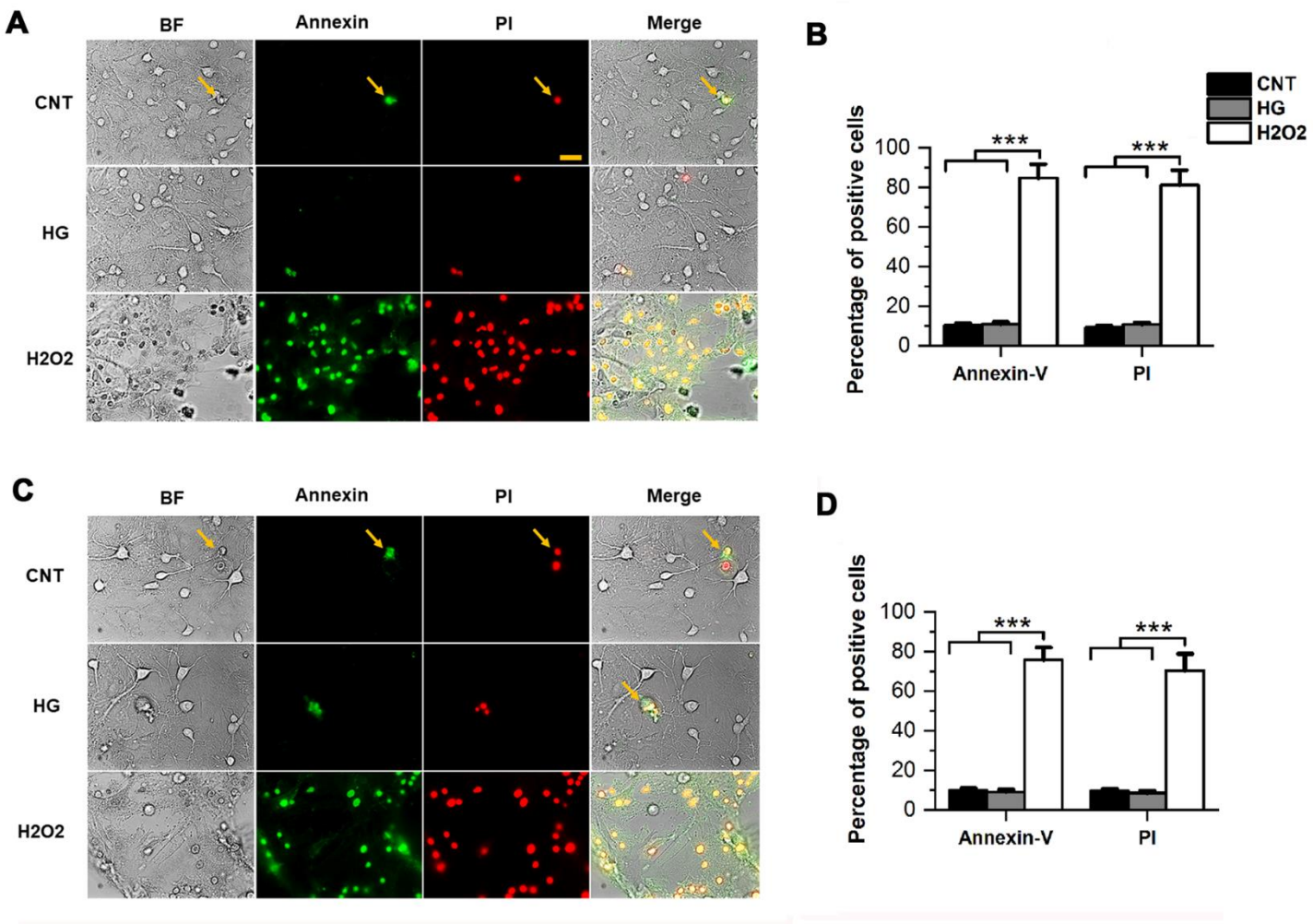


Figure 3.9. Percent of apoptotic/necrotic cells in primary cultured hippocampal neurons from WT and RAGE-KO groups. Representative images for annexinV-FITC and propidium iodide (PI), in control (CNT), high-glucose (HG), and 1mM H_2O_2 (as positive control) from **(A)** WT and **(C)** RAGE-KO cultured hippocampal neurons (P0-P2) (40x magnification). Yellow arrows show representative examples of an apoptotic/necrotic cell. Bar graphs summarize the percentage of annexinV- and PI-positive cells in CNT, HG, and 1 mM H_2O_2 from **(B)** WT and **(D)** RAGE-KO cultured hippocampal neurons respectively (n=4 dishes per condition). All data are represented as mean ± SEM. Means were statistically compared by two-way ANOVA followed by Sidak's multiple comparison test; *** $p<0.001$. Significant effect of H_2O_2 treatment on annexin-V ($F_{(2, 327)}$=906.3; $p<0.001$) and on PI ($F_{(2, 327)}$=868.2, $p<0.001$) positive cells. Scale bars represent 50 μm. BF: Bright Field

3.5 Discussion

This study provides novel evidence of RAGE-dependent hippocampal changes in high-glucose and diabetic conditions, which may contribute to the impairment of cognitive abilities in diabetic patients.

3.5.1 LTP impairment in diabetes

Type 1 and type 2 diabetes have both been associated with cognitive dysfunction (Kodl and Seaquist, 2008), which in animal models of diabetes are concomitant with biochemical and electrophysiological abnormalities in the CA1 region of the hippocampus, particularly with defects in LTP expression (Biessels et al., 1998; Lynch, 2004; Artola et al., 2005). Spatial learning and LTP expression in the CA1 region of the hippocampus were impaired in STZ-induced diabetic rats (Biessels et al., 1998; Artola et al., 2005).

The alterations in LTP observed in diabetic animal models can stem from both pre- and postsynaptic components (Trudeau et al., 2004). Impaired glutamate release was reported in the cerebral cortex of STZ-induced hyperglycemic rats (Guyot et al., 2000) and changes in PPF, a form of presynaptic plasticity indicative of the probability of neurotransmitter release (Trudeau et al., 2004), was shown to correlate with hippocampal LTP induction (Kleschevnikov et al., 1997). In our study, RAGE expression was required for both LTP impairment and increased PPF, indicating the RAGE-associated changes taking place during STZ-induced diabetes (insulin deficiency and hyperglycemia) were pre- and post-synaptic. The increase in PPF suggest that RAGE expression was required for the reduction in presynaptic neurotransmitter release, which would contribute to LTP impairment.

Statistical analysis of our LTP experiment also revealed a significant difference between the two genotypes. It has been previously reported that during CNS development RAGE is required for neurite outgrowth and neuronal differentiation (Wang et al., 2008), suggesting that RAGE-KO mice may have electrophysiological differences due to a developmental effect. Nevertheless, the STZ-induction of diabetes spared these mice from any further effect on LTP, indicating that RAGE expression is required for the induction of synaptic plasticity abnormalities in diabetic mice.

More importantly, we identified post-synaptic changes in AMPA receptors, which are essential for LTP induction (Isaac, 2003). These receptors, which are normally absent or electrophysiologically silent, are recruited to the membrane upon LTP induction. In diabetes, however, changes in receptor properties are shown as reduced [^{3}H] AMPA binding in various brain structures, including the hippocampus, in STZ-induced diabetic rats (Chabot et al, 1997; Gagne et al., 1997), and lower glutamate affinity for hippocampal AMPA receptors, supported by reduced GluA1 subunit immunoreactivity (Gagne et al., 1997). Furthermore, reductions in NMDA currents and NMDA receptor subunits (GluN1 and GluN2B) are reported in the hippocampus of STZ-induced diabetic animals (Gardoni et al., 2002; Nardin et al., 2016). However, GluN2A expression was increased in hippocampal synaptosomal fractions of non-obese diabetic (NOD) mice, which also showed impaired LTP expression in the CA1 region (Valastro et al., 2002).

3.5.2 RAGE signaling in hippocampal neurons

A strong association has been proposed between the activation of the AGE-RAGE pathway during diabetes and cognitive impairments characteristic of Alzheimer's disease (AD) (Srikanth et al., 2011). Treatment of hippocampal neurons or brain slices with AGEs decreases synaptic densities and impairs hippocampal LTP, effects that were largely RAGE-dependent (Zhang et al., 2014; Wang et al., 2018). How RAGE expression affects synaptic plasticity in diabetes, however, had not been studied until this work.

A key consequence of RAGE signaling is the activation of transcription factors—particularly NF-κB—and subsequent transcription of pro-inflammatory cytokines, RAGE up-regulation, and generation of more ROS (Granic et al., 2009). As in our model of STZ-induced insulin deficiency and hyperglycemia, it is reported that type 1 diabetes is linked to the increased expression/activation of NF-κB in the hippocampus (Patel et al., 2009).

Activation of the RAGE-NF-κB pathway induces oxidative stress, which may be the link between diabetes and synaptic transmission deficits as it affects the expression and function of glutamate receptors (Klann et al., 2011). In STZ-induced diabetic mice, as the level of oxidative stress increased a decline in expression of the AMPA GluA2 subunit was observed in the hippocampus (Pandey et al., 2015). Our data do not show a significant decrease in GluA2 subunits, although a

decreased trend was observed in WT but not in RAGE-KO STZ-induced diabetic mice. However, we did observe a significant reduction in the expression of the AMPA GluA1 subunit in STZ-induced diabetic mice that required RAGE expression, suggesting it may play a key role in synaptic alterations in diabetes.

The phosphorylation of the AMPA GluA1 subunit at Ser845 is key for surface expression of AMPA upon NMDA activation during activity-dependent LTP (Oh et al., 2006; Derkach et al., 2007). The latter is consistent with our findings showing reduced expression of the GluA1 subunit and pSer831 and pSer845 GluA1 in the hippocampi of WT STZ-induced diabetic mice, which correlates with the impaired LTP detected in these mice. Therefore, our findings may be the result of changes in subunit incorporation, phosphorylation state, and surface expression of AMPA receptors.

In addition to effects on AMPA receptors, we observed a decrease in neuronal excitability in cultured hippocampal neurons exposed to high-glucose conditions only in the WT group. This is consistent with other findings of reduced excitability of hippocampal neurons in hyperglycemia (Chandna et al., 2015b), oxidative stress (Pardillo-Diaz et al., 2016), and aging (Oh et al., 2010). None of the action potential or membrane property parameters that we studied showed changes that could underlie the reduction in excitability. Therefore, further research on ion channels not directly involved in spike generation (e.g. voltage-gated Ca^{2+} channels) should be considered.

3.5.3 MAPKs and modulation of synaptic transmission

To better understand the relationship between RAGE signaling and changes in AMPA receptor expression/function in STZ-induced diabetes, we first concentrated on the mitogen-activated protein kinase (MAPK) family, which regulate synaptic plasticity and signaling glutamate receptor trafficking (Gu and Stornetta, 2007). In fact, inhibition of the MAPK cascade causes a strong attenuation during LTP induction in the hippocampus CA1 region (English and Sweatt, 1997). All three members of the MAPK family (ERK, p38 and JNK) are involved in modulation of synaptic plasticity (Thomas and Huganir, 2004).

The MAPK cascade also regulates AMPA receptor trafficking in the hippocampus (Boudreau et al., 2007). ERK activation is suggested to mediate synaptic insertion of AMPA receptors during

LTP (Boudreau et al., 2007). Consistent with our findings, AMPA receptor insertion during LTP is associated with decreased p38 activity (Boudreau et al., 2007).

The role of JNK in AMPA receptor trafficking is, however, more controversial. During LTP, NMDA-induced JNK signaling mediates removal of GluA1- and GluA2-containing AMPA receptors (Zhu et al., 2005). However, metabotropic glutamate receptor-induced activation of calcium/calmodulin-dependent protein kinase (pCaMK)/pJNK and/or PKA/pJNK is thought to increase phosphorylation of GluA1-Ser831 and Ser845 (Ahn and Choe, 2009) and subsequently an increase in its surface expression (Oh et al., 2006). The latter parallels our findings showing a reduction in pJNK together with reduced pSer845 of the GluA1 AMPA receptor subunit, which should reduce AMPA surface expression.

Also comparable with our findings, a reduction in pJNK was reported in the hippocampus of STZ-treated rats while the pERK/total ERK ratio was unaffected (Dalli et al., 2018). However, reports by others show that phosphorylation of ERK1/2 and p38 were higher in the hippocampus of STZ-induced diabetic rats, while the level of pJNK was not changed (Jing et al., 2013). As in our study, pp38/total p38 ratio was higher in the hippocampus and cortex of STZ-induced rats (Liu et al., 2016).

Hyperglycemia-induced oxidative stress is one of the important upstream mediators of MAPK activation (Purves et al., 2001). As in our study, oxidative stress activated p38 in sensory neurons after exposure to high glucose *in vitro*, after STZ treatment *in vivo,* and in the sural nerves of type 1 and type 2 diabetic patients (Sharma et al., 2010). The increase in hyperglycemia-induced oxidative stress is indeed initiated/exacerbated by RAGE ligands, which phosphorylate and activate various protein kinases involving MAPKs and subsequently the NF-κB pathway (Zong et al., 2010).

The contribution of p38 signaling to the activation of NF-κB occurs through the phosphorylation of the transcriptionally active subunit of the NF-κB complex (Olson et al., 2007). Interestingly, NF-κB was shown to negatively regulate JNK (Lin, 2003) through growth arrest and DNA damage-inducing protein β (GADD45β), by binding to, and inhibiting, the JNK kinase, mitogen-activated protein kinase kinase 7 (MKK7 or MEK7) (Nakano, 2004). The latter supports our findings showing a decrease in JNK and MEK7 phosphorylation, along with an increase in NF-κB expression.

We also show a reduction in GluA1 subunit expression and phosphorylation, concomitant with reduced JNK phosphorylation and MEK7 expression, suggesting these mechanisms may contribute to hippocampal impairment in diabetes. The increase in phosphorylated and total p38 protein level, on the other hand, may lead to the generation of pro-inflammatory responses by regulating NF-κB activity, which further amplifies oxidative stress and promotes RAGE up-regulation—a cycle that can intensify diabetic complications and inflammation-induced tissue injury (Figure 3.10).

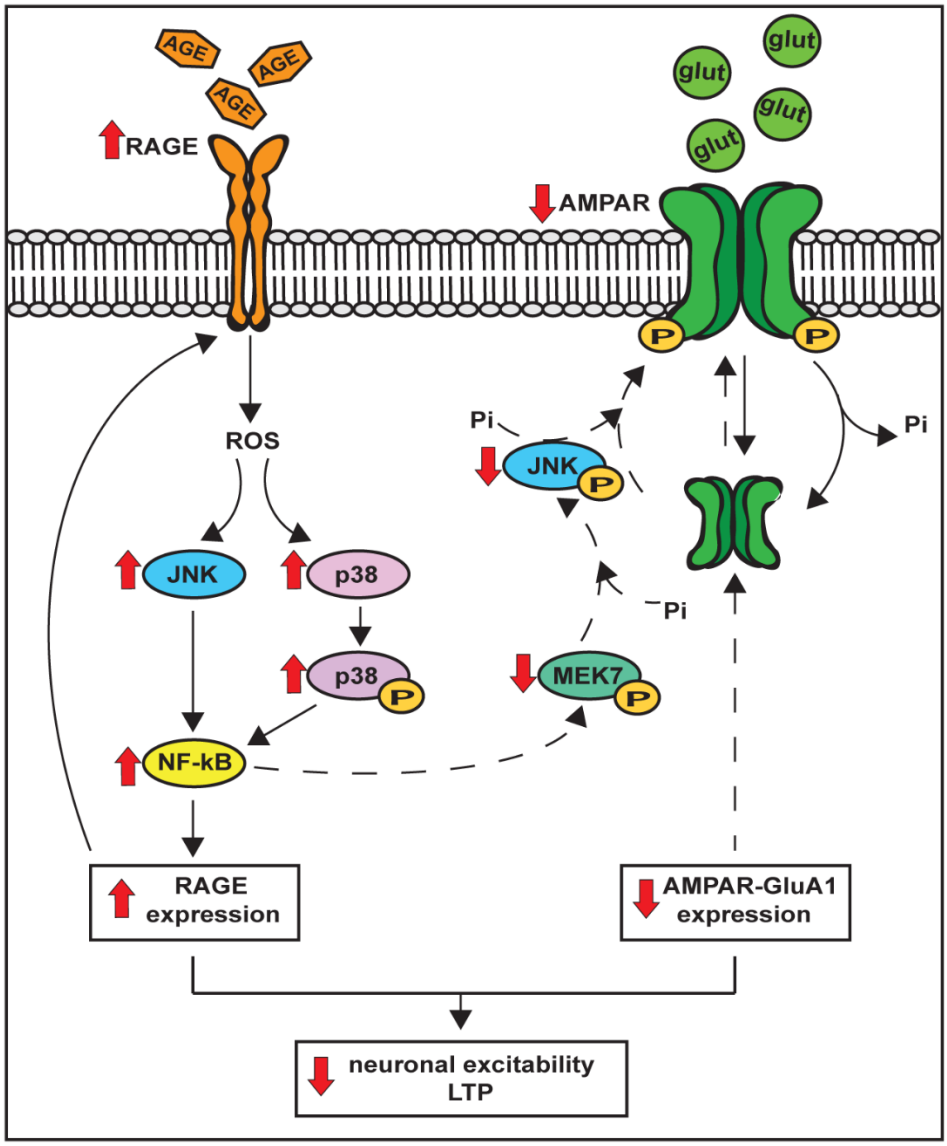


Figure 3.10. A schematic diagram showing the postulated mechanisms underlying the RAGE-dependent synaptic impairments in the hippocampus of diabetic mice. Activation of the RAGE pathways by its ligands (e.g. AGEs) in diabetes leads to oxidative stress, which in turn increases total JNK and p38 protein levels, as well as p38 phosphorylation. Increases in JNK and p38 result in increased activation of NF-κB, which further promotes RAGE up-regulation and amplifies oxidative stress. In addition, NF-κB negatively regulates JNK phosphorylation by inhibiting the JNK kinase, MEK7. Reduced JNK activity, due to reduced JNK phosphorylation, leads to decreased AMPA GluA1 subunit expression and phosphorylation and subsequent internalization of the receptor. These changes will result in decreased neuronal excitability and LTP impairment. Dashed lines indicate pathways that were downregulated/inactive in diabetic mice, and red arrows represent changes in protein detection by immunoblotting.

Although the main focus of this work was to investigate whether/how RAGE plays a role in some hippocampal complications of diabetes, it is important to note that the role and interplay of other possible factors also need to be taken into consideration in our model. Insulin, for example, is one of these factors. The interplay between hippocampal insulin receptors and RAGE would be worth investigating since MPAKs are shown to affect insulin receptors, and in turn, insulin therapy is found to affect the activity of MAPK signaling molecules (Iloun et al., 2018). Furthermore, insulin signaling is reported to affect glutamate receptor trafficking and synaptic plasticity in the hippocampus (Grillo et al., 2015). In our study, we generated a model of diabetes based on insulin deficiency and hyperglycemia, with levels of circulating insulin below the detectable range by ELISA. The fact that our insulin-deficient diabetic model was generated in both genotypes with similar insulin and glycemic parameters, allowed us to evaluate RAGE as a variable. However, considering that insulin can modulate synaptic transmission and plasticity in the hippocampus (Zhao et al., 2019), and that application of exogenous insulin (e.g. intraventricular and intraperitoneal) has provided some encouraging outcomes, particularly at the behavioral studies (Benedict et al., 2007; Shemesh et al., 2012), it becomes relevant to evaluate the effect of insulin on the RAGE-mediated deleterious effects of diabetes described here. The effects of exogenous insulin on hippocampal function and in the context of diabetes are contradictory (Maimaiti et al., 2016; Bell and Fadool, 2017) and further research will be required to evaluate the contribution of RAGE in not only models of insulin deficiency (such as STZ-induction), but also in insulin resistance and exogenous insulin administration.

CHAPTER 4

GENERAL DISCUSSION

Diabetes is a most common metabolic disorder with long-lasting complications and abnormalities in various body functions and systems. Micro- and macrovascular complications, as well as vasculature-independent cellular complications of diabetes have been well investigated in clinical and experimental diabetes. RAGE is shown to play a part in most diabetic complications. However, there is still the need for further knowledge on the role and/or the mechanisms of RAGE signaling in CNS complications of diabetes, particularly on those that impact quality of life in diabetic patients, such as impairments in learning and memory and cognitive function. In this dissertation, I tried to answer the question of whether RAGE is involved in cognitive dysfunction in diabetes and what RAGE-dependent mechanisms could underlie these detrimental effects of the disease.

4.1 Major findings

We found that STZ-induced diabetes caused a significant reduction in locomotor activity and an increase in anxiety-like behaviour in both WT and RAGE-KO mice. Our findings also showed that although recognition memory was spared in STZ-induced diabetes in both genotypes, spatial memory was impaired in STZ-induced diabetic mice in WT but not in the RAGE-KO group. This impairment in hippocampal-dependent spatial memory was consistent with impairment in synaptic plasticity, as measure by LTP and PPF, in the hippocampus and correlated with reduced expression and phosphorylation of the GluA1 subunit of the AMPA receptor in WT STZ-induced diabetic mice. These changes were associated with the activation of the MAPK pathway, i.e. increased total p38, pp38, and NF-κB and decreased pJNK and its kinase, MEK7. Consistently, in hippocampal cultures, high glucose caused a RAGE-dependent reduction of AMPA-evoked currents, as well as cell excitability, and an increase in cytosolic ROS.

4.2 STZ-induced behavioural abnormalities

Our findings show that STZ-induced diabetes causes a deficit in locomotor activity in both WT and RAGE-KO groups, shown as a significant reduction in distance traveled and movement time. This is consistent with previous reports indicating a decrease in locomotor activity in STZ-induced diabetic animals (Haider et al., 2013; Bădescu et al., 2016b; Bensaoula et al., 2016; Gao et al., 2018). Such reduction in locomotor activity is suggested to be associated with deficits in motor function due to dysregulation of the cholinergic system (Sherin et al., 2012). In particular, significant downregulation of acetylcholine esterase (AChE) and choline acetyltransferase (ChAT) and changes in the expression and binding properties of muscarinic and nicotinic acetylcholine receptors in the striatum are reported in STZ-induced diabetic rats (Sherin et al., 2012). In addition, the cerebellum, a brain area also involved in the control and execution of motor functions, has shown several alterations such as increased cellular degeneration, increased activation and proliferation of astroglia and microglia, loss of glutamate transporters on the astrocytes and subsequent glutamate excitotoxicity in STZ-induced diabetic rats with impaired locomotor activity (Nagayach et al., 2014). The associative interplay between these factors in the cerebellum is suggested to result in motor function deficits following diabetes (Nagayach et al., 2014). In our study, however, we cannot conclude if decreased locomotor activity observed in STZ-induced diabetic mice is due to motor function deficits or due to other factors such as higher anxiety or other effects associated with the STZ treatment.

We also observed a significant effect of the STZ treatment on decreased time spent in the center of the OF, which could be interpreted as an anxiety-like behaviour; however, we can not discard the possibility that it could be the result of lower locomotor activity. The results of the MWM test showing increased distance traveled and swimming velocity in STZ-induced diabetic mice, however, support an anxiety-like phenotype in this group as compared to age-matched controls. Increased anxiety-like behaviours have been previously reported in STZ-induced diabetic animals (Aksu et al. 2012; Gupta et al., 2014; Damian et al., 2014; Chu et al., 2017) and have been correlated with an increase in the HPA axis activity and glucocorticoid levels, lower levels of insulin and insulin-like growth factors (IGFs), reduced number of neurons, and changes in neurotransmitters, such as GABA and monoamines, in different regions of the brain such as prefrontal cortex (PFC), amygdala and the hippocampus in response to diabetes (Duarte et al.,

2000; Gomez et al., 2003; Antony et al., 2010; Shpakov et al., 2011; Aksu et al. 2012; Torres et al., 2013 ; Gupta et al., 2014; Soto et al., 2019).

The result of the NOR test, on the other hand, did not show any difference in the novel object recognition memory between STZ-induced diabetic mice and age-matched control in either genotype. This is inconsistent with previous reports that indicated impairments in novel object recognition memory, shown as a significant reduction in the discrimination index, following STZ-induced diabetes (King et al., 2013; Jabbarpour et a., 2014; Patel et al., 2016). Such discrepancy might be due to longer duration of STZ treatment (from eight to ten weeks) in the above-mentioned studies as compared to the present study (King et al., 2013; Jabbarpour et a., 2014; Patel et al., 2016). In this regard, the duration of diabetes is shown to have an impact on the severity of diabetic complications such as cognitive functions (Zilliox et al., 2016).

The rodent hippocampus is suggested to play a role in object recognition memory, although other brain regions, such as perirhinal cortex, are also reported to play important roles (Brown and Aggleton 2001). Hippocampal glutamate efflux and mean firing rate of CA1 neurons showed increases during NOR test session (Cohen et al., 2013), consistent with other reports that showed novelty-induced increases in hippocampal activity (Rutishauser et al., 2008; Cohen et al., 2013), suggesting a role for the hippocampus in recognition memory. On the other hand, reports are controversial regarding the effect of hippocampal damage on the NOR test. While some studies report that hippocampal lesions as small as 1% can affect the novel object recognition memory (Cohen et al., 2013), others show spared recognition memory following hippocampal damage (Winters et al. 2004; Forwood et al. 2005; Mumby et al. 2005), even after lesions that encompassed about 75 to 100% of hippocampal volume (Broadbent et al., 2004), suggesting lack of hippocampal involvement in NOR memory.

We did not conduct any experiments to investigate changes in hippocampal structural integrity, such as hippocampal volume and neuronal loss, and we cannot therefore come into any conclusion as to why recognition memory is spared in our STZ-induced diabetic mice. However, hippocampal damage by STZ-induced diabetes, reported previously (Pamidi et al., 2012), without any impact on the novel object recognition memory in our STZ-induced diabetic mice can suggest minimal or lack of hippocampal involvement in the task and/or larger involvement of other brain regions, such

as perirhinal regions, or the possibility of compensatory mechanisms by other structures within the medial temporal lobe (MTL) (Cohen et al., 2013), as discussed previously (Chapter 2).

In addition, there is no published evidence, to our knowledge, on the role of RAGE in recognition memory under normal or pathological conditions. However, since our findings show a similar trend in discrimination index in both WT and RAGE-KO groups, we can infer that recognition memory is independent of RAGE expression and/or signaling, at least during the period our experiments lasted.

Similarly, since our data shows a comparable trend in the parameters associated with locomotor activity in both WT and RAGE-KO groups, as reported previously (Sakatani et al., 2009), we can indicate that these parameters are also independent of RAGE expression and/or signaling. Anxiety-like behaviour, however, seems to be dependent on RAGE expression since there was a significant genotype difference between RAGE-KO and WT mice in the time spent in the center of the OF test.

Our hippocampal dependent-spatial memory tests, on the other hand, showed impairment in spatial memory in BM and MWM tasks in WT STZ-induced diabetic mice. This is consistent with other studies that show impairment in hippocampal-dependent spatial tasks such as MWM and BM in STZ-induced animal models of diabetes (Baydas et al., 2003b; Stranahan et al., 2008; Jolivalt et al., 2010; Babri et al., 2013; Enhamre-Brolin et al., 2013; King et al., 2013; Anderson et al., 2014; Ghasemi et al., 2016; Tender and Razdan 2017).

In the RAGE-KO group, however, there was no significant difference between STZ-induced diabetic group and the control in terms of spatial memory function, confirming that the effect of STZ on spatial memory differed significantly between the two genotypes.

Hippocampal-dependent cognitive impairments elicited by diabetes are suggested to be associated with morphological changes in pyramidal neurons (Magarinos and McEwen, 2000), decreased hippocampal neurogenesis (Stranahan et al., 2008), alteration in neurotransmitter synthesis or release (Trudeau et al., 2004), and electrophysiological dysfunction in the hippocampus, particularly defects in the expression of LTP (Biessels et al., 2002), as well as changes in the expression and function of glutamate receptors (Gispen and Biessels 2000; Trudeau et al., 2004). However, whether and if so how RAGE plays a role in these effects has not been investigated. Therefore, in order to better understand the mechanisms associated with hippocampal-dependent

spatial memory impairment observed in our WT STZ-induced diabetic mice and the role of RAGE in this effect, we evaluated LTP, as a robust measure of synaptic plasticity that underlies mechanisms associated with memory (Nabavi et al., 2014).

4.3 STZ-induced impairment in synaptic plasticity

The results of our hippocampal slice electrophysiology showed an impairment in LTP along with an increased PPF ratio in STZ-induced diabetic WT mice. Our data is consistent with a wide range of literature that shows impairment in LTP in animal models of diabetes (Izumi et al., 2003; Trudeau et al., 2004; Artola et al., 2005; Kamal et al., 2005; Stranahan et al., 2008; Reisi et al., 2010; Sasaki-Hamada et al., 2012).

As mentioned in Chapter 3, pre- and postsynaptic components can contribute to hippocampal LTP impairment during diabetes (Blundon and Zakharenko 2008; Costa et al., 2017). In the presynapse, alterations in the synthesis and release of neurotransmitters could underlie changes in LTP (Trudeau et al., 2004; Lovinger, 2010). In order to study and quantify these changes, PPF is considered a useful electrophysiological tool for detection of presynaptic changes, such as probability of neurotransmitter release, for example, increased PPF is indicative of decreased probability of neurotransmitter release and vice versa (Schulz et al., 1994 and 1997; Trudeau et al., 2004). In the case of a decreased probability of neurotransmitter release, there will be a small postsynaptic response during the first pulse, but the build-up of calcium in the presynaptic terminal will cause a higher probability of release on the second pulse, and thus, a higher PPF. In contrast, in the case of an increased probability of neurotransmitter release, available transmitters will be depleted during the first pulse, and therefore there will be less transmitter to be released during the second pulse, leading to a lower PPF (Volgushev et al., 1997). Thus, the increased PPF and impaired LTP observed in our study might be due, at least in part, to reduction in neurotransmitter release from the presynapse. The latter has indeed been reported in some brain regions in diabetic rodents, such as impaired glutamate transportation and release in response to STZ-induced hyperglycemia in the cerebellum and cerebral cortex of rats (Guyot et al, 2000; Trudeau et al., 2004; Nagayach et al., 2014).

On the other hand, lack of impairment in hippocampal LTP in our RAGE-KO model can be attributed, at least in part, to lack of changes in pre- and post-synaptic components, i.e. no changes in PPF nor in AMPA GluA1 subunit expression, phosphorylation, and function.

Inconsistent with our data from hippocampal slices that suggest a decreased probability of neurotransmitter release, our cultured hippocampal neurons exposed to high glucose condition showed an increased frequency of mEPSCs (Appendix 1, Figure A.2), which could suggest an increased probability of presynaptic transmitter release (Ju et al., 2016). This discrepancy can be attributed, at least in part, to the limitations of the culture conditions, especially since our cultured hippocampal neurons form *de novo* synapses that, although glutamatergic, may not mimic the properties of actual hippocampal CA3-CA1 synapses.

In the postsynapse, the impairment in LTP can be linked to the posttranslational modification of glutamate receptors (Trudeau et al., 2004). The latter is consistent with our finding showing that in the hippocampus of diabetic mice there was a reduction in expression and phosphorylation (Ser-831 and 845) of the GluA1 subunit of AMPA receptors. Indeed, phosphorylation of GluA1 on Ser-831 by CaMKII and on Ser-845 by PKA are important modifications that potentiate channel conductance and increase its open probability, respectively (Banke et al., 2000; Derkach, 2003). Furthermore, phosphorylation on Ser-845 increases GluA1 delivery to the plasma membrane modulating AMPA receptor surface expression during LTP (Ehlers, 2000; Oh et al, 2006), a mechanisms known to be triggered by NMDA receptor activation (Derkach et al, 2007). Thus, the significant decrease in the expression and phosphorylation of GluA1 subunit of AMPA receptor in our WT STZ-induced diabetic mice could explain the impairment observed in LTP due to changes in receptor function and trafficking, and therefore, in synaptic strength and plasticity (Chater and Goda, 2014).

It also needs to be mentioned that, consistent with our data from hippocampal tissues, we observed a decreased expression of GluA1 subunit of AMPA receptor (although not quite significant) in cultured hippocampal neurons exposed to high glucose condition in cells from WT mice (Appendix 1, Figure A.3). We also detected a reduction in the expression of GluN1 subunit of NMDA receptor in both WT and RAGE-KO hippocampal cultures exposed to high glucose condition (Appendix 1, Figure A.3). This reduction in GluN1 subunit of NMDA receptor was not accompanied by electrophysiological changes in NMDA receptor-evoked currents and/or ionic

charge (Chapter 3). In addition, hippocampal tissues showed no significant differences in the expression of NMDA receptor subunits in either WT or RAGE-KO STZ-induced diabetic mice (Chapter 3).

These discrepancies between cultured pyramidal neurons and actual hippocampal tissues, again, point to the limitations of culture conditions and highlight the need to validate results in actual tissues.

Similarly, the literature on the effect of diabetes on NMDA receptors is controversial. For example, an increase in the GluN2A subunit level in crude hippocampal synaptosomal fractions (Valastro et al, 2002) and a significant increase in expression of hippocampal GluN1, GluN2A and GluN2B subunits (Wang et al., 2019) are reported in STZ-induced diabetic rodents. Some reports, on the other hand, indicate reductions in NMDA receptor expression and function. For instance, STZ-induced diabetic rats showed a significant reduction in hippocampal content of the GluN1 subunit (Nardin et al, 2016), which indicates a reduction in the number of functional NMDA receptors (Salussolia et al., 2011). In addition, intracellular recordings from hippocampal slices of STZ-induced diabetic rats showed reduced NMDA-evoked currents in pyramidal neurons together with a significant decrease in GluN2B subunit immunoreactivity (Gardoni et al, 2002). In contrast, NMDA-mediated component of excitatory postsynaptic potentials was unaffected after STZ treatment, suggesting that NMDA receptor function remained intact in STZ-induced diabetes (Chabot et al, 1997).

Although research on the effect of diabetes on glutamate receptors expression and function is still ongoing and reports are controversial, factors such as duration of diabetes, age of animals at the onset of diabetes induction, and differences in the high glucose/diabetic models seem to play a role in such discrepancies.

Although our results in cultured hippocampal neurons exposed to high glucose condition are not in accordance with those of STZ-induced diabetic mice in terms of the NMDA receptor expression, we did observe the same effect of high glucose in cultured neurons or STZ-induced diabetes in both genotypes. In cultured hippocampal neurons NMDA receptor expression showed reduction in GluN1 subunit expression in both WT and RAGE-KO groups exposed to high glucose, while in hippocampal tissues there was no significant differences in NMDA subunits expression in either

WT or RAGE-KO STZ-induced diabetic mice. These results underscore the possibility that NMDA receptor expression and function is independent of RAGE expression and/or signaling.

4.4 STZ-induced RAGE-mediated effects

As discussed above, impaired hippocampal-dependent spatial memory, reduced LTP and increased PPF, and changes in AMPA receptor expression, phosphorylation and function were observed in STZ-induced diabetes or high glucose condition in WT but not in RAGE-KO mice. In addition, significant increase in RAGE expression was detected in the hippocampi of WT STZ-induced diabetic mice. These findings suggest a contribution of RAGE in mediating the STZ-induced abnormalities observed in our study.

One of the most likely signaling pathway candidates linking RAGE with synaptic impairment and cognitive dysfunction is the MAPKs pathway (Gu and Stornetta, 2007). As mentioned in Chapter 1, MAPKs (ERK, p38 and JNK) are not only part of a well-known downstream signaling pathway from RAGE (Yeh et al., 2001), but are also abundantly expressed in the synapses (Gu and Stornetta, 2007) where they regulate synaptic function and glutamate receptor trafficking (Gu and Stornetta, 2007).

Our findings show that although ERK and pERK levels were unaffected, total p38, pp38 and total JNK were increased and pJNK and its kinase, MEK7, were decreased in STZ-induced diabetic mice that had synaptic deficit and memory dysfunction.

Consistent with this, phosphorylation of JNK was significantly decreased in the hippocampus of STZ-induced diabetic rats while the pERK/total ERK ratio was unaffected (Dalli et al., 2018). In another study, however, phosphorylation levels of ERK and p38 were higher in the hippocampus of diabetic rats, while the level of pJNK was not changed (Jing et al., 2013). Pp38/total p38 ratio was also significantly higher in the hippocampus of STZ-induced diabetic rats (Liu et al., 2016).

Hyperglycemia-induced oxidative stress, initiated and/or exacerbated by RAGE-ligands binding, is one of the important upstream mediators of MAPKs activation (Purves et al., 2001; Price et al., 2004; Sharma et al., 2010) and is shown to phosphorylate and activate various MAPKs that subsequently activate NF-κB (Yeh et al., 2001; Hermani et al., 2006; Wang et al., 2008; Zong et al., 2010). For example, phosphorylation of p38 MAPK and the activation of NF-κB were

significantly up-regulated when macrophages were cultured with lipopolysaccharide (LPS) and HMGB1, two RAGE ligands (Qin et al., 2009). Similarly, Aβ-induced RAGE involvement led to the activation of ERK and JNK, and subsequently NF-κB (Origlia et al., 2009; Pan et al., 2013). On the other hand, NF-κB negatively regulates the activation of the JNK pathway (Lin 2003) through GADD45β which binds to, and inhibits, the JNK kinase MEK7 (Nakano 2004). Reduced JNK phosphorylation/activity can have an impact on AMPA receptor trafficking as reported previously and controversially (Zhu et al., 2005; Ahn and Choe, 2009). The NMDA-induced JNK signaling pathway, for example, mediated removal of GluA1-and GluA2L-containing AMPA receptors during LTP (Zhu et al., 2005), while mGluRs-induced activation of PKA/pJNK pathways increased phosphorylation of GluA1-Ser831 and Ser845 (Ahn and Choe, 2009), and subsequent increase in AMPA receptor surface expression (Oh et al., 2006). This is in congruent with our results that showed reduced pJNK in line with reduced phosphorylation of GluA1-Ser831 and Ser845.

Therefore, our findings showing an increase in cytosolic ROS production in cultured hippocampal neurons exposed to high glucose condition, and an increase in NF-κB levels along with significant decreases in pJNK, pMEK7 and GluA1-Ser831 and Ser845 phosphorylation in the hippocampi of WT STZ-induced diabetic mice are consistent with the reports in this field and can highlight the role of RAGE in mediating these effects, as none were observed in the RAGE-KO group.

It needs to be noted that there was a significant genotype difference in cytosolic ROS levels detected by CM-H_2DCFDA between WT and RAGE-KO hippocampal cultured neurons. Although in our study we did not explore the possible source of this difference, there are two main considerations that may be contributing to this effect. First, and as explained earlier in this thesis, is the physiological effect of RAGE during early stages of CNS development (Wang et al., 2008), although it is unclear how this could have an impact on basal redox state of culture hippocampal neurons. Second, a different level of cytosolic ROS between WT and RAGE-KO groups could indicate that RAGE, which is normally expressed in neurons at lower basal levels, is required to maintain basal redox state in neurons, and that lack of RAGE signaling may dysregulate signaling pathways involved in redox homeostasis. Indeed, differences in basal cytosolic ROS levels between WT and RAGE-KO mice have been previously reported in peripheral neurons, supporting

the idea that RAGE may play a physiological role in contributing to neuronal homeostasis (Chandna et al., 2015a).

In addition, CM-H_2DCFDA detects all individual hydrogen peroxide, superoxide anions, and the hydroxyl radicals, and the exact ROS profile cannot therefore be defined and distinguished. Some ROS species such as hydroxyl radicals have more deleterious effects (Yun et al., 2009) as compared to others such as superoxide anions, which play important roles in synaptic plasticity and memory by acting as a modulator of two kinases, CaMKII and PKA, which are involved in synaptic transmission (Hongpaisan et al., 2003 and 2004).

In addition to increased ROS, our study also showed reduced cell excitability in cultured hippocampal neurons exposed to high glucose condition in the WT group. Interestingly, MAPKs signaling pathway plays a role in neuronal excitability as well (Chen et al., 2017). P38 MAPK inhibition, for example, increased hippocampal pyramidal neuron excitability (Poolos et al., 2006; Jung et al., 2010), while inhibition of JNK had the opposite effect (Tai et al., 2017). Activation of p38 phosphorylates the voltage-gated sodium channel Nav1.6, which decreases its function (Wittmack et al., 2005), and impairs action potential generation in CA1 pyramidal neurons (Royeck et al., 2008). This is consistent with our findings that show a reduction in hippocampal neuronal excitability along with an increase in p38 phosphorylation/activity and reduced pJNK under high glucose/diabetic conditions, and further supports the role of RAGE in mediating impairment in hippocampal function.

4.5 Challenges

4.5.1 Effect of genotype

As this was the first work, to our knowledge, that investigated the role of RAGE in diabetes-induced hippocampal dysfunction, such as deficits in synaptic plasticity and cognitive impairment, there were not many studies available to compare and interpret our findings.

During the analysis of some of our data we encountered the effect of genotype at the animal and cellular levels, which indicate that in addition to the pathological roles played by RAGE in the CNS during diabetes, there are some effects that need to be further considered when modeling diabetes in RAGE-KO mice. As discussed previously, RAGE expression is required for neuronal

differentiation during CNS development (Wang et al., 2008). However, how this could explain the differences we observed between the two genotypes is rather complex. The fact that cytosolic ROS levels were elevated in RAGE-KO neurons respect to WT may imply that overlapping signals are recruiting similar pathway(s), which may interfere with the effects of diabetes. Therefore, our findings on the effect of genotype may point at the difficulty of targeting RAGE and its signaling pathway for the treatment of diabetes.

4.5.2 STZ-induced diabetic model

Another limitation is related to the drawbacks of chemically-induced diabetes, i.e. by STZ due to its potential toxicity to other organs. Administration of STZ has been associated with hyperalgesia, hepatotoxicity and nephrotoxicity as well as changes in the lungs, intestines and testes, of which some are not reversible with insulin treatment (Palm et al., 2004; Graham et al., 2011; Gvazava et al. 2018). Although peripherally administered STZ does not cross the BBB and, therefore, cannot affect the brain directly (Šerbedžija and Ishii, 2012), peripheral side effects of STZ on animal's well-being should not be overlooked. However, since our insulin deficient diabetic model was generated in both genotypes with similar insulin and glycemic parameters, RAGE can still be evaluated as a variable in our study.

4.5.3 Cell cultures exposed to high glucose

Although the cell cultures in our experimental groups were exposed to high glucose (25 mM) for a period of 1-2 weeks to mimic the chronic nature of diabetes, it needs to be noted that the glucose level usually increases gradually during the course of the disease in actual hyperglycemic state while our cultured hippocampal neurons were exposed to high glucose on the first day of culture. This points to another limitation of our cell culture model and emphasises the need to validate the results in actual hyperglycemic states in whole-animal models of diabetes, including pharmacologically induced and genetic models.

4.6 Future directions

Now that we have suggested a working model, the following experiments can be taken into consideration for future directions in this field.

1. The interplay between hippocampal insulin receptors and RAGE would be worth investigating since MPAKs are shown to affect insulin receptors, and in turn, inulin therapy is found to affect the activity of MAPK signaling molecules (Iloun et al., 2018). In addition, insulin signaling is reported to affect glutamate receptor trafficking and synaptic plasticity in the hippocampus (Grillo et al., 2015).

Hippocampal insulin receptor expression and/or function can be evaluated in both WT and RAGE-KO model in order to elucidate the role of the insulin receptor in some of the effects observed. Alternatively, the effect of insulin therapy can be evaluated in STZ-induced diabetic mice.

2. Glucocorticoid receptors (GCRs) are another strong candidate to be considered since they are not only abundantly expressed in the hippocampus and show significant upregulation under diabetes, but also affect synaptic plasticity and cognitive function (Stranahan et al., 2008). Although there is no data available regarding the interaction between RAGE and GCRs, blockade of GCRs was shown to improve synaptic plasticity and cognitive function under diabetes (Stranahan et al., 2008), similar to what we observed in STZ-induced diabetic mice in the RAGE-KO group. However, whether GCRs cause their negative effects independently or through interaction with other receptors such as RAGE needs further investigation.

3. Since our model suggests an important role of pp38 in activating the NF-κB and subsequent effects on MEK7 and pJNK, taking advantage of the knock-in $p38^{AF}$ mouse model, which harbours dominant-negative mutations of the activating phosphorylation sites of the p38, could help better elucidate the role of this MAPK signaling molecule in the effects observed under diabetes.

4. In addition to NF-κB, the role of other transcription factors such as cAMP-response element binding protein (CREB) can be evaluated as well, since CREB is also regulated by MAPKs and is involved in hippocampal synaptic plasticity and cognitive function (Benito and Barco 2010; Kandel 2012).

5. Looking further into other diabetes-induced hippocampal alterations such as hippocampal volume, neuronal density, morphological changes in pyramidal neurons, changes in glutamate synthesis and release and astroglial alterations, specially in STZ-treated RAGE-KO mice, could be of high importance as the role of RAGE has never been investigated in such diabetes-induced alterations.

6. Since we propose RAGE-induced oxidative stress as an important upstream mediator of the MAPK signaling pathway and the negative effects of MAPKs in this correlational model, it would be worthwhile to investigate the effect of antioxidant treatment on MAPKs activation and AMPA receptor expression and function.

In addition, looking further into the baseline differences in ROS level between WT and RAGE-KO hippocampal neurons, such as identifying the profile of oxidative stress and distinguishing between the different species of free radicals, would be beneficial in better understanding the role of oxidative stress in the effects observed.

7. Since the duration of diabetes and the age at the onset of diabetes play important roles in diabetic complications in animal models (Chen et al., 2005; Rajashree et al., 2011; Sasaki-Hamada et al., 2012), it would be valuable to test the proposed model after different durations of diabetes and different ages at the onset of STZ treatment.

8. The effects of RAGE knockout on CNS complications in type 2 diabetes would also be worth investigating, especially regarding the fact that type 2 diabetes is close to 90-95% of diabetic cases worldwide and that it is accompanied by reduced performance on multiple domains of cognitive function (Moheet et al., 2015).

4.7 Conclusion

Although the role of RAGE has been well investigated in peripheral complications of diabetes, much less is known about the contribution of RAGE to CNS complications of the disease. This work is the first, to our knowledge, that shows the role of RAGE in hippocampal synaptic deficit and spatial memory impairment under diabetes.

We postulate that RAGE-mediated oxidative stress under high glucose/diabetic condition induces increased phosphorylation and total p38 protein level as well as total JNK level, which result in increased activation of NF-κB and subsequent RAGE up-regulation and amplified oxidative stress. Activated NF-κB negatively regulates JNK phosphorylation by inhibiting the JNK kinase, MEK7. Reduced JNK activity due to reduced phosphorylation then leads to decreased AMPA GluA1 subunit expression and phosphorylation and subsequent internalization of the receptor. These changes will result in decreased neuronal excitability, synaptic impairments and spatial memory deficit observed in this study.

Although the main focus of this work was to investigate whether/how RAGE plays a role in hippocampal complications of diabetes, it is important to note that the role and interplay of other possible factors also need to be taken into consideration in this model for future investigations, as explained in the previous section. However, we hope that this primary model can provoke further understanding of CNS complications of diabetes and can serve as a start for developing new research in this area.

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APPENDICES

APPENDIX A

Role of RAGE signaling on hippocampal mEPSCs in cultured neurons exposed to high glucose condition

A.1 Introduction

Diabetes, a very common metabolic disorder characterized by hyperglycemia, has long been associated with pathological complications in the peripheral and central nervous system (American Diabetes Association 2010). Deficits in hippocampal-dependent cognitive functions such as learning and memory impairments, which can originate from defects in hippocampal synaptic plasticity has been widely reported in diabetes (Kodl and Seaquist 2008; Wrighten et al., 2009; Rostami et al., 2013, Moheet et al., 2015; Saedi et al., 2016). On the other hand, NMDA and AMPA subtypes of glutamate receptors, as the major excitatory receptors within the central nervous system, have been shown to play important roles in synaptic transmission during learning and memory processes (Trudeau et al., 2004). Findings in the diabetic hippocampus describe changes in AMPA and NMDA receptors which may underlie impaired synaptic plasticity found in diabetic models (Trudeau et al., 2004; Sasaki-Hamada et al., 2012). RAGE, on the other hand, has been identified to be actively involved in vascular and nervous system pathologies of diabetes (Ramasamy et al., 2005; Yamaguchi et al., 2009; Chen et al., 2012; Kanasaki et al., 2013; Manigrasso et al., 2014). However, if RAGE plays a role in mediating diabetes-induced changes in glutamate receptor expression and function has not been explored yet. Therefore, we hypothesize that RAGE expression/signaling under high glucose condition changes the expression and function of AMPA and NMDA subtypes of glutamate receptors.

Our findings show an increase in RAGE and a decrease in the expression of GluA1 subunit of AMPA receptor in cultured hippocampal neurons exposed to high glucose conditions in cells from WT mice, and a decrease in GluN1 subunit expression of NMDA receptor in both WT and RAGE-KO hippocampal cultures exposed to high glucose condition. In addition, the frequency of mEPSCs showed a significant increase in cultured hippocampal neurons exposed to high glucose condition from both WT and RAGE-KO mice.

A.2 Materials and Methods

A.2.1 Electrophysiological Recordings

A.2.1.1 Miniature Excitatory Postsynaptic Currents (mEPSCs)

Cultured hippocampal neurons maintained for 1–2 weeks in control and high glucose conditions were used for whole-cell patch-clamp recording. An Axopatch 200B amplifier (Molecular Devices, Palo Alto, CA) equipped with a 1 GΩ cooled head-stage feedback resistor and a Digidata 1400A analog-to-digital converter (Molecular Devices) were used for voltage clamp protocol, and pClamp 10 (Molecular Devices) and Origin 9.0 software (OriginLab Corporation, Northampton, MA, USA) were used for data acquisition and analysis. Patch pipettes were made using thin-wall borosilicate glass capillaries (World Precision Instruments, FL, USA) using a vertical puller (PC 10; Narishige Scientific Instrument Lab., Tokyo, Japan) and polished with a microforge (Narishige) to a final resistance of 3–8 MΩ when filled with intracellular recording solution. Recording electrodes were filled with the following intracellular solution (in mM): 65 KF, 55 KAc, 5 NaCl, 0.2 $CaCl_2$, 1 $MgCl_2$, 10 EGTA, 2 MgATP and 10 HEPES, and pH was adjusted to 7.2 with KOH (all from Sigma-Aldrich). Cultured neurons were perfused continuously at 1 ml/min with control perfusion solution consisting of (in mM): 140 NaCl, 5.4 KCl, 25 HEPES, 5 glucose, and 5 μg/ml phenol red; pH was adjusted to 7.4 with NaOH (all from Sigma-Aldrich); mEPSCs recordings were made in voltage clamp mode (V_m = −60 mV) using the whole-cell patch clamp technique. All the recordings were done in the presence of glycine (to enhance NMDAR opening), tetrodotoxin (to block Na^+-dependent action potentials), bicuculline (to block GABA receptors), and strychnine (to block glycine receptors). In order to distinguish the AMPA and NMDA receptor-mediated components of mEPSCs, 2-amino-5-phosphonovaleric acid (AP-5, 50 μm) was used to block the NMDA receptors.

A.2.2 Western blotting. For western blotting, whole cell extracts of hippocampal cultures maintained for 1-2 weeks in control and high glucose conditions were prepared using a 1% NP-40 lysis buffer containing protease inhibitor cocktail. At least three replicas were used per condition (control or high glucose), for both WT and RAGE-KO mice. Each replica was generated from 5 pups. Equal amounts of protein were loaded for each group and separated on 12% SDS polyacrylamide gels and electrotransferred onto a nitrocellulose membrane (Bio-Rad Laboratories,

Hercules, CA, USA). The membrane was then incubated with the following primary antibodies overnight at 4°C: rabbit anti-GluN1, anti-GluN2A, anti-GluN2B, anti-GluN2C, anti-GluN2D, anti-GluN3A and anti-GluN3B antibodies (1:1000, NMDA Receptor Antibody Explorer Kit, Alomone Labs), rabbit anti-GluA1, anti-GluA2, anti-GluA3 and anti-GluA4 antibodies (1:1000, AMPA Receptor Antibody Explorer Kit, Alomone Labs), rabbit anti-RAGE (1:1000; Abcam), rabbit anti-NF-kB p65 (1:1000, Abcam), rabbit anti-ADAM 10 (1:1000, Abcam) and mouse anti-α-tubulin (1:2000; Sigma) antibodies followed by horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (1:20000; Bio-Rad Laboratories). Protein signals were visualized using enhanced chemiluminescence reagents (Bio-Rad) and quantified by densitometry using ImageJ software (NIH, Bethesda, MD, USA).

A.2.3 Statistical Analysis. All values are reported as mean ± SEM and the significance threshold was set at 0.05 for all statistical tests performed. We used parametric t-test or non-parametric Mann-Whitney U test to compare two means, and two-way ANOVA to compare multiple means. All statistical analyses were carried out with GraphPad InStat 3.0 and Prism 8.0 (GraphPad Software Inc., La Jolla, CA, USA).

This work was approved by the University of Saskatchewan's Animal Research Ethics Board (Campanucci: protocol 20090082) and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

A.3 Results

A.3.1 High glucose increased the frequency of mEPSC in both WT and RAGE-KO neurons

To study synaptic glutamate receptors (AMPA and NMDA) formed *in vitro,* we recorded spontaneous mEPSCs from cultured hippocampal neurons from neonatal WT and RAGE-KO mice (P0-P2).

Our data showed no significant difference in the amplitude (Figure A.1 B), ionic charge (Figure A.1 C) and decay tau (Figure A.1 D) of AMPA as well as NMDA receptor-mediated components of mEPSCs between control and high glucose conditions in either WT or RAGE-KO group. There was, however, a significant main effect of high glucose treatment on the frequency of mEPSC (Figure A.2 A). Consistent with this, high glucose condition caused a significant increase in the frequency of mEPSC in both WT and RAGE-KO cultured hippocampal neurons (Figure A.2 A).

A.3.2 High glucose increased RAGE expression and decreased AMPA GluA1 subunit expression in cultured hippocampal neurons from WT mice, while NMDA GluN1 subunit expression was affected in both WT and RAGE-KO neurons

To evaluate if the observed changes in electrophysiological properties of cultured hippocampal neurons are correlated with changes in protein expression levels, western blotting was performed on whole cell extracts of hippocampal cultures maintained for 1-2 weeks in control and high glucose conditions in WT and RAGE-KO groups. We quantified expression of AMPA and NMDA receptor subunits as well as RAGE and RAGE-associated proteins (such as ADAM 10 and NF-kB) to determine changes in glutamate receptors expression and potential involvement of RAGE in mediating these alterations.

Immunoblotting analysis revealed both, an increase in RAGE and a decrease in GluA1 subunit of AMPA receptor in cultured hippocampal neurons exposed to high glucose condition in WT group (Figure A.3 A). High glucose, however, decreased the expression of GluN1 subunit of NMDA receptor in cultured hippocampal neurons from both WT and RAGE-KO mice (Figure A.3 C-D). We also observed an increased trend, although not significant, in both ADAM 10 and NF-kB expression in cultured hippocampal neurons exposed to high glucose condition (Figure A.3 A).

ADAM 10, however, did not show any increase in cultured neurons exposed to high glucose condition in the RAGE-KO group (Figure A.3 B).

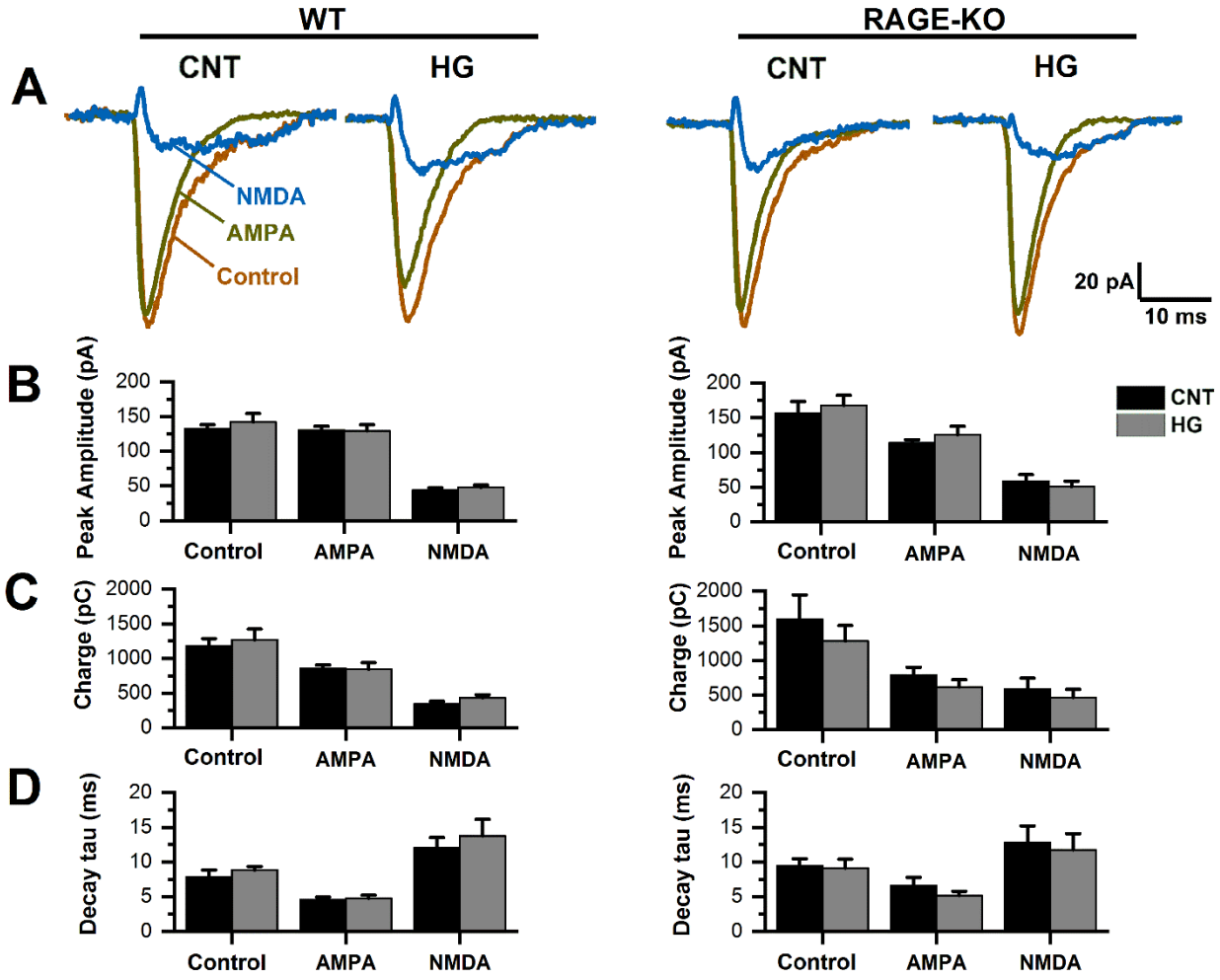

Figure A.1. High glucose condition did not change the characteristics of NMDA and AMPA receptor-mediated components of mEPSCs. (A) Representative traces from WT and RAGE-KO cultured hippocampal neurons (P0-P2) exposed to 5 mM glucose (CNT) and 25mM glucose (HG) showing the average of mEPSCs collected from one pyramidal cell in the absence (brown trace) and in the presence (green trace) of AP-5. The brown trace (control) arises from the coactivation of AMPA and NMDA receptors. The green trace is representative of AMPA receptor-mediated component of mEPSCs when the NMDA receptor-mediated component was blocked by AP-5 (50 μm). The blue trace shows the subtraction of the two traces, yielding the NMDA receptor component. Bar graphs summarize the mean **(B)** peak amplitude, **(B)** ionic charge and **(C)** decay tau of mEPSCs in the absence of AP-5 (control) and in the presence of AP-5 (AMPA) as well as in the subtracted trace (NMDA). Values are expressed as the mean ± SEM from WT CNT (n=10), WT HG (n=11), RAGE-KO CNT (n=9), and RAGE-KO HG (n=9). Means were statistically compared by two-way ANOVA, followed by Sidak's multiple comparison test.

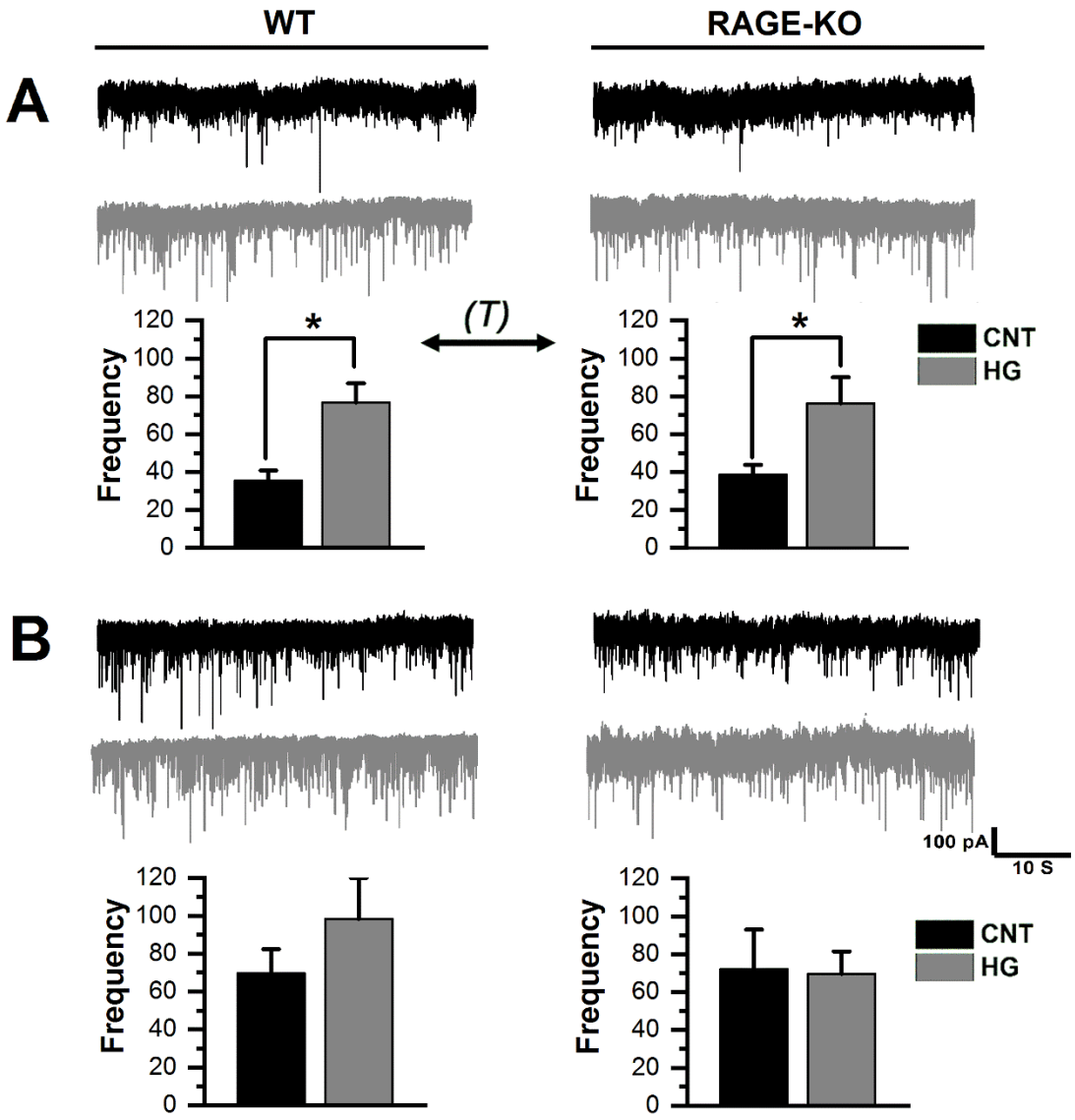

Figure A.2. High glucose condition caused a significant increase in the frequency of mEPSCs. Representative recordings of spontaneous mEPSCs from WT and RAGE-KO cultured hippocampal neurons (P0-P2) exposed to 5 mM glucose (CNT) and 25mM glucose (HG) before **(A)** and after **(B)** AP-5 (50 μm) treatment. Bar graphs summarize the mean frequency of mEPSCs in the absence **(A)** and in the presence **(B)** of AP-5. Values expressed as the mean ± SEM from WT CNT (n=10), WT HG (n=11), RAGE-KO CNT (n=9), and RAGE-KO HG (n=9); Means were statistically compared by two-way ANOVA, followed by Sidak's multiple comparison test; * $p<0.05$. *(T)*: Significant main effect of HG treatment ($F_{(1, 35)}=17.94$, $p<0.001$ in A).

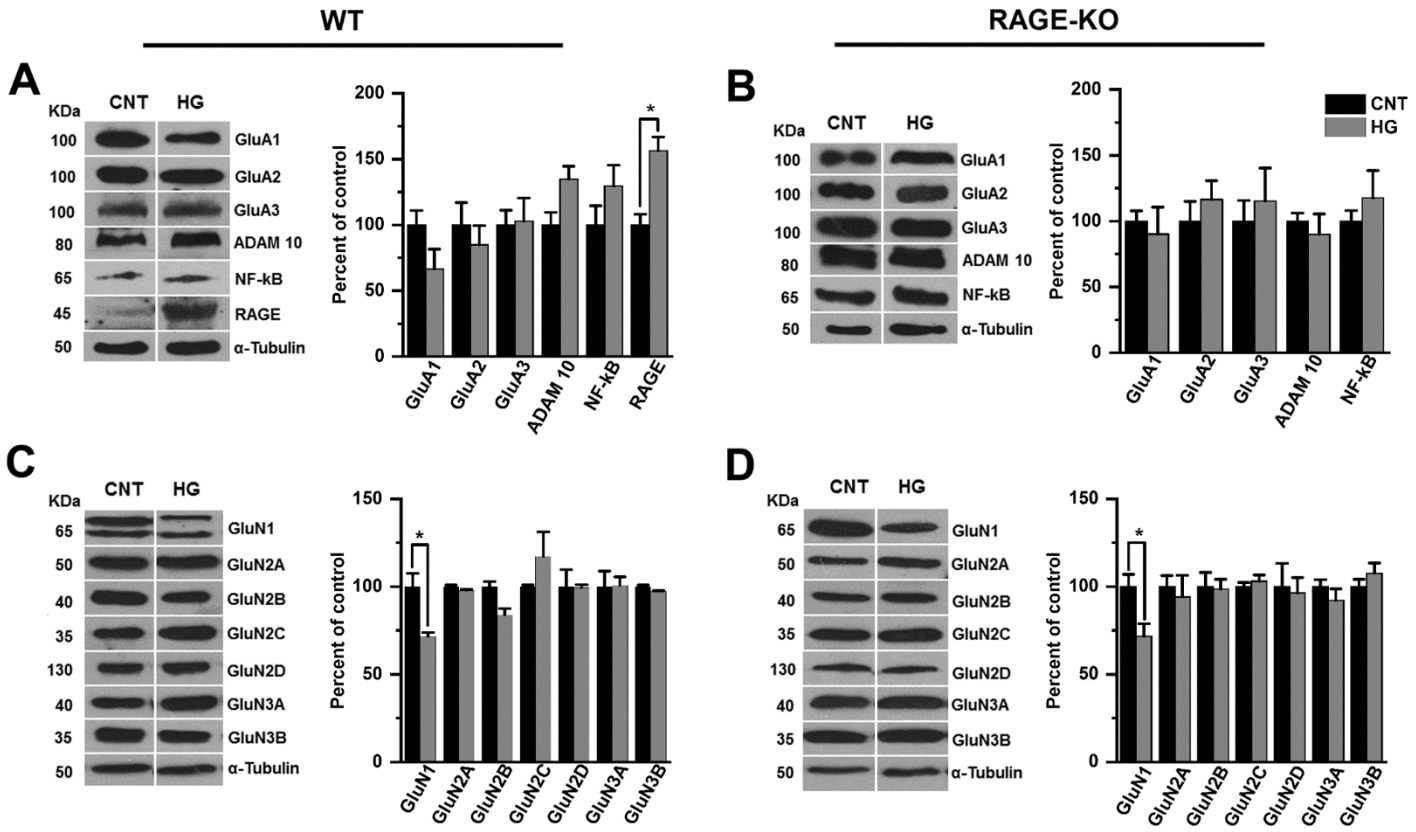

Figure A. 3. High glucose increased RAGE expression and decreased AMPA GluA1 subunit expression in cultured hippocampal neurons from WT mice, while NMDA GluN1 subunit expression was affected in both WT and RAGE-KO neurons. Representative immunoblots showing levels of AMPA receptor subunits and ADAM10, NF-kB and RAGE in whole-cell extracts of **(A)** WT and **(B)** RAGE-KO cultured hippocampal neurons as well as levels of NMDA receptor subunits in whole-cell extracts of **(C)** WT and **(D)** RAGE-KO cultured hippocampal neurons maintained in either 5 mM glucose (CNT) or 25 mM glucose (HG). Bar graphs show the mean ± SEM levels of each protein after normalization to tubulin expressed as a percentage of control (n=4 in each group). Means were statistically compared with the Mann-Whitney U test; * $p<0.05$. Protein samples for Western blotting were obtained from at least 3 independent sets of cultures, each culture was generated from the hippocampi of 5 neonatal (P0-P2) WT or RAGE-KO mice.

A. 4 Discussion

Glutamate receptors are shown to play important roles in controlling synaptic plasticity during learning and memory processes (Trudeau et al., 2004). Abnormal regulation of glutamate receptors thus appears to mediate impairments in synaptic plasticity and development of cognitive deficits in diabetes (Trudeau et al., 2004). In this regard, animal models of diabetes have shown electrophysiological and structural changes in AMPA and NMDA receptors (Valastro et al, 2002; Gardoni et al, 2002; Trudeau et al., 2004; Castilho et al., 2012; Viswaprakash et al., 2015; Nardin et al, 2016; Marshad et al., 2018; Wang et al., 2019).

Our results show a decrease (although not significant) in the expression of GluA1 subunit of AMPA receptor in cultured hippocampal neurons exposed to high glucose condition in WT group. This is consistent with studies that showed reduced level of GluA1 subunit of AMPA receptor in the hippocampus of STZ-induced diabetic rats (Gagne et al., 1997; Viswaprakash et al., 2015), and inconsistent with a study that showed increased protein expression of GluA1 subunit of AMPA receptor in the hippocampus of STZ-treated rats (Wang et al., 2019).

Our findings also show decreased expression of GluN1 subunit of NMDA receptor in both WT and RAGE-KO hippocampal cultures exposed to high glucose condition. Consistent with this, STZ-induced diabetic rats showed a significant reduction in hippocampal GluN1 subunit expression of NMDA receptor (Gardoni et al., 2002). On the contrary, increased expression of GluN1 subunit of NMDA receptor has been reported in a recent study in the hippocampus of STZ-induced diabetic rats (Wang et al., 2019).

Our data, on the other hand, did not show any changes in the electrophysiological properties of AMPA and NMDA-receptor mediated components of mEPSCs in cultured hippocampal neurons exposed to high glucose condition. There was, however, a significant increase in the frequency of mEPSCs in both WT and RAGE-KO cultured hippocampal neurons exposed to high glucose condition. Consistent with this, higher frequency of mEPSCs, indicative of increased probability of presynaptic neurotransmitter release (Ju et al., 2016), has been reported in dorsal motor nucleus of the vagus nerve (DMV) of hyperglycemic mice as compared to control, while no significant difference was found between the two groups in the amplitude of mEPSCs (Bach et al., 2015). Similarly, increased frequency of mEPSCs has been shown in kidney-related paraventricular nucleus (PVN) neurons of the hypothalamus in STZ-induced diabetic mice as compared with

controls, with no significant impact on the amplitude of mEPSCs (Jiang et al., 2013). However, AMPA receptor-mediated mEPSCs showed reduced amplitude and frequency in CA1 pyramidal neurons of the hippocampus in STZ-induced diabetic rats (Viswaprakash et al., 2015).

Overall, some previous findings are consistent with the data presented in the current chapter, but research on the effect of diabetes on glutamate receptors and how this affects synaptic plasticity is still ongoing and reports are controversial. The discrepancy between cultured neurons and actual hippocampal tissue might be a result of culture conditions, which points to the limitations of using cultured neurons to study brain-affecting diseases and highlights the need to validate results in actual tissues. However, since our findings of decreased GluN1 subunit expression of NMDA receptor, and increased frequency of mEPSCs were observed in neurons exposed to high glucose condition from both WT and RAGE-KO genotypes, we can infer that these effects could be independent of RAGE expression and/or signaling. On the other hand, decrease in GluA1 subunit expression of AMPA receptor, accompanied by significant increase in RAGE expression, in cultured hippocampal neurons exposed to high glucose condition in the WT group can suggest the role of RAGE in mediating this effect. However, further investigations are required to validate these results in animal models of diabetes.

Appendix B

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