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Influence of Advanced Glycation Endproducts on Neuronal Plasticity

Dissertation
zur Erlangung des akademischen Grades des
Doktor rerum medicarum (Dr. rer. medic.)
für das Fachgebiet Medizinische Physiologie und Pathophysiologie

vorgelegt
der Medizinischen Fakultät
der Martin-Luther Universität Halle-Wittenberg

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geboren am 24.09.1984 in Bautzen

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Eröffnung des Promotionsverfahrens am 15.09.2015

Halle (Saale), verteidigt am 18.12.2015

Abstract

Advanced glycation endproducts (AGEs) represent a posttranslational protein modification. They are generated by several subsequent chemical reactions of free reducing monosaccharides (= sugars) such as glucose or fructose with reactive side chains of proteins followed by oxidation, dehydration and condensation reactions, finally forming stable AGE-modifications. AGE-modified proteins accumulate in all cells and tissue as a normal feature of ageing, which can lead to non-degradable and less-functional proteins and enzymes. These irreversible non-enzymatic crosslinking reactions between sugars and proteins are a reaction referred as glycation. AGEs bind to the multi-ligand receptor of advanced glycation endproducts (RAGE). Binding of AGEs to RAGE activates several signaling pathways. Accumulation of AGEs and activation of RAGE mediated pathways can additionally induce inflammation and further pathophysiological processes such as neurodegeneration. In the present work the role of AGEs on neuronal plasticity was examined on the basis of the *in vitro* model PC12 cell line. In order to form AGEs the reactive carbonyl compound methylglyoxal (MGO) as physiological carbolite of glucose was used. On one hand, the real time cell analyzing show that the AGE-modified substrates such as extracellular matrix proteins and also the AGE-modified PC12 cells interferes with adhesion of PC12 cells. On the other hand, AGE-modification of cellular proteins reduces cell adhesion as well as NGF-induced neurite outgrowth. Moreover, the influence of receptor glycation on the high affinity NGF-receptor TrkA and the AGE-receptor RAGE at the binding affinity to their ligands was investigated. For that, the equilibrium dissociation constant (K_D) as measured quantity for the binding affinity of TrkA-receptor and RAGE to their ligands was quantified by surface plasmon resonance (SPR) spectroscopy. These binding affinities were compared with those after receptor glycation. Thereby, a glycation procedure directly on the receptor-coupled SPR-chip surface was established. Glycation of TrkA reduces affinity to NGF by the factor of three, a factor, leading to reduction of NGF-dependent neurite outgrowth in PC12 cells. Glycation of RAGE reduced binding affinity of AGEs about 6-fold even up to complete binding inhibition. Furthermore, activation of RAGE by binding AGEs does not show a neuro-protective effect but rather initiation of pro-inflammatory signaling pathway in the PC12 cell line. The present investigations have demonstrated that AGEs are negative prognostic markers for the neuronal plasticity.

Bennmann, Dorit: Influence of Advanced glycation endproducts on neuronal plasticity.
Halle (Saale), Univ., Med. Fak., Diss., 74 Seiten, 2015

Referat

Advanced glycation endproducts (AGEs) stellen posttranslationale Modifikationen dar. Sie werden durch eine Abfolge komplexer chemischer Reaktionen zwischen freien reduzierenden Monosacchariden wie Glukose oder Fruktose und reaktiven Seitenketten von Proteinen gebildet. Die Abfolge von Oxidations-, Dehydrations- und Kondensationsreaktionen bilden letztlich stabile AGE-Modifikationen. AGE-modifizierte Proteine akkumulieren in allen Zell- und Gewebetypen als alterungsbedingtes Charakteristikum und führen zu nicht-abbaubaren und weniger funktionsfähigen Proteinen. Diese irreversiblen nicht-enzymatischen Quervernetzungsreaktionen zwischen Zuckern und Proteinen werden als Glykierungsreaktion bezeichnet. AGEs binden an den multi-liganden Rezeptor für AGEs (RAGE). Die Bindung von AGEs an RAGE aktiviert bestimmte Signaltransduktionswege. Die Anhäufung von AGEs und die Aktivierung der von RAGE vermittelten Signalwege können Entzündungsreaktionen und pathophysiologische Prozesse wie neuronale Degenerationen hervorrufen.

In der hier vorliegenden Arbeit wurde die Wirkung von AGEs auf die neuronale Plastizität anhand des PC12 Zell- *in vitro* Model-Systems untersucht. Für die Bildung von AGEs wurde die hochreaktive Carbonylverbindung Methylglyoxal (MGO) verwendet, ein physiologisches Abbauprodukt von Glukose. Echtzeitzellanalysen zeigen, dass sowohl AGE-Modifikationen an Substraten, wie z.B. an Extrazellularmatrixproteinen, als auch an zellulären Proteinen die Zelladhäsion von PC12 Zellen einschränken. Zusätzlich reduzieren AGE-Modifikationen an zellulären Proteinen das Neuritenwachstum. Des Weiteren wurde der Einfluss der Rezeptorglykierung auf dessen Ligandenbindungsverhalten am Beispiel des hochaffinen NGF-Rezeptors TrkA und des AGE-Rezeptors RAGE untersucht. Hierfür wurden zunächst die Bindungsaffinitäten der Liganden zu ihren entsprechenden Rezeptoren mittels Oberflächenplasmonenresonanzspektroskopie (SPR) quantifiziert und mit den Bindungsaffinitäten der Liganden nach Rezeptorglykierung verglichen. Hierbei wurde eine Glykierungsmethodik direkt an der rezeptorgekoppelten SPR-Chipoberfläche etabliert. Die Glykierung des TrkA -Rezeptors reduziert die Affinität für NGF um den Faktor drei, was an PC12-Zellen eine Reduktion des NGF-abhängigen Neuritenwachstums auslöste. Die Glykierung von RAGE verminderte die Bindungsaffinität von AGEs um das 6-fache bis hin zur vollständigen Hemmung der Rezeptor-Ligandenbindung. Die Aktivierung von RAGE in PC12 -Zellen zeigte keinen neuroprotektiven Effekt, sondern führte zum Auslösen eines pro-inflammatorischen Signalweges.

Die hier vorgelegten Untersuchungsergebnisse führen zu dem Schluss, dass AGEs als negative prognostische Marker der neuronalen Plastizität einzustufen sind.

Contents

List of abbreviations

AGE/ AGEs	Advanced glycation endproducts
AGE R	Advanced glycation endproducts receptor
APS	ammonium persulfate
BBB	blood brain barrier
BCA	bicinchoninic acid
BcI2	B-cell lymphoma 2
BSA	bovine serum albumin
CEL	carboxyethyllysine
CML	carboxymethyllysine
ECM	extracellular matrix
EDC	1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide
ERK	Extracellular-signal Regulated Kinase
FITC	fluorescein isothiocyanate
GLUT	glucose transporter
HBS	HEPES Buffered Saline
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMGB1	High-Mobility-Group-Protein B1
HRP	horseradish peroxidase
Ig CAM	immunoglobulin cell adhesion molecule
MAPK	Mitogen-activated protein kinase
MGO	methylglyoxal
MTT	3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCAM	neuronal cell adhesion molecules
NGF	nerve growth factor
NHS	N-Hydroxysuccinimide
NF-κB	nuclear factor nuclear factor 'kappa-light-chain enhancer of activated B-cells
PFKFB	Phosphofructokinase-2/Fructose-2,6-bisphosphatase
PI	propidium iodide
PI3K	phosphatidylinositol 3-kinase

PLCγ	Phospholipase C (isotype γ)
RAGE	Receptor for Advanced glycation endproducts
RAS	Rat sarcoma
ROS	reactive oxygen species
RTCA	real-time cell analyzer
SPR	surface plasmon resonance
sRAGE	soluble receptor for Advanced glycation endproducts
TBS	Tris-buffered saline
TEMED	N,N,N‘,N‘-Tetramethylethylendiamine
Tris	Tris(hydroxymethyl)aminomethane
TrkA	Tropomyosin receptor kinase

1 Introduction

1.1 Advanced glycation endproducts (AGEs)

1.1.1 Formation of Advanced glycation endproducts

In 1912 Camille Maillard incubated glucose with amino acids and observed the formation of yellow-brown pigments after a certain time due to non-enzymatic glycosylation reaction. This browning reaction is called Millard reaction according to his discoverer [1]. Glucose and the free reactive amino groups of the proteins lead in this first reaction, a nucleophilic addition reaction, to an unstable compound known as a Schiff base, implemented within couple of minutes up to couple of hours. The Schiff base is the correlate of the yellow-brown pigments, which Milliard observed. The amount of the Schiff base is directly dependent of the glucose concentration, thus the product decays within couple of minutes, if glucose is detracted or its concentration is decreased in the reaction [2], [3]. Therefore the reaction is highly reversible and can undergo rearrangement over several days to form the more stable Amadori-type product. Thus the process to the Amadori- type product is less reversible, it accumulated within days up to weeks on proteins. One well-characterized example of this product type is hemoglobin A_{1C}, the adduct of glucose with the N-terminal valine amino group of the β-chain of hemoglobin. Practical application occurs the measurement of hemoglobin A_{1C} at the therapy control of diabetics, which draws conclusions about the blood glucose level over the last weeks [4]. The primarily Amadori-type product rearranges and reacts, over several chemical reactions such as dehydration, oxidation and condensation reactions, to the endproducts of the milliard reaction, the advanced glycation endproducts (AGEs), a reaction referred also to glycation reaction. A schematic overview of the Milliard reaction is shown in figure 1. This process of non-enzymatic protein glycation differs from enzyme-dependent *N*- or *O*-glycosylation, the latter being a reversible process whereby proteins are modified at specific residues to effect signal transduction such as phosphorylation. Glycated proteins, in contrast, can further evolve over time, undergoing irreversible complex rearrangements and polymerization to yield cross-linked proteins. Highly reactive dicarbonyl compounds are generated during rearrangement of Amadori products to AGEs. Glyoxal, methylglyoxal and 3-deoxyglucosone being the most studied AGE-dicarbonyl precursors [5]. As side products during the conversion of the Amadori products, reactive oxygen species (ROS) are generated [6].

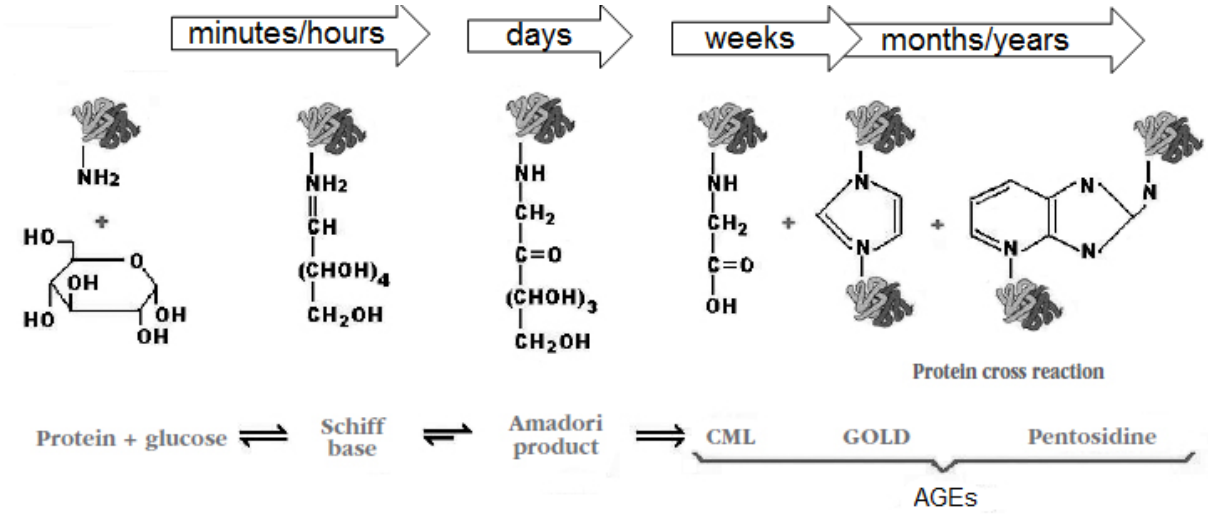

Figure 1: **Stages of glycation reaction on the example of glucose**.
Glycation reaction or also called Millard reaction is schematically illustrated. Regarding, intermediate step of rearrangement from Amadori products over o-oxoaldehydes to AGEs is not shown. CML = carboxymethyllysine; GOLD = glyoxal lysine dimer; AGEs = advanced glycation endproducts. Provided by [7].

Due to their synthesis pathway a high variety of different, heterogeneous and complex forms of AGEs exists. They are classified in three main groups: First, fluorescent crosslinking AGEs e.g. pentosidine; second, non-fluorescent crosslinking AGEs e.g. furosine or imidazolium dilysine cross-links and third; non-crosslinking AGEs, which include the frequent and well characterized AGEs, carboxylmethyllysine (CML), carboxylethyllysine (CEL). They are generated by the reaction of Nε-amino group of lysine with the reactive dicarbonyl compound glyoxal to CML or with methylglyoxal to CEL. Figure 2 shows an overview of the variety of advanced glycation endproducts (AGEs).

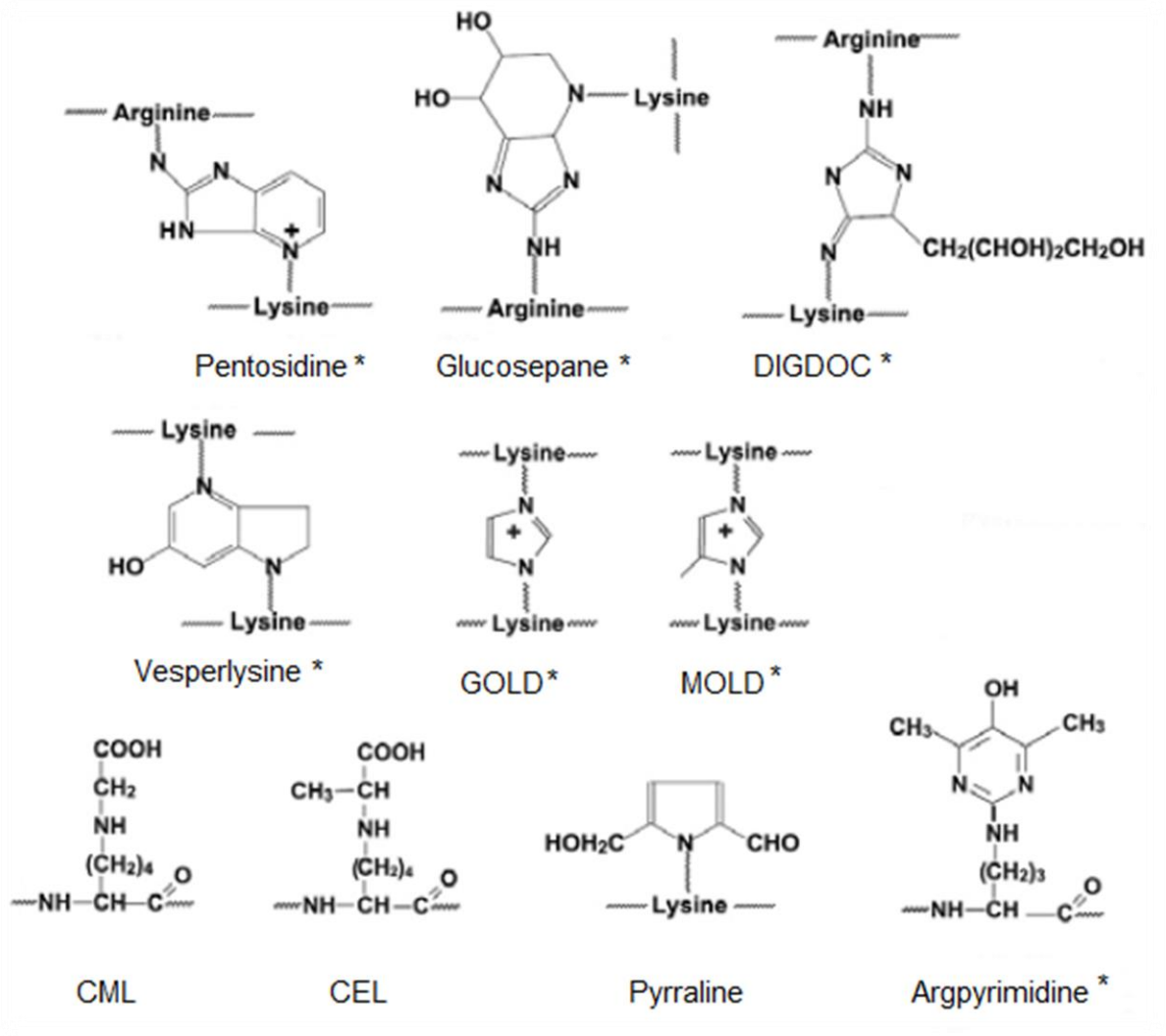

Figure 2: **Chemical structures of main AGEs**

All of these compounds are derivatives of lysine and arginine residues in protein. The selection illustrates the structure variety of AGEs e.g. short-chained carbonyl structure until fluorescent heterocycles or heterogeneous crosslinks. Fluorescent AGEs marked by *. CML = carboxymethyllysine, CEL = carboxyethyllysine, GOLD = Glyoxallysine dimer, MOLD = methylgyloxallysine dimer DIGDOC = 3-desoxyglucosone derived imidazolium cross-link. Adapted from [8].

1.1.2 Metabolism of AGEs

The majority of AGEs in the human body are endogenously generated, but AGEs can also be generated exogenously by heating food or in smoke of cigarettes. Therefore AGEs can ingest by nutrition or by smoking. AGEs do not underlie the enzymatic metabolism, only the kidney is able to filtrate and excrete AGEs from the human body. The degradation of AGEs, which allows the excretion by kidney, is mostly unknown. Macrophages are involved at the formation of a “second generation AGEs”. They degrade AGEs to more soluble peptides, which can leave the cells and are transported by the bloodstream to be finally excreted by the kidney. The receptor AGE-R1 mediates the uptake of AGEs into tissue macrophages [9], [10]. It has been shown that the filtration rate of creatinine is higher than for AGEs, evidence that not all AGEs are filtrated [11], [12]. In consequence, the amount on circulating AGEs increases during ageing and especially for patients with renal insufficiency. AGEs precursors and AGEs underlie the following four metabolism pathways and are represented in the schematic overview in figure 3. First, endogenous AGEs and their precursors modify intracellular proteins, including those which are involved in regulation and gene transcription [9]. Second, precursors of AGEs leave the bloodstream or the cells by diffusion and modify extracellular matrix proteins thereby signaling between matrix and cells can change. Third circulating AGEs and precursors modify proteins in the bloodstream and alter their functions [13]. Finally, the fourth possibility, they bind to an AGE-receptor and activate receptor mediated signaling pathways. In the context of these metabolism pathways, the accumulation of AGEs during ageing promotes and accelerates a couple of pathological processes such as diabetes mellitus, renal failure, cardiovascular diseases, rheumatoid arthritis and neurodegeneration e.g. during Alzheimer´s disease. Therefore AGEs present not only a normal ageing phenomenon but rather contribute to pathophysiological processes.

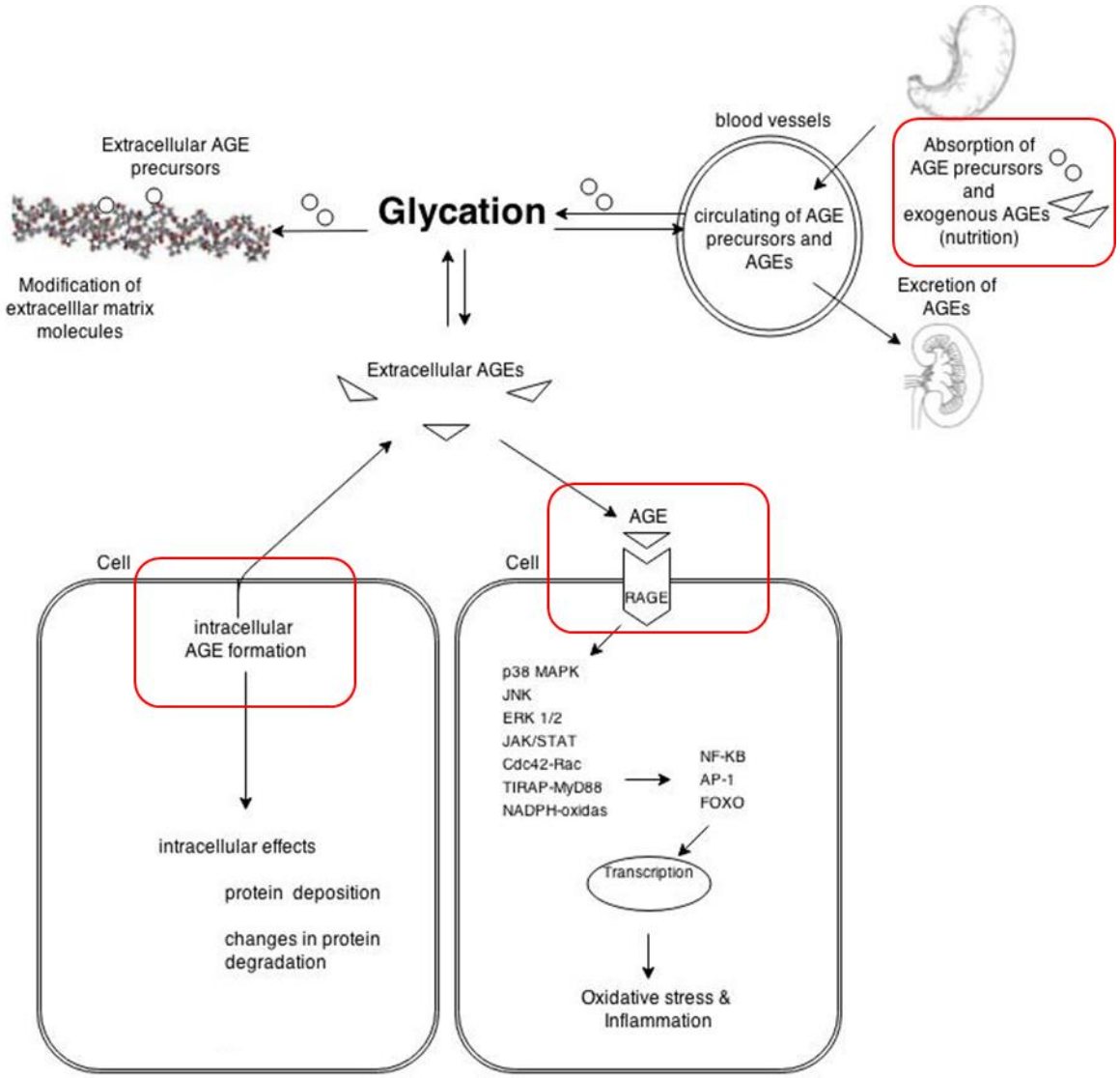

Figure 3: **Metabolism and circulation of AGEs *in vivo.***

Cycle of exogenous and endogenous AGEs and receptor-dependent pathways activated by AGEs. A substrate excess of AGEs leads to a RAGE overexpression and promotion of RAGE-dependent pathways. Adapted to [10].

1.1.3 Pathological properties of AGEs

There exist several pathways by which AGEs can affect tissue functions. On the one side AGEs are able to generate complex protein cross-links, which cannot be cleaved by the human body since no appropriate enzymes are existent. This can alter normal functions of proteins such as change in molecular conformation, alteration in enzyme activity, changes in the clearance and interference with receptor recognition to mention only a few selective examples. The majority of all proteins, which underlie AGE-formation, are stable and long-lived proteins such as collagen, cartilage tissue, and lens matrix [14]–[17], although affecting short-lived proteins could be not excluded [18],[19].

On the example of collagen, the AGE-induced pathological crosslinks lead to increased stiffness and consequently to a decreased elasticity of the fibers [20],[21]. Metalloproteinases, which are responsible for the cleavage of collagen, cannot degrade affected AGE-modified collagen [22]. This accelerates not only ageing processes but also changes the collagen remodeling e.g. with simultaneous dysfunction collagen synthesis can result in a fibrosis or sclerosis. This effect of accumulation of AGEs affect also other pathological processes e.g. progression of diabetes mellitus, arteriosclerosis or the development of cataract [23],[24]. Diabetes mellitus patients have a higher blood glucose concentration than non-diabetics. Thus glucose is the main substrate for the AGE-formation, it is supposed that more AGEs are generated in the tissues of diabetes patients. Recent studies could be confirmed this hypothesis. They have shown that the concentration of AGEs in the serum is significantly higher than in healthy individuals [25]. Diseases such as nephropathy, retinopathy, neuropathy and angiopathies occur during later ageing. Patients, suffering from diabetes mellitus, develop these diseases significantly rather than non-diabetics, which results also in an earlier, accelerated ageing process [26]–[28]. All these diabetic long-term consequences are accompanied with arteriosclerotic vessel lesions [26], [29]. AGEs seems to play an important role during the development and acceleration of these arteriosclerotic vessel lesions. *In vivo* studies have shown that AGEs accumulate in atherosclerotic plaques [25], [30]. Moreover, immune-histological studies of kidney tissue of diabetic rats have shown an accumulation of AGEs in the glomerular basal membrane, in the mesangium, in the podocytes and the tubules system in comparison to non-diabetic rats [29]. Proximate studies showed that an AGEs enriched nutrition of non-diabetic rats result also in a basal membrane hyperplasia, mesangium expansion and a glomerular hypertrophy, which resemble a diabetic nephropathy [11], [31]. As already indicated, AGEs influence also the development of retinopathy. It has been shown, that AGEs of diabetic patients accumulate in the blood vessels along the human retina, which could not be observed in non-diabetics [32], [33]. Moreover, AGEs seems to be involved during the development of cataract [16], [17]. Crosslinks of lens proteins, triggered through the accumulation of AGEs, is assumed to be the main mechanism of the changes in the lens during development of cataract [34].

In all these cases, AGEs affect cells and tissues directly by irreversible binding proteins and AGE accumulation in and on the tissue. Furthermore, AGE deposits have been detected in skin, lung, intestine, intervertebral discs [25] and the pathological lesions of Alzheimer's disease [35] and Parkinson's disease [36]. The relevance of AGEs during development of age-related neurodegenerative diseases will be discussed in section 1.3.4. Anyway, in the last decades the intensive investigation of pathophysiological

relevance of AGEs in ageing and age-related diseases increased. According to this, AGEs are already discussed as “glycotoxins” [37].

On the other side, it is stated that reactive oxygen species (ROS) are generated during the AGE-formation, which lead to oxidative stress. ROS are endogenously or exogenously produced free oxygen radicals such as peroxide, hydroxyl radicals or alkoxy radicals, which can directly damage the cells by oxidation of proteins and lipids or indirectly by activation of specific signal transduction pathways [38]–[40]. Several oxidative protective systems exist in the human body as defense of oxidative stress such as superoxide dismutase, catalase or glutathione peroxidase [38], [41], [42]. The increased generation of ROS during AGE-formation leads to an imbalance of oxidative species and the capacity of oxidative protective systems and finally AGE-formation induces oxidative stress [9], [43]. The modification by ROS results in a loss of functions of proteins, lipids, and DNA. These effects accumulate during ageing and leads finally to dysfunction. For example, oxidation processes of the lens matrix are another reason for the changes of the lens during the development of cataract besides the AGE-accumulation [44].

AGE-interaction with AGE–receptors on the cell surface is another possibility to interfere with cell functions. AGEs cannot be cleaved by enzymes, but receptors for binding of AGEs exist in the human body to remove them from the extracellular tissues. The effect of AGEs on cells is regulated by activation of receptor-dependent signal transduction cascades. AGE-receptors represent a heterogeneous group, which are present on the surface of different cell types such as endothelial cells, vascular smooth cells or macrophages. Several cellular types of receptors react with AGEs such as AGE-R1, AGE-R2, AGE-R3, scavenger receptors and including receptor for glycation endproducts (RAGE) [9]. Modified AGE-proteins are recognized by RAGE and promotes several pathways such as inflammatory response mainly by activation of nuclear factor κB (NF-κB), apoptosis or oxidative stress [45], [46]. RAGE is the most prominent described AGE-receptor and it is assumed that AGE-RAGE interaction plays the major role of activation many signaling pathways in an AGE-related matter [47]. It is supposed that activation of RAGE by AGEs causes the main pathogenic of vascular complications in context of arteriosclerosis and diabetic mellitus [9], [10].

1.2 Receptor for Advanced glycation endproducts (RAGE)

RAGE was identified and characterized in 1992 as a receptor for AGEs with a molecular weight of approximately 45 kDa [48]. RAGE belongs to the immunoglobulin (Ig) superfamily and shares homology to other immunoglobulin cell surface proteins, such as

NCAM or MUC18 [48]. Since these proteins are cell adhesion molecules, an involvement of RAGE in cell adhesion was proposed [49]. Functionally, RAGE is involved in lung homeostasis, bone metabolism, in the adaptive and innate immune response, and in several neuronal functions such as nerve regeneration, migration and neuronal outgrowth [9], but the whole physiological function of the multi-ligand receptor RAGE is still not completely understood. The expression of RAGE begins already during embryogenesis. RAGE mRNA expression is first detectable in the early blastocyst stage and increases during gastrulation [9]. RAGE is highly expressed in embryonic cells, but at low levels in a wide range of differentiated adults cell types e.g. in endothelial cells [50], lung epithelial cells [51], in the immune system on monocytes and macrophages [52], [53], in neuronal cells [54], and in smooth muscle cells [55]. Interestingly, in mature type I pneumocytes, RAGE represent a higher level than in other resting cells. RAGE consists of a N-terminal extracellular domain, including typical immunoglobulin domains a V-type domain and two constant domains, and C-terminal arranged in a single transmembrane/intracellular domain [56]. The V-type is responsible for ligand binding [9], [57]. The intracellular domain is required for a ligand-induced intracellular transduction pathway. Deletion of this domain blocks the introduction of signaling pathways [58]. Based on alternative splicing, 20 different variants of RAGE are described. The major splicing variant of full length RAGE (fl-RAGE) mRNA is soluble RAGE (sRAGE) [59], lacking C-terminal transmembrane/intracellular domain, illustrated in figure 4. These, both human variants have also been detected at protein level *in vivo* [47]. The soluble form of RAGE is secreted. Interestingly, RAGE is able to bind diverse ligands of several distinct families, not only specific AGEs. RAGE binds HMGB1/Amphoterin, an intracellular DNA-binding protein and members of the S100 protein/calgranulins family, small calcium binding proteins, whose binding to RAGE induces a pro-inflammatory gene activation [60]. Regarding to the development of Alzheimer´s disease, β-amyloid has been identified as a binding partner of RAGE and RAGE mediated the tracking of β-amyloid protein through the blood-brain barrier [61]. Also matrix proteins such as collagen I or IV are bound by RAGE [47]. Under stress conditions RAGE or the soluble form are precise up-regulated [9], [47]. Activation of RAGE leads dependent on the cell type and tissue localization to enhanced cell survival, angiogenesis or neurite outgrowth but leads also to autophagy, inflammation, decrease of proliferation [58], [62]–[65]. In general, RAGE plays a predicted role, in regulating the metabolism, inflammation and stress responses.

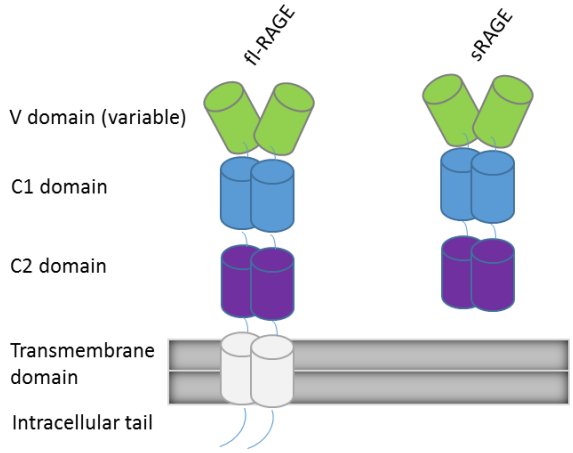

Figure 4: **Schematic representation of the two main RAGE isoforms.**
Full-length RAGE (fl-RAGE) and soluble isoform of RAGE (sRAGE) are shown.

1.2.1 RAGE-mediated signal transduction

AGEs are able to stimulate RAGE expression in form of a positive feedback [66], [67]. Increased AGE-levels in tissues correlate with enhanced gene expression of RAGE e.g. in podocytes of diabetic patients, endothelial and smooth muscle cells in the frame of nephropathy [68], and also in heart tissue of diabetic rats [67], [69]. RAGE triggers oxidative stress and activates the transcription factor NF-κB. This one dislocates into the cell nucleus and stimulates NF-κB-dependent gene expression such as cytokines, vasoconstrictor Endothelin-1, coagulation factors or leucocytes adhesion molecules, thereby hemostasis functions are disrupted. In addition, in several cell types AGEs activates mitogen activated protein kinases (MAPK) pathway (MAPK p38 or p42/p44) as well as c-Jun N-terminal kinase (JNK) or JAK-STAT (JAK=Januskinase and STAT=Signal Transducers and Activators of Transcription pathway), which induce also the distribution of cytokines and growth factors [9], [47]. It is assumed that RAGE is involved in the development and progression of diabetes and arteriosclerosis. Interestingly, RAGE-deficient mice are viable and are resistant to arteriosclerosis and have less neurodegeneration [70], [71].

1.2.2 Ligands-specific RAGE-mediated effects in neuronal environment

RAGE plays a double-edged role in neuronal environment, dependent on its interaction partner or of the pathogenic state of the neuronal cells. Amyloid β peptide, which is transported through the blood brain barrier mediated by RAGE, binds to RAGE on neurons and microglial cells. On microglial cells, amyloid β mediated RAGE activation increases cell proliferation and migration but in contrast on neurons, RAGE activation by binding amyloid β peptide induce oxidative stress and activate NF-κB pathway [72]. The interaction between HMGB1 and RAGE promotes neurite outgrowth of cortical cells, indicating RAGE is a promoter for neuronal development [73]. S100B as binding partner promotes several concentration-dependent effects. Nanomolar concentrations of S100B mediates cell migration or neurite outgrowth as well as increased ERK activation in astrocytes by several RAGE dependent pathways. However increasing micromolar concentrations of S100B induces RAGE-dependent apoptotic pathways [74]. S100B and HMGB1 can activate RAGE together and co-regulate neurite outgrowth and cell survival by activation of transcription factor NF-κB and increased expression of anti-apoptotic protein Bcl-2 (B-cell lymphoma 2). [75]. During different pathogenic conditions, RAGE is up-regulated in neuronal cells, e.g. in diabetic neuropathy [47], [76]. The RAGE mediated NF-κB pathway is also up-regulated in diabetic neuropathy and activation of RAGE appears to be responsible for the loss of pain perception [77]. AGEs as prominent binding partners of RAGE could be detected in endoneurial and epineurial cells, surrounding cells of peripheral nerve cells and are involved in binding to RAGE in chronic inflammatory demyelinating polyneuropathy [78], [79].

1.3 Glycation on neuronal tissue

1.3.1 Glucose as the major “fuel” for the brain

Glucose is the main energy substrate for the brain. Although the brain represents only two percent of the mass of the body weight, it uses about two-thirds of daily fed carbohydrates. This corresponds to about 130 g of glucose. [80]. The supply and disposal is ensured by a number of specific transport processes through the blood brain barrier. The essential element of the blood-brain barrier are endothelial cells with their tight junctions [81]. Furthermore, supporting cells such as astrocytes (90%) and pericytes (10%) enclose endothelial cells. These components form the human blood-brain barrier and with its highly selective filter effect. The cell-cell interactions between endothelial cells, pericytes and astrocytes are as tight as nowhere else in tissue cells. In general, the blood brain barrier is a highly selective filter function to protect the brain from toxins,

germ-ridden and transmitters [82]. This protective function impairs also the medication for neurodegenerative diseases. Glucose passes the blood brain barrier via GLUT transporter. GLUT 1 in the endothelial cells and astrocytes transports glucose from bloodstream to the astrocytes. Interestingly, astrocytes store a several-fold higher glucose level than neurons, despite their actually less energy requirement compared to neurons [83], [84]. Astrocytes display a high glycolytic rate and metabolize glucose to pyruvate, which is transformed to lactate for a shuttle-mediated transfer to the neurons, where it is used as energy substrate for citrate cycle and mitochondrial respiration. Nevertheless neurons can uptake glucose as well by GLUT 3 transporter [85]. However, glycolysis appears to be limited in neurons by restriction of the enzyme Phosphofructokinase-2/Fructose-2,6-bisphosphatase (PFKFB). This key enzyme of glycolysis catalyzes the phosphorylation of fructose-6-phosphate to fructose-2,6-bisphosphate. PFKFB is highly expressed in astrocytes and strongly restricted in neurons by continuously proteosomal degradation, which impairs the stimulation of glycolysis in neurons. Anyway, neurons increase the ability of glucose utilization rate under several condition such as high brain activity [86], [87] or a high lactate blood concentration [88].

The GLUT-1-mediated glucose uptake into the brain is an insulin independent process. This transporter is an uniporter, which enables a carrier-mediated transport along a concentration gradient without energy requirement, called as facilitated diffusion [89], [90]. With this type of transport system the brain directs the energy supply of the body. The brain uptakes first its required energy in form of glucose, before the energy requirement of the peripheral tissues is satisfied (selfish brain theory). Figure 5 summarizes the GLUT-mediated glucose transport into the brain.

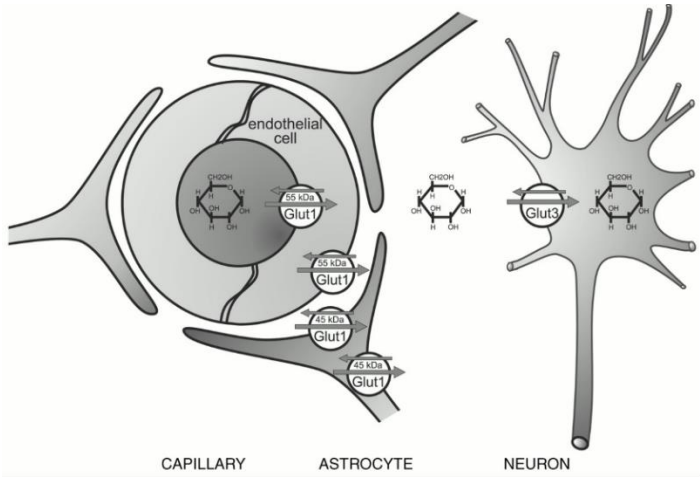

Figure 5: **GLUT-mediated glucose transport into the brain.**

Schematic presentation of distribution of GLUT transporter on blood brain barrier, astrocytes and neurons for verification glucose supply of neuronal tissues. Taken from [91].

1.3.2 Methylglyoxal - a highly reactive glucose carbolite

Methylglyoxal (MGO) is a side product of the glycolysis. MGO is a highly reactive α-oxoaldehyde formed in a significant amount, approximately 0.1%-0.4% of glucose flux [92] as a normal byproduct of glucose metabolism and can be formed as an intermediate of glycation reaction. Its structure is shown in figure 6. MGO is formed from glyceraldehyde-3 phosphate (GAP) and dihydroxyacetone (DHAP). In rat muscles, the non-enzymatic formation rate from GAP and DHAP to MGO could be determined with 0.1 mM per day [93], [94]. MGO shows about 20,000 times more reactivity than glucose in glycation reactions and thus it has a short half-life time *in vivo*, indicating 90 up to 99% of MGO is bound on macromolecules such as proteins [92]. It leads to the formation of several AGEs with changes in the protein functions. Moreover, AGE-modified proteins interact with AGE receptor RAGE with downstream inflammatory effects. MGO reacts preferably with the basic amino acids lysine or ariginine to well-known and well-characterized carboxyethyllysine or argpyrimidine [95], but can modify also aromatic amino acids such as thryptophane to β-carboline [96]. Most of these AGE products are fluorescent, thus, increase of AGE can be detected by fluorescence spectroscopy. This diagnostic technique is in the skin analysis in age- or disease-related matter already established.

At first in *Escherichia coli* could be detected a physiological concentration of MGO reached to 20 µmol [97], [98]. In the presence of a constantly high concentration of glucose in *Escherichia coli*, the MGO concentration also increased until glyoxalase I removes MGO [98], [99]. This ubiquitous enzyme mediate also in humans the main detoxification pathway for MGO to a reduced non-toxic level. Cerebrospinal fluid levels of MGO have been investigated to a concentration of 10 to 20 µM [100], cellular level of MGO are supposed in a lower µM range [101]. It is important to understand that this pathway does not produce any ATP, this pathway does not replace glycolysis but runs simultaneously to glycolysis and is only initiated with an enhanced concentration of sugar phosphates, e.g. glucose-6-phosphate. One believed purpose of the methylglyoxal pathway is to help release the stress of an elevated sugar phosphate concentration.

It is known that higher concentration of MGO correlates with greater accumulation of AGEs in all tissues including the brain over time [100], [102], [103]. Higher serum concentration of MGO is associated with poorer cognitive function and lower brain volumes [104]. In this context, it could be shown that methylglyoxal levels in Alzheimer´s disease patients were twofold higher than in the ones of the control group [100]. Additionally, it could be demonstrated that glyoxalase I is up-regulated in Alzheimer´s disease patients, but the glyoxalase system is limited in neurons. Up-regulating the glycolysis rate results in an enhanced concentration of MGO, if the capacity of the

glyoxalase system is exploited then the toxic effect of MGO increases. Since, MGO has been identified as a reactive neurotoxic intermediate of AGE-formation, statistical surveys on humans were carried out to determine the correlation between MGO–induced AGE-formation and degenerating effects of brain tissue, but the investigation on molecular level are still missing.

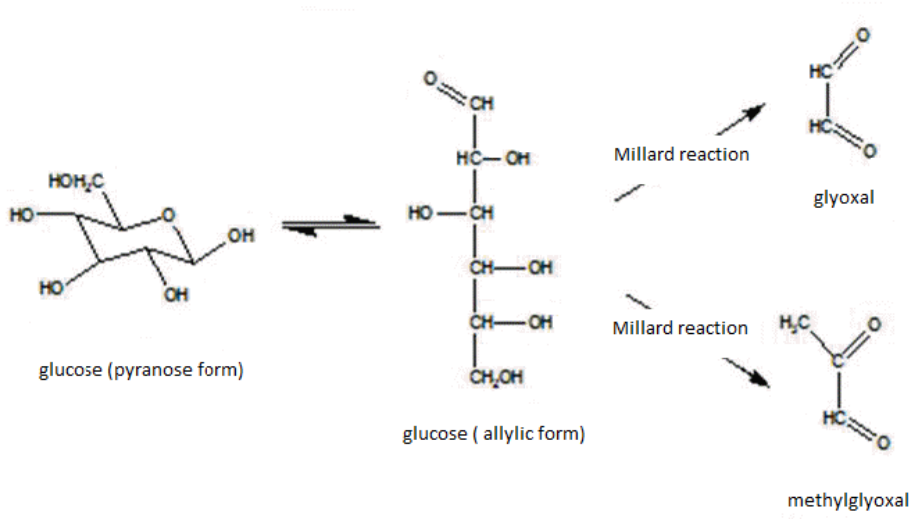

Figure 6: **Non-enzymatic conversion from glucose to α-oxoaldheydes.**
The α-oxoaldheydes are intermediates of glycation (Milliard reaction), which reacts finally irreversibly with proteins to AGEs, called glyoxal or methylglyoxal. The detailed formation of these intermediates is still not completely known. Besides this reaction methylglyoxal is a side products of glycolysis.

1.3.3 Cell adhesion and cell-matrix adhesion

Cell adhesion is essential for proliferation, differentiation, cell survival and function of cells as well as for cellular communication as it occurs in many physiological processes of a multicellular organism. Adhesion is defined as the specific receptor-mediated contact between cells or between cells and the surrounding extracellular matrix (ECM) [105]. These interactions have on the one hand a mechanical function, which are important for the cell migration, tissue integrity such as elasticity and tensile strength. On the other hand, the adhesion receptors also act as a signal transmitter. They are able to receive signals from the immediate environment and to transfer them into the interior of the cell and thus, they serve as adhesion receptors. Through activation of cell adhesion molecules may include intracellular restructuring of the cytoskeleton and the initiation of signal transduction processes, which thus e.g. can also lead to a change in gene expression [106]. A number of adhesion molecules associate with members of the cytoskeleton and / or intracellular signaling molecules, such as protein kinases, phosphatases or adapter proteins. However, they even can represent a kinase or

phosphatase or laterally, associated with other receptor proteins such as receptor tyrosine kinases [106]. Cell adhesion receptors are a heterogeneous group transmembrane glycoproteins, which can be divided in four groups, selectins, cadherins, molecules of the immunoglobuline family (Ig CAMs) and integrins [106], [107]. Selectins, cadherins and Ig CAMs are mainly important for formation of cell-cell contacts and communication; integrins interact with the ECM [107]. Cell-matrix interactions capture a central role at migrating cells, differentiation of neurites by neurite outgrowth as well as in their targeted navigation. In the tissues cells are tight connected to the extracellular matrix, which consists of complex extracellular macromolecules. The heterogeneous population of extracellular matrix proteins include numerous proteoglycans belonging (so far identified) nineteen collagens [108],[109], eleven laminin isoforms [110], fibronectin, nidogen, elastin ,and a large number of further components. They are important for the supramolecular organization of the extracellular matrix, which is based on further molecular interactions. In general, the extracellular matrix is responsible for the structural and functional organization of cells and for the mechanical stability of tissues.
Prominent AGE-modifications of ECM proteins are known e.g. for skin containing collagen, which decrease skin elasticity and increase its auto-fluorescence and are also known for lens matrix proteins in a disease-related matter.

1.3.4 Neuronal plasticity

Neural plasticity refers to the ability of neural structures to adapt, depending on their usage. This may relate to individual nerve cells or extend to all areas of the brain. Until a few decades ago it was assumed that the brain is a rigid system, but recently research showed that the brain is quite plastic and adaptable. An element of the neuronal plasticity is the structural plasticity. Synapses underlie structural plasticity when axons or dendrites degenerate and / or be stretched out in different directions. Neurogenesis is found in humans only in two areas of the brain, the olfactory *bulb* and the *dentate gyrus* of the hippocampus, also part of the structural plasticity. Here current research projects engage, which attempt to generate new neurons from pluripotent stem cells to replace damaged tissue [111].
Neuronal structures need a high energy level for processes such as learning and memory. Therefore the brain keeps a lot of workflows alive, for instance maintenance of action potentials or restoration ion gradients, uptake and recycling neurotransmitter or postsynaptic excitation transfer. For these functions neurites play an important role. The entirety of nerve processes, dendrites and axons, are called neurites. Neurites serve as recording stimuli and transfer them from neuron to neuron. Despite the importance of the

dendrites of neurons it is only little known about growth of dendrites, their orientation and branching *in vivo*.

Neurite outgrowth serve as marker for neuronal plasticity. To ensure the functionality of neurons e.g. neurite outgrowth, an intact cell adhesion is required. For investigations, cell adhesion and neurite outgrowth were used as marker for neuronal plasticity. While cell adhesion describes cell-cell interaction and adherence of cells to an extracellular matrix, containing collagen, laminin and/or fibronectin, neurite outgrowth describes the growth of dendrites and axons of neurons. Neurite outgrowth is mediated by activation of TrkA receptor, a receptor tyrosine kinase of the Trk family. TrkA receptor is activated by binding the nerve growth factor (NGF) [112]. The extracellular part of the TrkA receptor consists of an embedded in two cysteine-rich clusters leucine-rich domain followed by two immunoglobulin-like (Ig) domains [113]. The binding occurs mainly via the Ig-C2 domain [114]. TrkA binds NGF with a stoichiometry of 2:2, which means that two TrkA receptors can bind an NGF-dimer (figure 7). The binding of the ligand induces dimerization [115]. NGF is described as neuronal survival factor for sympathetic and sensory neurons [116]. After binding on the high affinity receptor TrkA, NGF promotes differentiation, survival and neurite outgrowth [117]. Several activated pathways are schematically illustrated in figure 7. Moreover, the transduction pathway of NGF/TrkA plays a central role during the development of the nervous system at several ontogenetic stages [117]. Activation of TrkA by binding of NGF activates MAPK, RAS, phoshoinositide 3-kinase (PI3K) and phospholipase Cy1 pathways [112]. The neuronal plasticity is age-dependent. It increases from birth to adult but during ageing the neuronal plasticity decreases [118]. Although a decreased level of NGF in aged rats has been determined [119], little is known about age-related changes of the NGF/TrkA pathway.

The influence of glycation on neuronal plasticity in an age-related context is not investigated yet. However, glycation on proNGF leads to reduced availability of NGF, followed by an imbalance of proNGF/NGF, which leads to an inhibition of the NGF-mediated neuroprotection [120]. Further studies, in relation to neurodegenerative diseases, have shown that glycation induces the formation of β-sheet structures in β-amyloid protein, α-synuclein, transthyretin (TTR), copper-zinc superoxide dismutase 1 (Cu, Zn-SOD-1) or prion proteins. Aggregation of β-sheets in each case creates fibrillar structures, probably causing Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, familial amyloid polyneuropathy or prion diseases [121]. These findings demonstrated that glycation is not a negligible factor in neurodegeneration. Highlighting the involvement of AGEs in neurodegeneration using Alzheimer´s diseases as example. Alzheimer´s disease is the most common neurodegenerative diseases of aged people with almost 50% over 85 years [122]. Characteristic features represent loss of memory,

recognition ability and final loss of speech as well as basic motoric and social skills. The dysfunctions interfere with degeneration of nerve tissue, especially in the hippocampus and the forebrain. Histological findings are characterized by destruction of nerve cells and protein deposition, described as β amyloid plaques. Glycated β -amyloid protein could be already detected in amyloid plaques of patients, suffering from Alzheimer´s disease [7], [123]. Glycated-amyloid β may exacerbate neurotoxic effect. Mice with glycated amyloid β show increased loss of cognitive functions compared to mice with amyloid β with reduced glycation level [124]. Patients with diabetes mellitus and Alzheimer´s disease showed higher AGE levels and up-regulated RAGE compared to patients only with Alzheimer´s disease. Anyway, there exist different cases for development Alzheimer's diseases but AGEs appear to present one distinct factor.

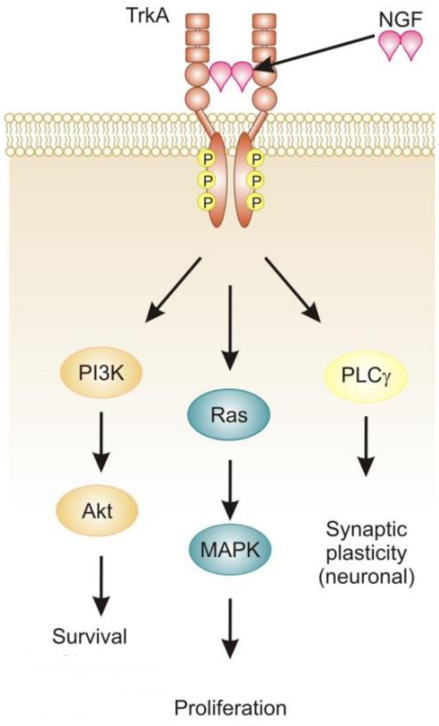

Figure 7: **NGF-mediated pathway on high affinity receptor TrkA.**

NGF binding as dimer on TrkA receptor stabilizes TrkA receptor dimer and triggers receptor *trans*-auto-phosphorylation. Phosphorylation and activation of the receptor dimer initiate several signaling pathways for cell protection and suppression of apoptosis-induced cell death. Taken from [125].

2 Aims of work

Advanced glycation endproducts (AGEs) represent a non-enzymatic posttranslational modification, generated by a chain of chemical reactions of free reducing sugars and free exposed amino acid residues. AGEs accumulate in all tissues as a normal feature of ageing. Their role in peripheral age-related diseases such as diabetes mellitus or arteriosclerosis is already extensively investigated. It is also described that AGEs interfere in pathogenesis of neuronal disorders but a detailed characterization of molecular mechanism of involvement by AGEs is still missing. Therefore this thesis aims to investigate the influence of AGEs on the neuronal plasticity, as an indicator for neuronal health. For induction of glycation reaction a physiologically high reactive AGE-precursor was used: methylglyoxal. Firstly cell adhesion and neurite outgrowth of *in vitro* model, PC12 cells to glycated extracellular matrix proteins (ECMs) should be analyzed by real-time cell analyzing. In the second part, the testing condition should be reversed and cell adhesion and neurite outgrowth of glycated PC12 cells to ECMs should be analyzed. Neurite outgrowth should be induced by NGF-mediated activation of the MAPK signal transduction pathway. In frame of analyzing neurite outgrowth, the influence of AGEs on the activation of this signal pathway should be also examined.

AGE-modifications concern also cell surface proteins. Therefore third aim of this thesis was to quantify the binding affinities by determination of the equilibrium dissociation constant after receptor glycation, using high affinity NGF-receptor TrkA and AGE-binding receptor, RAGE.

RAGE, plays a particular role in neuronal disorders by induction several receptor-mediated pathways. One frequently studied ligand of RAGE is glycated bovine serum albumin (glycated BSA), thus by binding to RAGE the activation of the described signaling pathways could be detected. In this context, the last part of this thesis aims to quantify the binding of glycated-BSA to RAGE for the first time. Additionally, the influence of activation of RAGE by binding glycated-BSA on neuronal plasticity is an aim of this thesis. Hence the glycated-BSA dependent activation of MAPK pathway should be analyzed on the *in vitro* model.

The resulting findings from this study should form the basis for a better understanding of the effects of AGEs in and on neuronal cells with regard to neural disorders.

3 Material and Methods

3.1 Chemicals, instruments and consumables

All chemicals were obtained, unless stated otherwise, by Roth, Merck or AppliChem. Used instruments or consumables were specified in the part of methods.

3.2 Antibodies

All primary or secondary antibodies were obtained, unless stated otherwise, by abcam (Cambridge, United Kingdom).

3.3 Receptors and ligands

Receptors	
Human TrkA receptor with C-terminal His-tag	Sino Biological Inc., Beijing, China
Human recombinant Receptor for Advanced glycation endproducts (RAGE) with C-terminal Flag-tag	MyBioSource, San Diego , USA

Ligands	
Recombinant human β-Nerve growth factor (rh β-NGF)	ImmunoTools, Friesoythe, Germany
Bovine serum albumin (BSA) Glycated BSA with glucose as glycation reagent Glycated BSA with fructose as glycation reagent Glycated BSA with ribose as glycation reagent	Produced and provided by Kathleen Jacobs and Alexander Navarette Santos of research laboratory “Herz- und Thoraxchirurgie”, medical faculty of Martin Luther university, Germany

3.4 Prepared reagents

Luminata Forte Western HRP Substrate	Merck Millipore, Darmstadt, Germany
Page Ruler^{TM} prestained protein ladder	Thermo Fisher Scientific, Waltham, USA
Protease InhibitorCocktail	Sigma-Aldrich, St.Louis, USA
Sodium orthovandate	Sigma-Aldrich, St.Louis, USA

3.5 Commercialized kits

Annexin V-FITC apoptosis detection Kit	Abcam, Cambridge, United Kingdom
In vitro Toxicology Assay Kit, MTT based	Sigma-Aldrich, St. Louis, USA
Pierce BCA Protein Assay Kit	Thermo Fisher Scientific, Waltham, USA

3.6 Buffers and Solutions

All buffer and solutions for universal usage are listed here and all other used buffers for experimental specific usage are described in the corresponding part of methods.

PBS for cell culture

150 mM NaCl
3 mM KCl
8 mM Na_2HPO_4
1 mM KH_2PO_4 pH 7.4

Blocking solutions

0.5% - 5% BSA in TBST
3% gelatin in PBS

TBS buffer	TBST buffer
730 mM NaCl 27 mM KCl 4 M Tris, pH 7.5	TBS buffer + 0.1% Tween 20 (v/v)

10x Running buffer for SDS-PAGE
0.25 M Tris pH 8.8 1.92 M glycine 1 % SDS (w/v)

Resolving gel (8%)	Stacking gel (4%)
25% Rotiphorese® Gel 30	0.4 ml Rotiphorese® Gel 30
1.5 M Tris/HCL pH 8.8	0.5 M Tris/HCL pH 6.8
0.2 % SDS (w/v)	0.2 % SDS (w/v)
120 µl 10% APS	12 µl 10% APS
12 µl TEMED	3 µl TEMED
7.2 ml distillated water	1.85 ml distillated water

3.7 Cell biological methods

3.7.1 Cultivation of PC12 cells

Rat adrenal medulla pheochromocytoma cells (PC12) are solution cells [126], which were cultured in RPMI-1640 with 5% heat-inactivated fetal calf serum (FCS), 10% horse serum (HS) and 2 mM L-glutamine in humidified 5% CO_2 atmosphere at 37°C in the cell culture incubator Hera cell (Heraeus Holding GmbH, Hanau, Germany). The cultures were also supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (all materials from PAA, Cölbe, Germany). The cells were split according to confluence every third or fourth in a ratio 1:3 up to 1:4. Cells were harvested by centrifugation at 110 g for three minutes and washed twice with sterile PBS (pH 7.4) (cp. 3.6).

3.7.2 Matrix AGE-modification

Cell culture plates were coated with collagen IV or laminin (20 µg/ml for 2 hours; OMNI LIFE Science, Bremen, Germany). Plates were AGE-modified with the reactive carbonyl compound 1.0 mM methylglyoxal (Sigma-Aldrich, St. Louis, USA) for 4 hours at 37°C. Some plates were divided into two parts and only one half of each plate was AGE-modified as described above. Finally plates were thoroughly washed twice with sterile PBS (pH 7.4) (cp. 3.6).

3.7.3 Cellular AGE-modification

When cells reached 80% confluence, methylglyoxal (MGO) was added at several final concentrations of 0.1 mM, 0.3 mM or 1.0 mM to serum-free cell culture media for 4 hours in a humidified 5% CO_2 atmosphere at 37°C. Untreated PC12 cells cultured under serum-free conditions were used as control. After the incubation time, cells were harvested, centrifuged at 110 g for three minutes and washed twice with sterile PBS (pH 7.4) or were cultured under serum containing conditions.

3.7.4 Flow cytometry

Flow cytometry is a laser-based biophysical technology, which allows cells to classify dependent of their structure, form, size and coloring in different cell types and cell states. Therefore suspended cells pass in a single cell stream of fluid, were scanned by a light ray and induce, according to morphology, several effects to draw conclusions from their properties. For detection by flow cytometry 5×10^6 PC12 cells were used at the Accuri 6 flow cytometer (BD Biosciences, Heidelberg, Germany).

3.7.5 Detection of AGE-modification by flow cytometry

For the detection of AGE modification of PC12 cells, AGE-modified PC12 cells were harvested and fixed by 4% paraformaldehyde/PBS for 10 minutes at 4°C. Cells were permeabilized with 0.1% PBS-tween for 2 minutes at 4°C. Non-permeabilized or permeabilized cells were then blocked with PBS/0.3 M glycine/5% FCS for 15 minutes at 4°C. Anti-CML26 antibody, was added at a concentration of 0.5 µg/ml for one hour at 4°C. This antibody detects specific AGEs on lysine residues, carboxylmethyllysine (CML) and carboxylethyllysine (CEL). CML and CEL applies as biomarker for AGE-formation [127], [128]. Afterwards cells were incubated with the Delight 488-fluorescence labeled secondary anti-mouse antibody. (Conjugated with fluorescent dye Alexa Fluor®488, Invitrogen, Darmstadt, Germany) for one hour at 4°C. Cells were washed twice between all steps. However finally cells were re-suspended in sterile PBS (pH 7.4) and the fluorescence of the labeled antibody was measured by Accuri C6 flow cytometer using the channel F1 for green fluorescence light detection (533 nm). In the forward-light scattering display a threshold (discriminator) was set, which excludes all these impulses such as impurities or not desired particles e.g. cell debris.

3.7.6 Apoptosis assay

For analyzing interfering of AGE-modification with the live cell states, an apoptosis assay is performed. Therefore PC12 cells were labeled with annexin V-FITC (green) and propidium iodide (PI) (red). Cells were analyzed using the Accuri C6, a dual-laser flow cytometer. Annexin V-FITC and PI marked cells were excited using a 488-nm laser light. Fluorescence emission was detected at 533 nm (F1 channel for green fluorescence) and at 585 nm (F2 channel for red fluorescence). For dual labeling, fluorescence emissions of individual fluorophores were corrected by spectral overlay using electronic compensation. Additionally, a threshold was set in the forward-light scattering display (cp. 3.7.5).

3.7.7 Tryphan blue staining

Glycation reagent methylglyoxal is cytotoxic in higher concentration. Therefore the cell viability of AGE-modified PC12 cells is analyzed. Moreover, the cell viability of PC12 cells in presence of glycated BSA was also analyzed.

Immediately after AGE-modification of PC12 cells, the cells were centrifuged at 110 g for 3 minutes and washed with PBS (pH 7.4). Cells were re-suspended in PBS and mixed

1:1 with trypan blue dye 0.4% (Invitrogen, Darmstadt, Germany). Cells were counted using an automated cell counter (Countess; Invitrogen, Darmstadt, Germany).

3.7.8 MTT assay

The MTT assay is a colorimetric assay for evaluating cell metabolic activity. Evidence of cell viability is based on the reduction of yellow, water-soluble dye 3- (4,5-dimethyl-2-yl) -2,5-diphenyl (MTT) in a blue-violet, water insoluble Formazan by NAD(P)H-dependent cellular oxido-reductase enzymes. Cell viability of AGE-modified PC12 cells and of PC12 cells in presence of glycated BSA was also analyzed.157 cells/mm^2 of AGE-modified PC12 cells were cultured on laminin (20 µg/ml) coated 96 well plates (Corning, Tewksbury, USA) for 48 hours or 157 cells/mm^2 unmodified PC12 cells were cultured on laminin (20 µg/ml) coated plates in presence of 100 ng/ml NGF for 48 hour and were supplemented with 50 µg/ ml or 500 µg/ml glycated BSA. 10% of culture medium volume of 3-[4,5- dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) was then added to all cultures and incubated for three hours at 37°C. Formazan crystals were dissolved by adding MTT solubilization solution equal to original culture medium volume and were spectrophotometrically analyzed at a wavelength of 570 nm according to the manufacturer´s instructions of MTT based *in vitro* toxicology kit.

3.7.9 Micrographs

Micrographs were taken visual control for cell viability after glycation procedure. PC12 cells were modified by MGO induced glycation such as described in 3.7.3. After 4 hours, cultures were fixed with 4% paraformaldehyde/ PBS for 10 minutes, stained with crystal violet and micrographs were taken.

3.7.10 Micrographs of neurite outgrowth

In each case micrographs of neurite outgrowth were taken as visual control. 5x10^3 PC12 cells were cultured on laminin (20 µg/ml) coated plates in presence of 100 ng/ml or 33 ng/ml NGF or for analyzing neurite outgrowth on AGE-modified ECMs. Furthermore, 157 cell/mm^2 PC12 cells were cultured on AGE-modified and non-modified laminin (20 µg/ml) coated plates (cp. 3.7.2) in presence of 100 ng/ml NGF. However after 48 hours, cultures were fixed with 4% paraformaldehyde/ PBS for 10 minutes, stained with crystal violet and micrographs were taken.

3.7.11 Real time cell analyzing

A real-time cell analyzer (RTCA) (OMNI Life Science, Bremen, Germany) was used to quantify cell adhesion and neurite outgrowth. The RTCA allows to measure the behavior of live cells in real-time, based on the impedance measurement with sensor electrodes at several signal frequencies. The measurement principle is implemented by the interaction between the cells and biosensors. Therefore micro-titer plates, called E-plates, are coated by a gold layer, which are connected by microelectrodes. Thus changes in the cell-electrodes interaction is continuously detected by a changed impedance. The change of the electrical impedance is expressed as cell index and represents the status of the cell. Cell morphology, cell adhesion, cell development and proliferation influence the cell index. Moreover, our working group could show that the cell index also correlates with the length of the neuritis. Therefore the cell index can also be used as an indicator for neurite outgrowth. The experiments were occurred by the XCELLigence RTCA (Roche, Basle, Schwitzerland) using PC12 cell, which were seeded with a density of 157 cells/mm^2. For maintenance the cultivation conditions the RTCA experiments were carried out in the Hera cell incubator.

3.7.12 Cell adhesion of PC12 cells by Real-time cell analyzer (RTCA)

E plates were coated with 20 µg/ml laminin or collagen IV in PBS for 1 hour at 37°C. After coating, the E plates were washed with PBS and blocked with 0.5% BSA for 30 minutes at room temperature. After two further washing steps with PBS, PC12 cells were seeded onto coated E plates and cell adhesion was continuously monitored for 4 hours.

3.7.13 Differentiation of PC12 cells by Real-time cell analyzer (RTCA)

For analyzing neurite outgrowth, PC12 cells were seeded on laminin coated (20 µg/ml) E-plates (OMNI Life Science, Bremen, Germany) as described under 3.7.11 cells were then monitored continuously for 48 hours in the absence or presence of 100 ng/ml NGF by the xCELLigence system. Neurite outgrowth of PC12 cells on glycated ECM proteins were also monitored by RTCA, using AGE-modified laminin coated E plates for seeding cells.

3.7.14 Preparation of cell extracts

Cell pellets were solubilized at 4°C for 15 minutes in buffer containing 150 mM NaCl, 50 mM Tris, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and in presence or absence of 1% Triton X-100

at pH 7.4 with freshly supplemented protease inhibitor cocktail (PIC; diluted 1:500). After centrifugation at 16,600 g for 10 min, supernatants were collected as cytosolic fraction. Cell pellet was collected as membrane fraction.

3.8 Protein biochemical methods

3.8.1 Determination of protein concentration

Protein concentrations were determined using 25 µl of 1:10 diluted cell lysate (cp. 3.7.14) by bicinchoninic acid (BCA) method. The procedure was occurred in according to manufacturer´s instructions, whereby 96 well plates were used. Plates were evaluated by the microplate reader Multiskan EX (Thermo Fisher Scientific, Rockford, USA) at 560 nm. For a precise determination of protein concentration a threefold measurement was carried out.

3.8.2 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

The one-dimensional SDS gel electrophoresis was used to separate the PC12 cell lysate containing proteins. Polyacrylamide gels consisting of 8% resolving gel, covered by 4% stacking gel, which serves to concentrate the protein sample. The electrophoresis was occurred under denaturing and reducing conditions in SDS-PAGE chambers (VWR, Radnor, USA). Proteins were denaturized by mixing with one fold volume of 5x reducing Laemmli loading dye and followed by incubation for 4 minutes at 94°C on the heating block Thermomixer Univortemp (Universal Labortechnik GmbH, Leipzig, Germany). 25µg protein amount was loaded onto the SDS-polyacrylamide gel. In addition, the Page Ruler^{TM} prestained protein ladder was loaded as protein standard. Electrophoresis was run at 50 V (constant voltage) through the stacking gel and with an increased voltage of 120 V for separation in the resolving gel for around 45 minutes using 1x running buffer.

3.8.3 Western Blot

Samples were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membrane using tank blot method. Therefore a transfer buffer, containing 150 mM glycine, 20 mM Tris/HCl (pH 8.3) and 10% Ethanol 96% (v/v), was used. The blotting process was occurred with a voltage of 300 V for one and half hour under continuously stirring. During the blotting process the blot chamber (VWR, Radnor, USA) was cooled down to avoid overheating. Afterwards a reversible Ponceau dye was performed with a

red colored solution, containing 2% Ponceau S (w/v), 30% TCA (w/v), 30% sulfosalicylic acid (w/v) to control the efficiency of the transfer.

3.8.4 Dot Blot

Dot Blot analysis represents a simplification of the western blot analysis, in this work using for single protein samples to clarify the glycation status. 20 µg of protein samples were spotted on a dry nitrocellulose membrane and were incubated in absence or presence of 1.0 mM MGO for 4 hours.

3.8.5 Chemiluminiscence Detection

The transferred proteins were detected by specific antibodies in a traditionally two-steps process, whereby the secondary antibody was linked to the reporter enzyme, horseradish peroxidase. This enzyme cleave a chemiluminescent agent, and the reaction product luminesce in proportion to the amount of protein. But at first, to avoid unspecific binding of the antibodies, the blots were blocked with 3% gelatin for 30 minutes at room temperature. The nitrocellulose membrane of the Dot Blot analysis were blocked with 5% BSA. Blots were incubated with primary antibodies, solubilized in TBST buffer, overnight at 4°C. AGE-formation was detected using a lysine-metabolized AGE-specific monoclonal antibody, anti-CML26 antibody at a concentration of 0.1 µg/ml. This antibody detects specifically CML and CEL, but mention also other types of AGE can be formed, which were not considered in this analysis. For the detection of the NGF-induced activated pathway, antibodies to ERK1/2 and phospho-ERK1/2 were used at a concentration of 0.1 µg/ml. After washing twice with TBST Subsequently, the blots were incubated with the secondary antibodies for one hour at room temperature. Proteins of interest were detected by addition of Luminata Forte Western HRP Substrate, according to the manufacturer's instructions and visualized by exposing blots for 10 to 120 seconds using a BioRad Imager system (BioRad, München, Germany).

3.8.6 Coomassie staining

20 µg of the protein samples were separated on 8% SDS-polyacrylamide gel. The gel was stained with 1% Coomassie brilliant blue (Serva Electrophoresis GmbH, Heidelberg, Germany) in 10% acetic acid, 40% ethanol and 50% H_2O for 20 minutes. Afterwards the SDS polyacrylamide gel was washed by an aqueous solution of 7.5% acetic acid and 5% ethanol for 24 hours with continuous shaking and at least three solvent changes to ensure an adequate removal of unspecific background coloring.

3.8.7 Production of glycated BSA

For the procedure 4 solutions were prepared. For each solution 1 mM endotoxin free BSA was dissolved in 50 mM potassium phosphate buffer, supplemented with 1 mM EDTA, pH 7.3. 0.5 M D(-) glucose, D(-) fructose and D(-) ribose were separately added to each BSA solution. The fourth solution without additions was used as control. The four preparations were incubated at 50°C for 40 days. After incubation, the glycated BSA and BSA control solutions were dialyzed against double distillated water for 72 hours. Each solution was lyophilized, resolved by sterilized PBS, pH 7.4 and adjusted to a concentration of 10 mg/ml (150 µM). This procedure was realized by Kathleen Jacobs and Alexander Navarette-Santos.

3.8.8 Surface plasmon resonance (SPR)

Receptor binding interactions were performed using a surface plasmon resonance (SPR) Biacore 2000 or Biacore 3000 system (GE Healthcare, Freiburg, Germany). Surface plasmon resonance represents a technique to measure biomolecular interactions in real-time based on a quantum-mechanical phenomenon on the sensor chip surface [129]. The biosensor chip exist of a glass surface coated with a thin gold layer. Polarized light is total reflected on the sensor surface and induces a surface plasmon resonant wave. This SPR wave is changing through mass bound and this SPR effect is expressed in an attenuated total reflection, which is detected by a biosensor. The most common biosensor chip, the CM5, carries a carboxy-methyl dextran molecules on the gold surface. This dextran film forms a hydrophilic matrix allowing to bind covalently non-denatured biomolecules. The other interaction partner pass in free solution over the surface. The biosensor measures the changes of mass on the surface during the interaction of the mobile with immobilized interaction partner, expressed in response units (RU). The signal strength correlated proportional to the bounded mass on the surface. 1 RU corresponds to 1pg/mm^2 mass bound to the sensor surface.

3.8.9 NGF /TrkA kinetics

NTA sensor chips (GE Healthcare, Freiburg, Germany) were used for coupling of the C-terminal his-tagged TrkA receptor. Immobilization of the histidines coupled receptor relies on a NTA-chelated nickel atom allowing a orientating of the biomolecule in a homogenous way with a free N-terminal site for the ligand interaction. Each flow cell was activated individually by injecting 500 µM $NiCl_2$ in HBS buffer for 1 min at 20 µl/min followed by injecting 0.2 M EDC/0.05M NHS (GE Healthcare, Freiburg, Germany) for 7

min at 5 µl/min. 50 µg/ml TrkA receptor was injected for 1-5 min at flow rate 2 µl/min until desired level of immobilization was reached. Around 400 response units (RU) ± 150 RU were coupled, which is equivalent to an amount of 400 pg receptor /mm^2 ± 150 pg/mm^2. This amount corresponds to the calculated analyte binding capacity (theoretical RU) of the chip. The real-time kinetics measurements were performed with several different immobilized TrkA flow cells for independent measurements. Excess reactive esters were blocked using 1 M ethanolamine (GE Healthcare, Freiburg, Germany) for 7 min at 5 µl/min. A non-receptor coupled flow cell provided as appropriate reference surface. As running buffer 10 mM HEPES, 150 mM NaCl, 50 µM EDTA, 0.005% Tween 20, pH 7.4 (HBS buffer) at a flow rate of 5 up to 10 µl/min was used. Binding isotherms were determined at 23°C.

To measure the kinetics of NGF (ImmunoTools, Friesoythe, Germany) over TrkA on the chip, a threefold serial dilution of NGF (0, 0.06, 0.2, 0.6, 1.85, 5.5, 16, 50 nM) on three independent TrkA immobilized surfaces were injected for 3 min at 30 µl/min, also allowing a 30 s dissociation phase. The measurements conditions were chosen in this way to minimize potential mass transport affects. Finally, the dissociation constant was calculated by three separate experiments on three flow cells. Surface was regenerated in two steps firstly with a 1:1 mix of running buffer pH 9.3 and 10 mM NaOH for 1 min at 10 µl/min and as second step with 10 mM NaOH for 10 s at 30 µl/min. The sensogram data from real-time bimolecular interaction analysis were evaluated with the BIAevaluation software.

3.8.10 RAGE/glycated BSA kinetics

A classical CM5 sensor chip (GE Healthcare, Freiburg, Germany) was used for coupling of RAGE. Therefore, the Biacore surfaces were coupled by a standard covalent amine coupling method, which allowed a more stable coupling. Each flow cell was activated individually by injecting 0.2 M EDC/0.05 M NHS for 7 min at 5 µl/min. 50 µg/ml of RAGE, solubilized in sodium acetate buffer, pH 4.5, was applied for 1-3 min at 2 µl/min until desired level of immobilization was reached, according of the calculated theoretical RU_{max} (final level of 400RU+/- 250 RU). Excess of reactive esters was blocked using 1 M ethanolamine for 7 min at 5 µl/min. A non-receptor coupled flow cell provided the appropriate reference surface. The HBS buffer (10 mM HEPES, 150 mM NaCl, 0.005% Tween 20, pH 7.4) was used as running buffer at a flow rate of 5 up to 10 µl/min. Binding isotherms were determined at 23°C.

Glycated BSA (fructose-BSA, glucose-BSA or ribose-BSA) solubilized in PBS buffer, was used as ligand for RAGE. To measure the kinetics of glycated BSA over RAGE on the chip serial dilutions of glycated BSA (0, 1.85, 5.5, 16, 30, 50, 75, 150 µM) were

injected for 6 min at 15 µl/min on the RAGE immobilized surfaces, also allowing a 30 s dissociation phase. The measurements conditions were chosen in this way to minimize potential mass transport affects. The dissociation constant was calculated by three separate experiments on three flow cells. Surfaces were regenerated by 10 mM NaOH for 32 s at 15 µl/min. The sensorgram data from real-time bimolecular interaction analysis were evaluated with the BIAevaluation software.

3.8.11 Glycation of receptors on SPR surface

Receptors were immobilized, as described above. Glycation was performed by injection of 0.1 mM methylglyoxal for 10 hours at 1 µl/min for RAGE receptor or 20 hours at 1 µl/min for TrkA receptor, respectively at 23°C. Only chemically bonded methylglyoxal in form of glycation endproducts remained, the rest of sticky methylglyoxal was removed by injecting HBS buffer at 10 µl/min for 5 min. For detection of AGE-modifications on the coupled receptors a polyclonal anti-AGE antibody, which recognize a wide range of AGE-modification.

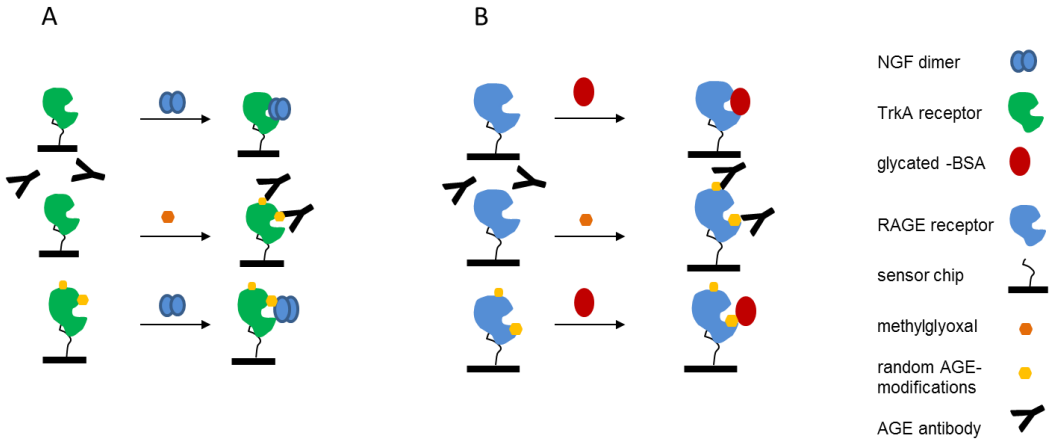

Figure 8: **schematic presentation of receptor-ligand binding before glycation and after glycation of the receptor.**

Receptor is coupled on chip surface. Ligands pass at the current flow over the chip for ability to bind the receptors. After measurement, chip surface was regenerated by removing ligand from receptor. Receptors were directly glycated on chip surface. Surface was washed for removing unbounded glycation agent, methylglyoxal and ligand was allowed to pass for binding on receptor. A) NGF-TrkA binding is shown and B) Glycated-BSA-RAGE binding before glycation, at detection of AGE modifications by AGE antibody and after glycation and ligand-receptor interaction after glycation.

4 Results

4.1 Glycation of extracellular matrix proteins interferes with neuronal plasticity

Ageing is associated with decreased regenerative capacity of the nervous system. One prominent molecular event during ageing is the accumulation of advanced glycation endproducts (AGEs). Accumulation of AGEs has been identified in different human brain regions and compartments in an age-related manner. AGE-formation occurs in presence of physiological concentrations of glucose or fructose over a long time. In the framework of this thesis glycation was induced by the reactive dicarbonyl specie methylglyoxal (MGO), which occurs as a physiological carbolite of glucose and it is a common agent to shorten the glycation process *in vitro*. Long-lived proteins e.g. extracellular matrix proteins serve as ideal substrates for glycation reactions.

4.1.1 Reduced cell adhesion of PC12 cells on glycated extracellular matrix proteins

Extracellular matrix is a complex environment of several types of protein filaments and essential for the anchoring of the cells and the shape consistency of the tissue. These extracellular matrix proteins (ECM proteins) play a decisive role in process of cell adhesion for nervous system development and regeneration. We used PC12 cells, which are non-adherent cells, form aggregates and adhere on several ECM components such as collagens or laminins. Cell adhesion of PC12 cells on unmodified laminin or collagen IV was compared to those of glycated laminin or collagen IV. First, glycation of laminin and collagen IV was analyzed by dot blot (figure 9). Detection with monoclonal CML26 antibody, a specific AGE-antibody for carboxymethyllysine and carboxyethyllysine, showed a strong glycation signal for laminin and also for collagen IV in comparison to their unmodified pendants. The unmodified ECMs showed also a low detection signals for AGE-modification. ECM proteins were successfully glycated by using methylglyoxal.

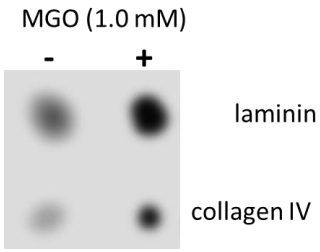

Figure 9: **Glycation of ECM proteins using MGO.**
20 µg laminin or collagen were spotted on a nitrocellulose membrane. The right part of the membrane was incubated with 1.0 mM MGO for 4 hours. AGE-formation was detected by dot blot analysis using monoclonal CML26 antibody.

Then, cell adhesion of PC12 cells on glycated collagen IV and laminin were quantified by monitoring in real-time. After 4 hours of real-time analysis, cell adhesion was quantified (figure 10). The glycation of collagen IV resulted in 80% reduction of cell adhesion. The cell adhesion of PC12 cells on glycated laminin declined even by 90%.

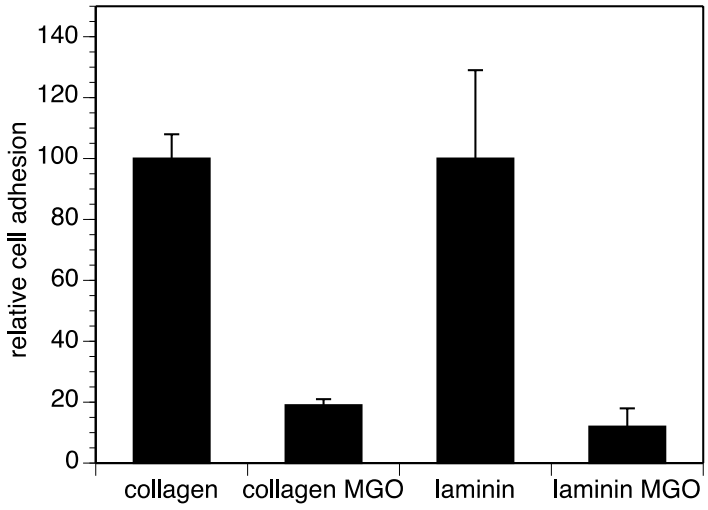

Figure 10: **Cell adhesion of PC12 cells on glycated ECM proteins**
20 µg laminin or collagen were spotted on a nitrocellulose membrane. The right part of the membrane was incubated with1.0 mM MGO for 4 hours. Each bars represent three independent measurement carried out in triplicates (*p<0.0001; n=9).

4.1.2 Impaired neurite outgrowth on PC12 cells to glycated ECM proteins

Cell adhesion is a crucial presupposition for neurite outgrowth and neuronal regeneration. NGF induces neurite outgrowth in PC12 cells, described by Pollscheit *et al.* [130]. PC12 cells were cultured on unmodified laminin or collagen IV and on glycated laminin or collagen IV in presence of NGF for over 48 hours. A plate, coated with ECM

proteins, was divided in two parts and one side was incubated with 1.0 mM MGO. Cell growth and differentiation on the border of glycated ECM protein to unglycated ECM protein was then visualized by representative micrographs. A drastic reduction of cell adhesion on glycated laminin or collagen IV compared to unglycated ECM proteins could be confirmed (figure 11 A). However, glycation appears not to influence the neurite outgrowth (figure 11). Note that PC12 cells, cultured on collagen IV, showed a stronger branching of neurites than on laminin, disregarding glycation (figure 11 B).

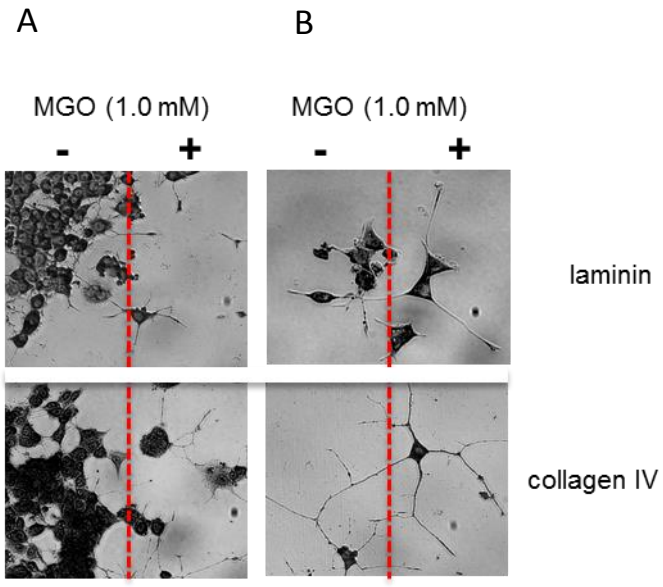

Figure 11: **Cell adhesion and neurite outgrowth at the border of glycated ECM proteins.**
Representative micrographs of PC12 cells taken at the border (red line) of glycated laminin or collagen IV coated cell culture plates. Three independent experiments were performed. A) Adherence of PC12 cells was preferably observed on unglycated ECM proteins (left side). B) Neurite outgrowth of single PC12 cells on the border of glycated ECM proteins or unglycated ECM proteins are represented.

4.2 Glycation of PC12 cells interferes with neuronal plasticity

Beside matrix proteins also cell surface proteins and intracellular proteins can be modified by AGEs. Methylglyoxal is as physiological intracellular carbolite compound [104], which reacts as glycation argent. Additionally, methylglyoxal is membrane permeable fluid and therefore it can be used as *in vitro* glycation agent of PC12 cells.

4.2.1 Validation of glycation on PC12 cells

PC12 cells were incubated in presence of serum free medium with several concentrations of MGO (0.1 mM, 0.3 mM or 1.0 mM) for 4 hours. PC12 cells under serum free condition served as control. After lysis, the glycation of intracellular proteins was analyzed by Western blot. For detection of AGE-modifications (glycation) the monoclonal CML26 antibody was used. The treatment with 0.1 mM MGO on PC12 cells does not lead to glycation of intracellular proteins (figure 12). With increasing concentration of MGO (0.3 mM up to 1.0 mM) a stronger going signal could be detected by Western blot analysis, whereas no distinctive bands can be identified. A variety of intracellular proteins appears to be glycated, an indication delivers the broad smear over the entire lane (figure 12).

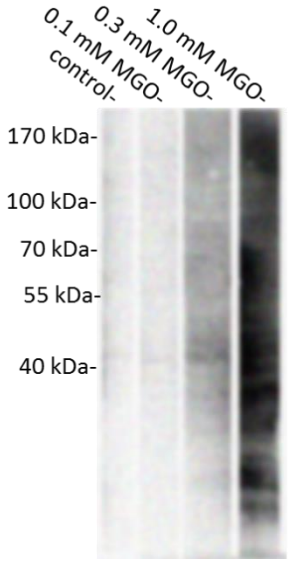

Figure 12: **Western blot analysis of Glycation of PC12 cell lysate.**

PC12 cells were incubated with PBS (control), 0.1 mM MGO, 0.3 mM MGO, 1.0 mM MGO for 4 hours. Cells were harvested, washed and solubilized. Protein concentration of the cytosolic fraction was determined by BCA test. 20 µg of each protein sample was subjected to SDS-PAGE-gel electrophoresis. Proteins were blotted and detected using monoclonal CML26 antibody. Representative Western blot of three independent performed experiments are shown.

The glycation of PC12 cells was quantified by flow cytometry. MGO-treated, PC12 cells were permeabilized for analysis of intracellular glycation. In order to verify glycation on the cell surface, analysis by flow cytometry was also performed with non-permeabilized cells. For detection AGE-modification, monoclonal CML26 antibody was used, which was bound by a fluorescent labeled secondary antibody. The fluorescence of the control cells corresponds to their own auto-fluorescence, emitted by cytoplasmic fluorochromes.

Permeabilized PC12 cells after treatment with 1.0 mM MGO has been shown 4 times higher fluorescence intensity than PC12 cells after treatment with 0.3 mM or 0.1 mM MGO and 1.0 mM MGO treated cells displayed an increase of fluorescence intensity by 30 times compared to the control cells (figure 13 A) This analysis of flow cytometry confirmed the result of the Western Blot analysis that intracellular proteins have been glycated after treatment with a concentration of 1.0 mM MGO. The fluorescence intensity of non-permeabilized cells increased continuously with enhancing concentration of MGO (0.1 mM up to 1.0 mM) until it has almost doubled (with a concentration of 1.0 mM MGO) compared to the control cells (figure 13 B). Comparing permeabilized and non-permeabilized cells after 1.0 mM treatment, the fluorescence intensity of permeabilized cells occurs a 12-fold increase by comparable fluorescence of the control cells for both sets of measurement. It appeared that a high amount of intracellular proteins modified by MGO related to the less protein content on the cell surface. The quantification of all fluorescence data is shown in figure 13.

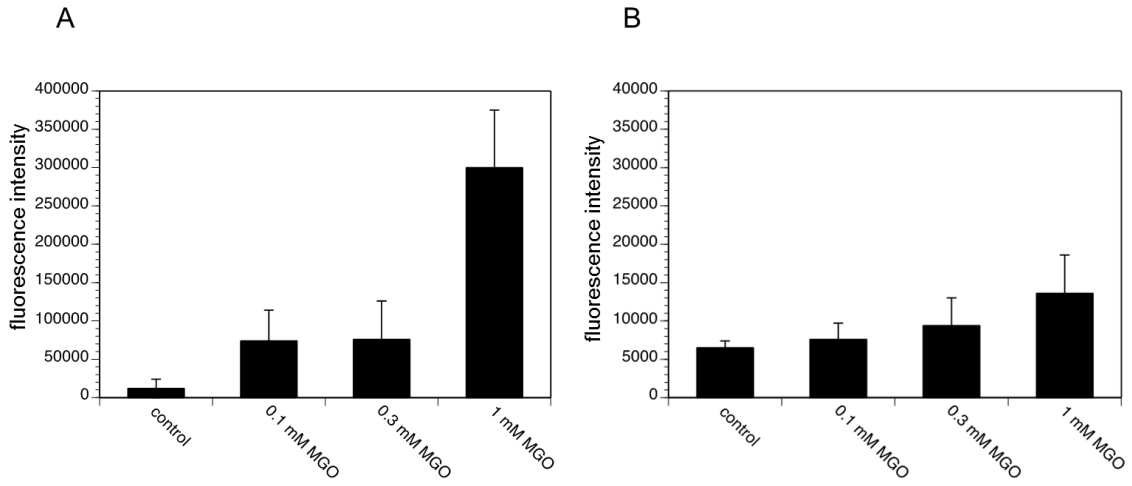

Figure 13: **Analysis glycation of PC12 cells by flow cytometry.**

PC12 cells were incubated with PBS (control), 0.1 mM MGO, 0.3 mM MGO, 1.0 mM MGO for 4 hours. A) Permeabilized cells and B) non-permeabilized cells were toughly washed to remove MGO. Cells were incubated with monoclonal CML26 antibody for an hour. The secondary antibody was fluorescence marked by Alexa Fluor®488. In each case, 10,000 cells were analyzed by flow cytometry. Bars represent standard derivation of three independent measurement carried out in triplicates.

4.2.2 Cell viability of PC12 cells

Cell viability bases on live cell properties as endocytosis, enzymatic activity, integrity of the cell membrane or propagation. Because each method has weaknesses, several detection methods were used, including microscopic and flow cytometric based methods. First, micrographs were taken and the cell morphology was compared (figure

14 A). There were no obvious differences in cell morphology after treatment of MGO up to a concentration of 1.0 mM MGO. Furthermore a tryphan blue exclusion assay was performed. This is a diffusion-based assay, in which the dye is able to pass through perforated cell membranes. However, living cells are scarcely stained. The amount of stained cells was relatively constant with increasing concentration of MGO (up to 1.0 mM) comparable to the untreated control cells (figure 14 B). To quantify membrane integrity of PC12 cells more in detail, a vital fluorescent double staining for the simultaneous detection of apoptosis and necrosis was additionally performed with propidium iodide (PI) and Annexin V. One early event in the early apoptosis is the translocation of phosphatidyl-serine from the inner to the outer membrane facing the cell surface. Phosphatidyl-serine can be detected by the phosphatidyl-binding protein Annexin V. Thus, Annexin V-stained cells were classified as apoptotic. Cells stained with Annexin V and PI indicated dead cells, while PI-stained cells were classified as necrotic. PC12 cells and glycated PC12 cells (0.1mM, 0.3mM or 1.0mM MGO) were labeled with Annexin V and PI. No obvious difference in the distribution of the cells could be observed between glycated PC12 cells and unglycated PC12 cells (figure 14 C). 87% of control cells are alive and 11% are classified as early apoptotic cells and less than 1% as late apoptotic cells. The number of necrotic cells is negligible. The values of glycated PC12 cells are similar to the control cells but it appeared that 1.0 mM MGO treated PC12 cells incline more to apoptosis with 15% early apoptotic cells than less glycated cells or than the control cells. Thus, it could be resumed that 1.0 mM MGO should be the maximal concentration for treatment on PC12 cells for 4 hours.

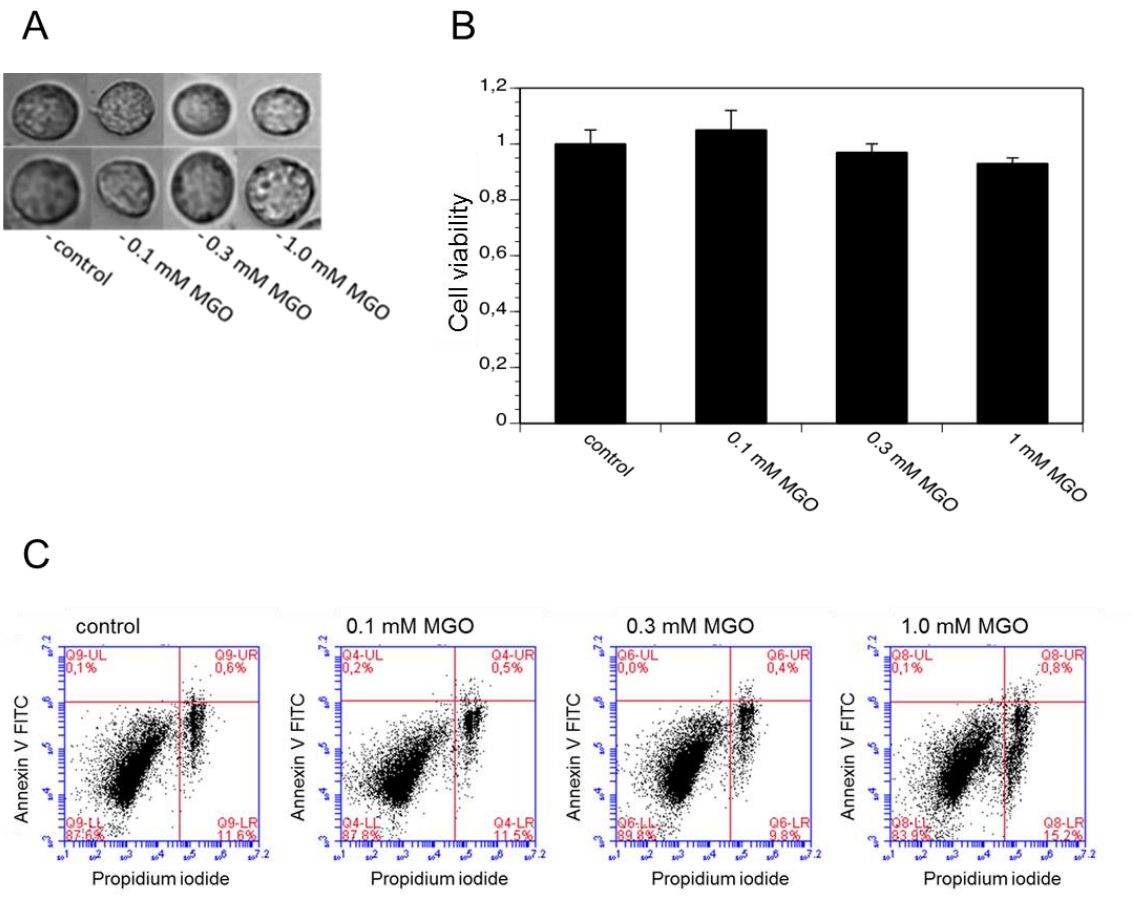

Figure 14: **Cell viability of PC12 cells after inducing glycation by using MGO.**

PC12 cells were incubated with PBS (control), 0.1 mM MGO, 0.3 mM MGO, 1.0 mM MGO for 4 hours.

A) Representative micrographs of typical glycated and unglycated PC12 cells are shown.

B) Tryphan blue staining of PC12 cells. Cell viability of unglycated cells (control) was set to 1 and cell viability of glycated PC12 cells were calculated in relation to the control. Bars represent standard derivation of three independent measurement carried out in quadruplicates.

C) Representative analysis of PC12 cells by flow cytometry. PC12 cells were stained with Annexin V (A) and propium iodide (PI). LL=live (A-;PI -) ;LR= apoptotic cells (A+; PI -); UR= later stage of apoptotic/death cells (A+; PI +); UL= necrotic cells (A-; PI+). Three independent measurements were performed carried out in triplicates.

4.2.3 Reduced cell adhesion of glycated PC12 cells

Cell surface proteins are involved in cell adhesion and they can be also affected by glycation. Cell adhesion of glycated PC12 cells was quantified by real-time analysis. Therefore, PC12 cells were glycated by 1.0 mM MGO. Plates were coated with laminin and cells were analyzed in real-time for 4 hours. A reduction of cell adhesion about 83% of glycated PC12 cells to laminin compared to unmodified PC12 cells was observed. Cell surface proteins appear to be glycated and interfere with the cell adhesion to the extracellular matrix.

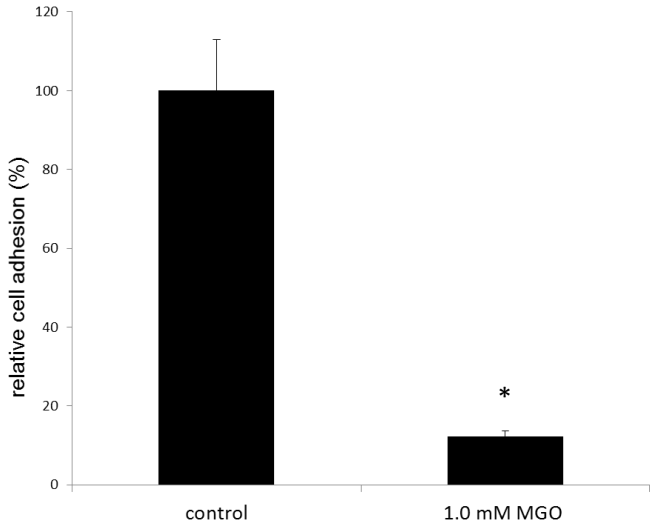

Figure 15: **Cell adhesion of glycated PC12 cells.**

PC12 cells were incubated with 1.0 mM MGO for 4 hours. Cells were cultured on laminin (20µg/ml) coated E- plates. Cell adhesion was monitored after 4 hours. Bars represent standard derivation of three independent measurement carried out in triplicates (*p< 0.005; n=9).

4.2.4 Reduced neurite outgrowth of PC12 cells

PC12 cells are well described *in vitro* model for investigate neurite outgrowth as an indicator for regeneration capacity. PC12 cells were cultured in presence of 1.0 mM for 4 hours. Glycated PC12 cells and unmodified PC12 cells (control) were then cultured in presence of nerve growth factor (NGF) as described in Pollscheit *et al.* and monitored for 48 hours in real-time [130]. As expected, a prominent neurite outgrowth was observed for unmodified PC12 cells after 48 hours but a dramatic decrease of neurite outgrowth was monitored of glycated PC12 cells. Quantification is shown in figure 16. This result shows that cellular proteins interfere with NGF-mediated neurite outgrowth.

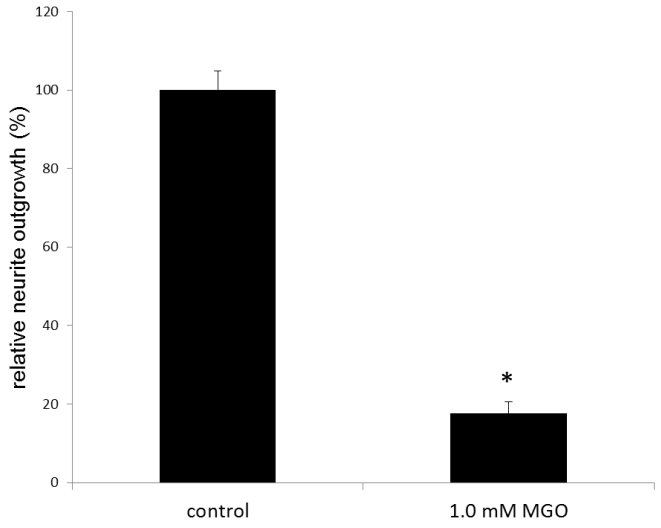

Figure 16**: Real-time analysis of neurite outgrowth of glycated PC12 cells.**
PC12 cells were incubated with 1.0 mM MGO for 4 hours. Unmodified PC12 cells serve as control. Cells were cultured on laminin (20µg/ml) coated E- plates. Neurite outgrowth was induced by application of 100 ng/ ml NGF. Neurite outgrowth was continuously quantified over 48 hours by real-time cell analyzer (RTCA) as described in Pollscheit et al. (2012). Total neurite outgrowth of control cells was set to 100%. Bars represent standard derivation of three independent measurement carried out in triplicates (*p< 0.0001; n=9).

4.2.5 Analysis of NGF-mediated mitogen-activated protein kinase pathway

NGF promotes cell survival, neurite outgrowth and differentiation via the high affinity TrkA receptor. Binding of NGF to TrkA receptor activates the mitogen-activated protein kinase (MAPK) pathway. Signal cascade of MAPK pathway included the activation several proteins. One essential step is the activation of ERK1/2 by phosphorylation. To elucidate the underlying molecular effects of MGO-mediated glycation on the NGF signal transduction pathway, the activation of ERK1/2 was analyzed. Therefore a Western blot was performed for ERK1/2 and its activated form phoshoERK1/2 of glycated PC12 cell lysate. Unglycated NGF-treated PC12 cells served as positive control as described by Kontou *et al.* [131] and unstimulated PC12 cells serve as negative control. All PC12 cells expressed ERK1/2 (figure 17 A). PhoshoERK was detected only in presence of NGF in unglycated cells but phoshoERK1/2 could not be detected in glycated PC12 cells neither in presence nor absence of NGF. Results shows that NGF-mediated signal transduction pathway is disrupted by glycation. In second set of experiments, PC12 cells, after glycation with 1.0 mM MGO, were cultured under cell culture condition for 72 hours (figure 17 B). However, analyzing activation of ERK1/2 after 72 hours of recovering a relatively light signal for phoshoERK1/2 is observed for glycated PC12 cells in presence

of NGF compared to the cells of positive control. Additionally, micrographs were taken of unglycated and glycated PC12 cells after 72 hours recovering in presence of NGF to visualize recovering of neurite outgrowth (figure 17 C). Cells in presence of NGF show prominent neurites. On the contrary, the glycated cells show only basic approach of neurites.

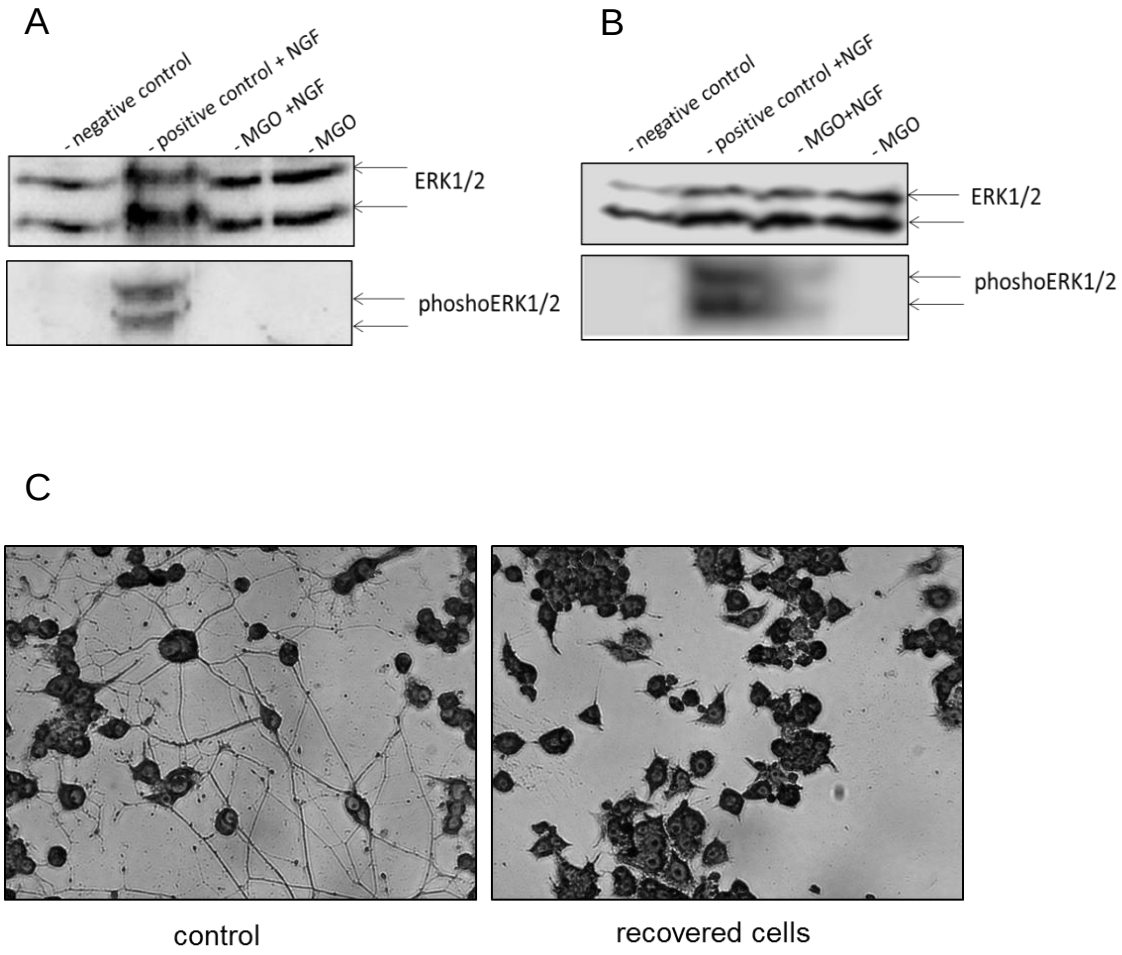

Figure 17: **ERK 1/2 activation of glycated C12 cells.**

PC12 cells were incubated with 1mM MGO for 4 hours and then stimulated with 100ng/ml NGF. Cells were harvested, washed and solubilized. Protein concentration of the cytosolic fraction was determined by BCA test. 20 µg of each protein sample was subjected to SDS-PAGE-gel electrophoresis. Proteins were blotted and detected using monoclonal ERK1/2 and phoshoERK1/2 antibody. Detection of ERK1/2 serve as control for equal loading. Detection of phoshoERK1/2 using for analyzing ERK1/2 activation. PC12 cells in absence of NGF serve as negative control and PC12 cells in presence of NGF serve as positive control. Representative Western blots of three independent performed experiments are shown. A) Activation of ERK 1/2 was analyzed after MGO-induced glycation. B) Activation of ERK1/2 was analyzed after MGO-induced glycation and further 72 hours culturing under cell culture conditions. C) Micrographs of NGF-induced neurite outgrowth on PC12 cells after 72 hours (left side) compared to 72 hours recovered cells (right side).

4.3 Glycation of TrkA receptor induced a threefold reduction of NGF-binding

Binding of NGF to the high affinity neurotropin TrkA receptor promotes neurite outgrowth [112]. Since analysis of glycated PC12 has been shown that cells do not respond to NGF by activation of ERK1/2, receptor-ligand binding could be interfere by glycation. Additionally, the analysis of glycated PC12 cells by flow cytometry has been shown that cell surface proteins are glycated. Therefore, the affinity of receptors-ligand interaction in real-time was quantified by optical surface plasmon resonance (SPR). TrkA receptor was immobilized by C-terminal His-tag onto a Ni-NTA sensor chip to create a directed immobilization of TrkA receptor with a free N-terminal binding domain. Approximately, 400 response units (RU) ± 150 RU were coupled, which is equivalent to an amount of 400 pg receptor/mm^2 ± 150 pg/mm^2. A series of measurements was performed by using NGF concentrations between 0.06 nmol/l and 50 nmol/l (figure 18 A). An equilibrium dissociation constant K_D of (1.47± 0.2)*10^{-10} M was calculated. This value corresponds exactly to previously published values by Hempstead *et al.* [132], Mahadeo *et al.* [133] and Woo *et al*. [134]. TrkA receptor has been then glycated directly on the sensor chip using 0.1 mM MGO and a flow of 1 µl/min for 20 hours. Glycation of TrkA receptor could be verified by SPR (~ 350 RU response) using a polyclonal anti-AGE antibody, which can detect a high variety of AGE-modifications (figure 18 B). In the next series of experiments, the measurements of NGF kinetics were repeated on the glycated TrkA receptor. After glycation a series of measurements using NGF-concentration between 0.06 nmol/l and 50 nmol/l was again performed (figure 18 C). Glycation of TrkA receptor resulted in a threefold reduction of K_D. The equilibrium dissociation constant of the glycated TrkA receptor was calculated as K_D (4.57± 0.9)*10^{-10} M.

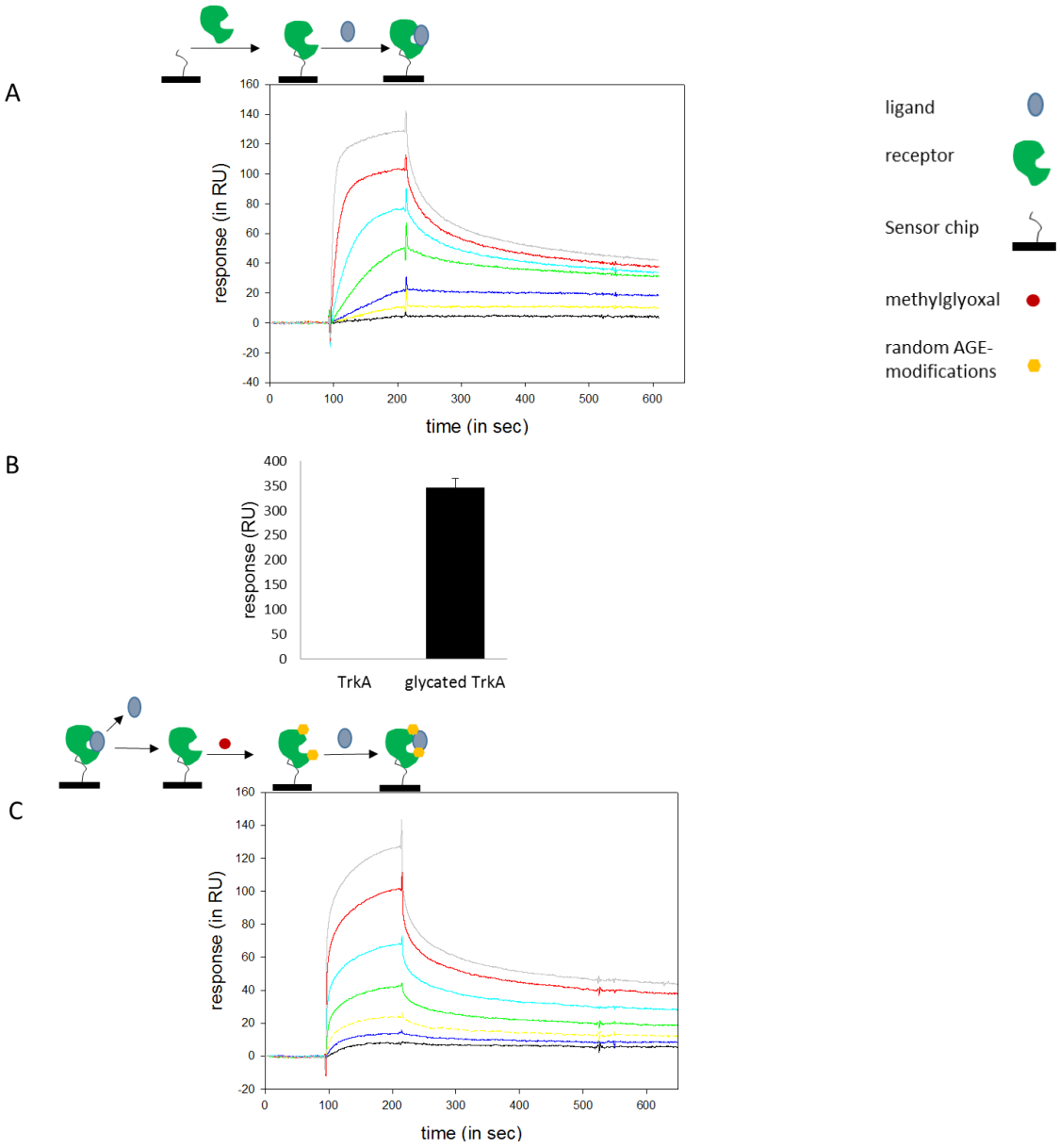

Figure 18: **Kinetic analysis of NGF-binding to the glycated TrkA receptor.**

A) Kinetic analysis of NGF-binding to the high affinity TrkA receptor. NGF (--0.06, --0.2, --0.6, --1.85, --5.5, --16, --50 nM) was injected and allowed to bind to coupled TrkA receptor. A representative measurement of binding kinetics with association and dissociation of NGF to TrkA receptor is shown. The dissociation constant was calculated by BIAevaluation program yielding a K_D value of (1.47±0.2)*10^{-10} M from three independent measurements.

B) Anti-AGE antibody binding to glycated TrkA receptor. Anti-AGE antibody was used at 1 µg/ml in HBS running buffer, pH 7.4. No binding of anti-AGE antibody before glycation of TrkA receptor could be measured (TrkA). Glycation of TrkA receptor yielded ~350 RU (corresponding to 350 pg antibody binding/mm^2). Standard derivation is calculated from three independent measurements.

C) Kinetic analysis of NGF-binding to glycated TrkA receptor. Coupled TrkA receptor was glycated by MGOl for 20 hours at 23°C directly on the sensor chip surface. NGF (0, 0.06, 0.2, 1.85, 5.5, 16, 50 nM) was injected and allowed to bind to the glycated TrkA receptor. One representative measurement of binding kinetics with association and dissociation of NGF to TrkA is shown. The dissociation constant was calculated by BIAevaluation program yielding a K_D value of (4.57±0.9)*10^{-10} M from three independent measurements.

4.3.1 Threefold reduction of NGF results in decreased neurite outgrowth

A threefold reduced NGF-binding to TrkA receptor can influence the neurite outgrowth. Therefore, NGF-mediated neurite outgrowth of PC12 cells was quantified as described previously in Pollscheit *et al*. [130]. Neurite outgrowth was compared in presence of 100 ng/ml NGF and 33 ng/ml. Stimulation with 100 ng/ml NGF was used as reference concentration. The NGF concentration was then reduced by the factor of three to 33 ng/ml. A prominent neurite outgrowth including branching of neurites on the cells was observed after 48 hours in presence of 100 ng/ml NGF (figure 19 A). However, cells in presence of 33 ng/ml rarely showed neurite outgrowth and represent an early stage of growing neurites without branching (figure 19 B). Neurites are visualized by micrographs, representative ones are shown in figure 19.

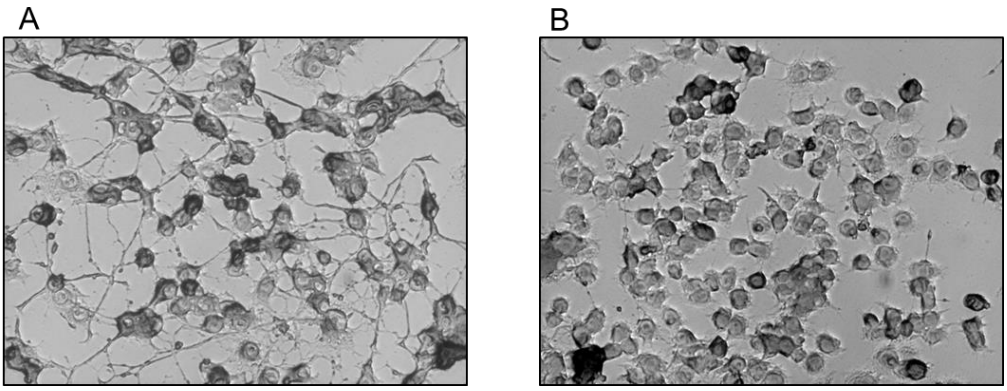

Figure 19: **NGF-mediated neurite outgrowth of PC12 cells.**
5* 10^4 PC12 cells were seeded on laminin coated plates. Representative micrographs after 48 hours in culture of three independent experiments were shown.
A) Cells were cultured in the presence of 100 ng/ml NGF.
B) PC12 cells were cultured in the presence of 33 ng/ml NGF, a threefold reduced concentration of NGF in comparison to A.

4.4 Analysis of the binding affinity of glycated BSA to RAGE

Albumin represents the major circulating blood plasma protein and is AGE-modified during ageing and age-related manner [13], [135]. Recently studies described glycated BSA also as a ligand for RAGE and stimulate RAGE mediated effects.

4.4.1 Analysis of glycation of BSA

Since glycated BSA (AGE-BSA) has been described as a ligand for RAGE, commercially acquired BSA was used for glycation by either glucose, fructose or ribose. The glycated BSA was provided by Kathleen Jacobs and Alexander Navarette-Santos, members of

the research laboratory" surgery of heart and chest" in the Medical faculty of the Martin-Luther university. A high variety of AGE-modifications could be formed during the incubation time of 40 days. These indefinite glycated BSA solutions were referred by their glycation argents, glucose-BSA (G), fructose-BSA (F), or ribose-BSA (R). For an imagination of the highly cross-linked AGE-modifications, the glycation of BSA was analyzed by SDS-PAGE. Each lane was loaded with 20 µg of BSA, glucose-BSA, fructose-BSA or ribose-BSA. Coomassie staining revealed a distinct band pattern for the size of BSA in all lanes, (figure 20). The distinct band of ribose-BSA is particularly slighter, which refers to less amount of unmodified BSA and high amount of AGE-modifications or crosslinks of BSA. The glycated BSA showed a broad smear (> 130kDa) indicating a high amount of AGE-modifications. In addition, a high-molecular smear could be detected in all glycated samples, which also indicates AGE typical protein crosslinks. The unglycated BSA appears to exhibit also BSA crosslinks, thus BSA could be observed over a molecular weight of 250 kDa. Analysis of the AGE-modifications were performed in cooperation with K. Jacobs.

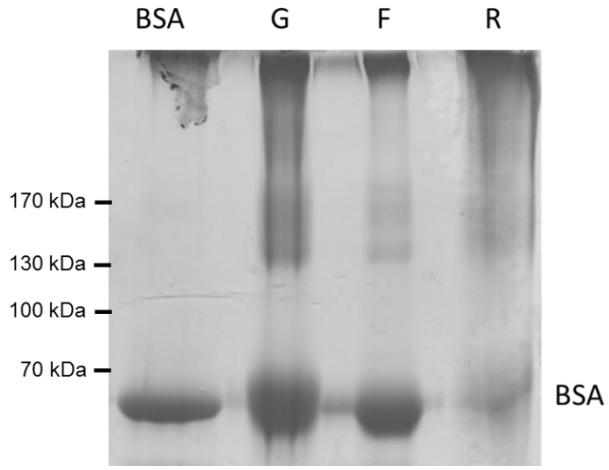

Figure 20: **Validation of glycated BSA.**
Validation of BSA modification. 20 µg of each BSA solution was subjected to 8% SDS gel electrophoresis and stained with Coomassie brilliant blue. Unglycated BSA (BSA) served as control. Note that glucose-BSA (G), fructose-BSA (F) and ribose-BSA (R) smeared in the high molecular weight over 130 kDa.

4.4.2 RAGE binds glycated BSA

The multi-ligand receptor RAGE has been at first described as receptor for AGEs. Glycated BSA has been proposed as potential ligand for RAGE, although no detailed binding analysis has been described. The binding affinity of glycated BSA modified by glucose (glucose-BSA), fructose (fructose-BSA) or ribose (ribose-BSA) to RAGE was analyzed by SPR. Therefore, RAGE was immobilized on a carboxymethylated CM5

sensor chip using a standard covalent amine coupling method. Around 400 response units (RU) ± 250 RU were coupled, which is equivalent to an amount of 400 pg receptor/mm^2 ± 250 pg/mm^2. In a first set of experiments, the three glycated BSA were analyzed as ligands separately for their binding to RAGE over a range of concentrations (ng up to µg) with a flow rate of 15 µl/min. At a concentration 15 µmol/l the relative binding of glycated BSA was compared. Glucose-BSA appears to be the RAGE ligand with the highest affinity. As expected, the unglycated BSA, under the same measurement conditions, did not show any affinity to RAGE. Binding affinity of fructose-BSA was nearly comparable to BSA-glucose, whereas the affinity of ribose-BSA was only 30% compared to glucose-BSA. Binding affinities are presented in figure 21.

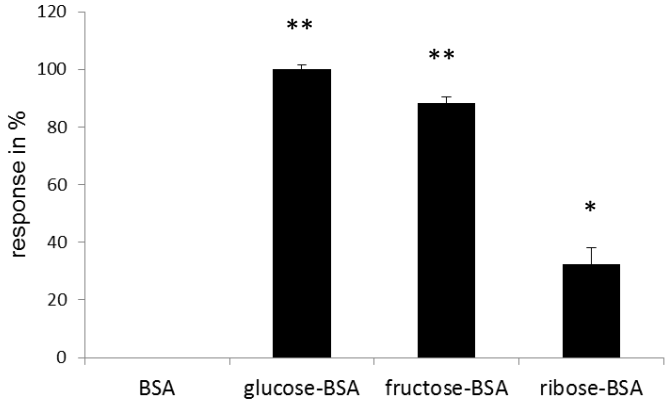

Figure 21: **Determination of potential binding partners to RAGE.**
15 µM BSA at a flow rate of 15 µl/min was allowed to bind to coupled RAGE receptor. Glucose-BSA exhibits the strongest binding and was set to 100%. Percentage of binding to RAGE of fructose-BSA and ribose-BSA were calculated in comparison to glucose-BSA. Bars represent standard derivation of three independent measurements carried out in triplicates (*p<0.001%, **p<0.0001 referred to BSA).

Consequently, glucose-BSA and fructose-BSA are regarded as major RAGE ligands. Their affinity to RAGE were determined in further experiments. A series of measurements using glycated BSA concentration between 1.85 µmol/l and 150 µmol/l was performed (figure 22). Regarding to the reduced affinity of ribose-BSA compared to glucose-BSA, K_D for ribose-BSA was significantly higher expected than for glucose-BSA or fructose-BSA. Thus, measurements of binding kinetics of ribose-BSA were limited by the maximally achieved ribose-BSA concentration (150 µM), the K_D and binding kinetics could not be determined. An equilibrium dissociation constant K_D of (1.39± 0.2)*10^{-5} M for glucose-BSA was calculated and an equilibrium dissociation constant of (1.95± 0.7)*10^{-5} M for fructose-BSA. K_D values indicate that glycated BSA binds as low affinity ligands to RAGE. Figure 22 shows measurements of binding kinetics to RAGE with association and dissociation of glucose-BSA or fructose-BSA.

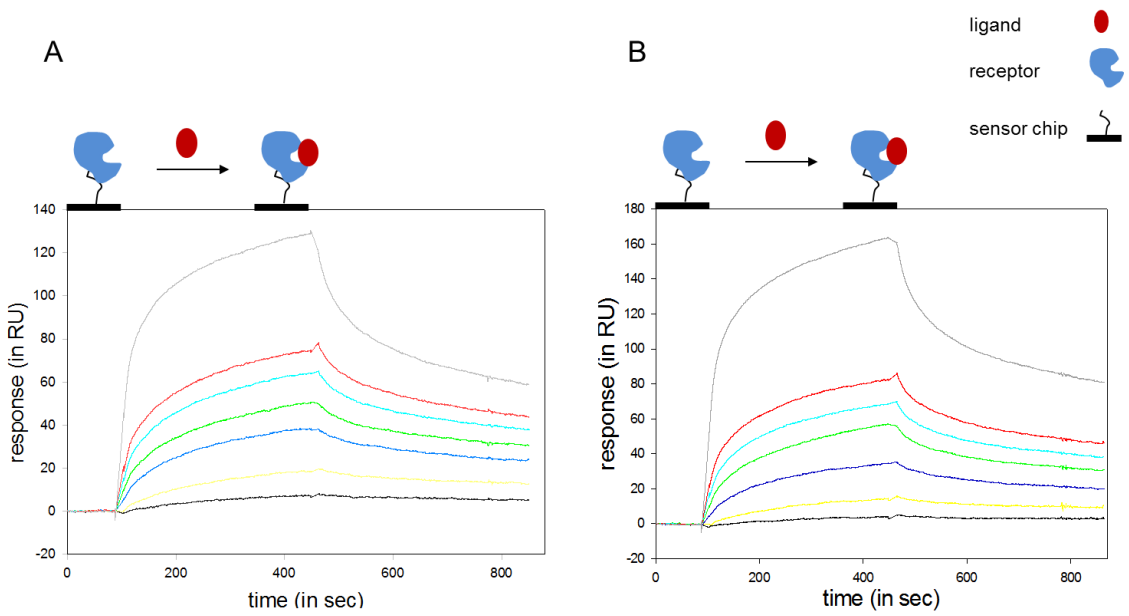

Figure 22: **Kinetic analysis of glycated BSA to RAGE.**

Glycated BSA (--1.85, --5.5, --16, --30, --50, --75, --150 µM) was injected and allowed to bind to coupled RAGE receptor. One representative measurement of binding kinetics with association and dissociation of each glycated BSA to RAGE receptor is shown.

A) Glucose-BSA was used as ligand partner. The dissociation constant was calculated by BIAevaluation program resulting in a K_D value of (1.39±0.2)*10^{-5} M from three independent measurements.

B) Fructose-BSA was used as ligand partner. The dissociation constant was calculated by BIAevaluation program resulting in a K_D value of (1.95±0.7)*10^{-5} M from three independent measurements.

4.4.3 Glycation of RAGE interferes with binding of glycated BSA

Also RAGE can be glycated and therefore, RAGE was modified by MGO-induced glycation. Binding affinity of glucose-BSA or fructose-BSA was analyzed after modification of RAGE by glycation. RAGE (400 response units (RU) ± 250 RU) was directly glycated on the sensor chip using 0.1 mM methylglyoxal and a flow of 1 µl/min for 10 hours. Glycation of RAGE could be verified by SPR (~ 320 RU response) using a polyclonal anti-AGE antibody (figure 23 A). Then, the kinetic measurements of glucose-BSA and fructose-BSA to glycated RAGE were repeated using glycated BSA concentration between 1.85 µmol/l and 150 µmol/l. Binding of glucose-BSA was strongly inhibited, whereas binding of fructose-BSA was completely abolished, no association and dissociation could be observed. We calculated a binding affinity for glucose-BSA with a K_D of (7.62± 0.4)*10^{-5} M. Thus, the equilibrium dissociation constant of glycated RAGE is decreased by a factor of 5.7 compared to unglycated RAGE (figure 23 B). Since fructose-BSA did not bind to glycated RAGE, no equilibrium dissociation constant could be calculated (figure 23 C).

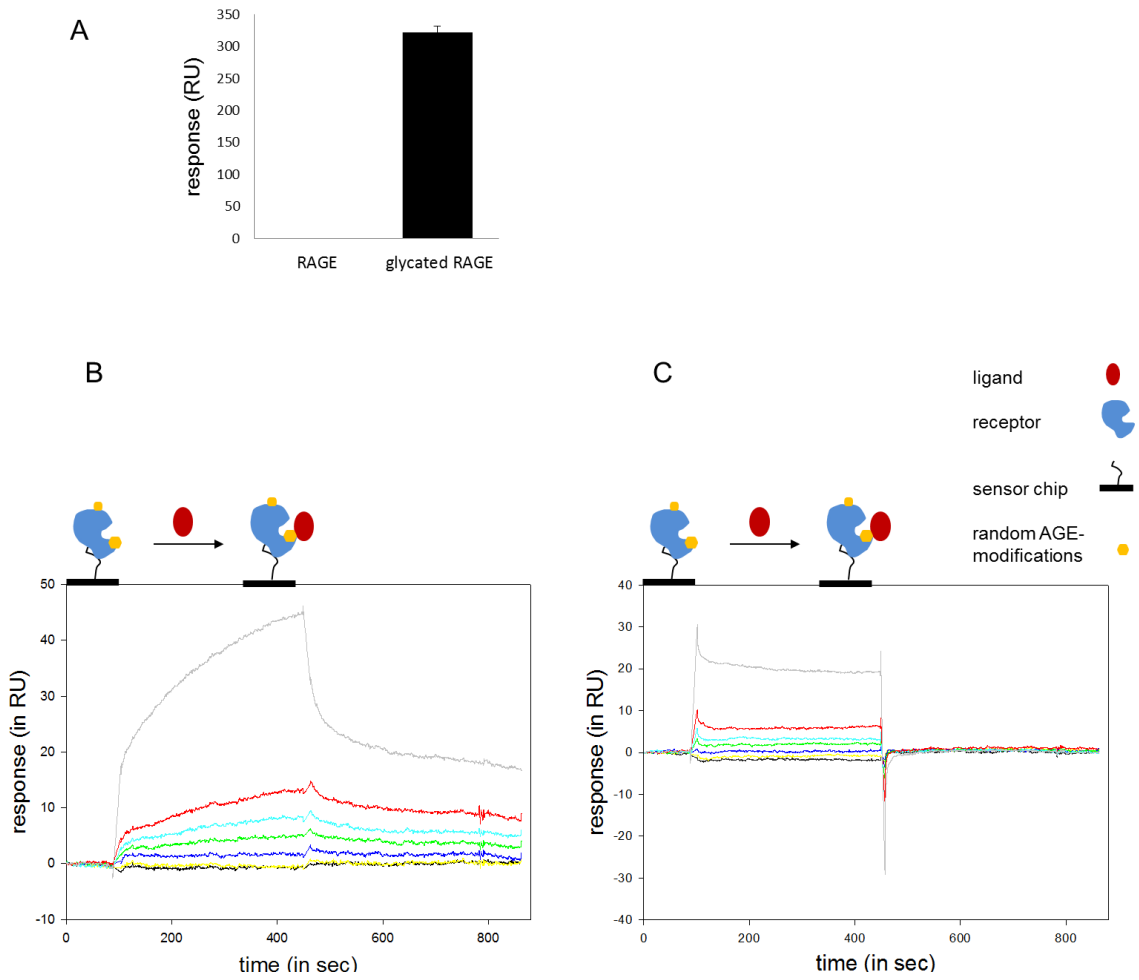

Figure 23 . **Kinetic analysis of glycated BSA to glycated RAGE.**

A) Anti-AGE antibody binding to glycated RAGE receptor. Anti-AGE antibody was used at 1 µg/ml in HBS running buffer, pH 7.4. No binding of anti-AGE antibody before glycation of RAGE could be measured (RAGE). RAGE receptor was glycated by methylglyoxal for 10 hours at 23°C directly on the CM5 sensor chip surface. Glycation of RAGE resulted in a response of ~320 RU (corresponding to 320 pg antibody binding/mm^2). Standard derivation is calculated from three independent measurements.

Glycated BSA (--1.85, --5.5, --16, --30, --50, --75, --150 µM) was injected and allowed to bind to the glycated RAGE receptor. One representative measurement of binding kinetics with association and dissociation of glycated BSA to glycated RAGE receptor is shown.

B) Glucose-BSA was used as ligand partner. The dissociation constant was calculated by BIAevaluation program resulting in a K_D value of (7.62±0.4)*10^{-5} M from three independent measurements, which corresponds to a 5.7-fold reduction of binding affinity.

C) Fructose-BSA was used as ligand partner. No binding of fructose-BSA to RAGE was detectable after glycation of RAGE receptor in three independent measurements.

4.4.4 Detection of RAGE on PC12 cells

RAGE is expressed in the nervous system [9]. Therefore, PC12 cells were analyzed by Western blot analysis for RAGE. Cytosolic fraction and membrane fraction were analyzed. RAGE antibodies detect two distinctive bands with 42 kDa and 45 kDa in the membrane fraction. The characteristic bands of RAGE are displayed in figure 24.

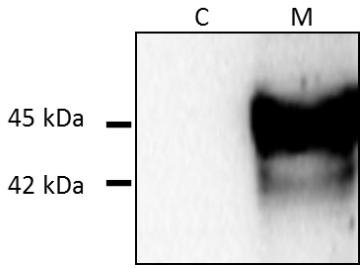

Figure 24: **Kinetic analysis of gycated BSA to glycated RAGE.**

RAGE expression in PC12 cells. 15 µg of a membrane fraction of PC12 cells (M) was subjected to 10% SDS gel electrophoresis and transferred to a nitrocellulose membrane. RAGE was detected by using a polyclonal anti-RAGE antibody. A predicted size of 42 or 45 kDa for RAGE was confirmed. No RAGE was detected in the cytosolic fractions (C).

4.4.5 Glycated BSA does not interfere with cell viability

Glycated BSA binding to RAGE activated several transduction pathways such as NF-κB/JNK signaling pathway or MAPK pathway [135]–[138]. Since PC12 cells possess endogenous RAGE, the influence of glycated BSA on PC12 cells was analyzed. Glucose-BSA or fructose-BSA has been identified as adequate ligand partners for RAGE. Therefore, glucose-BSA, fructose-BSA or control BSA (500 µg/ml) was added to the cell culture medium of PC12 cells and cells were monitored for 48 hours. Cell viability was quantified by detection of mitochondrial activity by formazan (MTT assay). The MTT assay shows a comparable mitochondrial activity of PC12 cells in presence of glucose-BSA or fructose-BSA cells with BSA as control (figure 25). The cell morphology of PC12 cells possesses no obvious difference. PC12 cells in presence of glycated BSA (up to a concentration 500 µg/ml) do not appear to influence the cell viability after 48 hours.

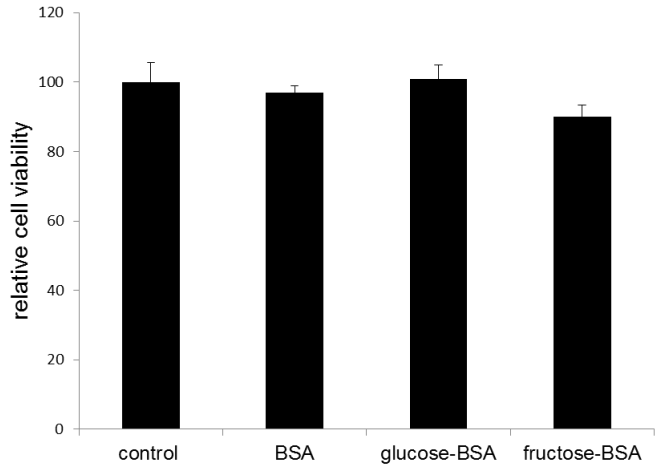

Figure 25**: Cell viability in presence of glycated BSA.**

A) PC12 cells were cultured on laminin-coated plates in the presence of 100 ng/ml NGF and supplemented with 500 µg/ml BSA, glucose-BSA or fructose-BSA for 48 hours. Cell viability was measured by MTT assays. Cell viability of untreated PC12 cells in presence of NGF was set to 100% (control). Cell viability in the presence of BSA, glucose-BSA or fructose-BSA was calculated in percent of control. Bars represent standard derivation of mean values of three independent measurements. A tryphan blue assay, which was additionally performed, displays the same result, data not shown.

4.4.6 Glycated BSA does not interfere with neurite outgrowth.

Neurite outgrowth is initiated by activation of the MAPK pathway. Since it is described that glycated BSA by binding to RAGE activated MAPK pathway, neurite outgrowth of PC12 cells was analyzed in presence of glycated-BSA or control-BSA in real-time for 48 hours. Therefore, 500 µg/ml glucose-BSA, fructose-BSA or control-BSA were added. PC12 cells in presence of NGF serve as positive control for neurite outgrowth. As expected, control cells in absence of NGF did not show neurite outgrowth, but for PC12 cells in presence of NGF neurite outgrowth is monitored (figure 26 A, B). Interestingly, PC12 cells, cultured in presence of glycated-BSA (glucose-BSA or fructose-BSA) without NGF showed also no neurite outgrowth (figure 26 A). In the next set of experiments, cells were supplemented with glycated–BSA as well as NGF as initiator for neurite outgrowth. Neither addition of 500 µg/ml glucose-BSA nor 500 µg/ml fructose-BSA had an enhancing effect on NGF-induced neurite outgrowth (figure 26 B). Real-time analysis of neurite outgrowth was quantified and displayed in figure 26.

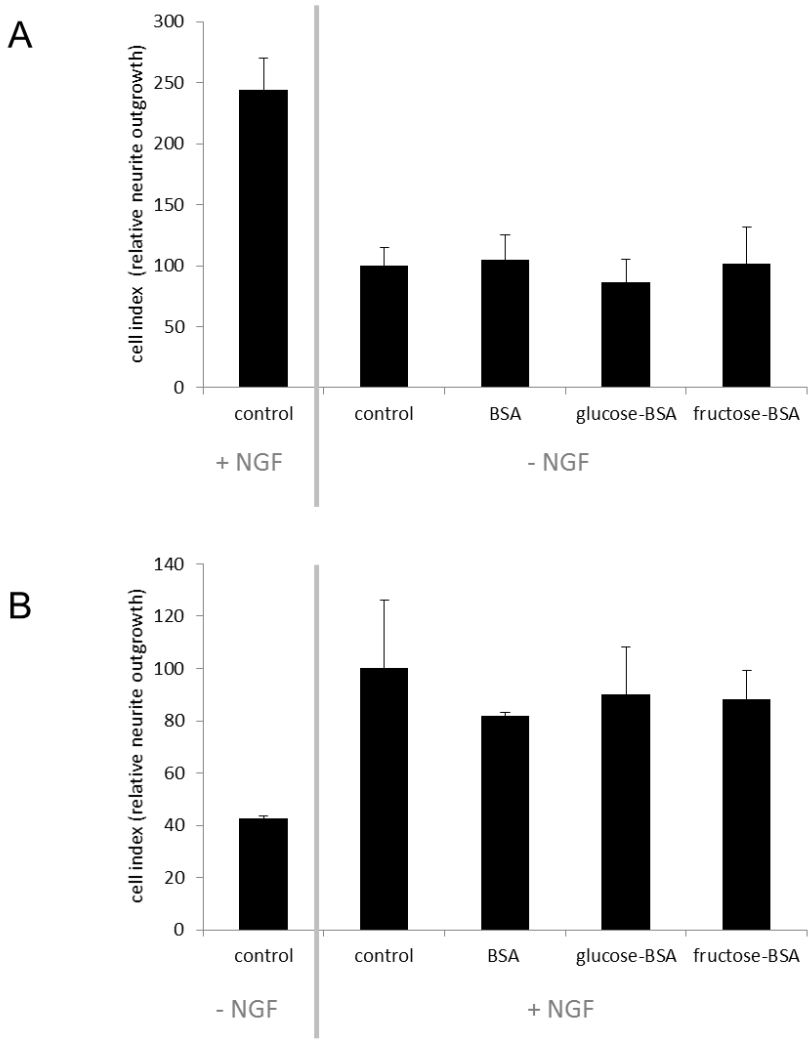

Figure 26: **Neurite outgrowth in presence of glycated BSA.**

PC12 cells were cultured on laminin-coated E-plates and neurite outgrowth was induced by adding 100 ng/ml NGF. Neurite outgrowth was continuously quantified over 48 hours by RTCA as described in [130].
A) Neurite outgrowth of PC12 cells in addition of glycated BSA, but in absence of NGF. PC12 cells supplemented with unglycated BSA in absence of NGF was set to 100% (control -NGF) and compared with cells in presence of NGF as positive control (control +NGF). 500 µg/ml BSA, glucose-BSA ad fructose-BSA were added to PC12 cells in absence of NGF. Neurite outgrowth for each condition is expressed as percent compared to control-NGF. Bars represent standard derivation of mean values of three independent experiments carried out in triplicates.
B) Neurite outgrowth of PC12 cells in addition of glycated BSA, but in presence of NGF. Neurite outgrowth in the presence of NGF was set to 100% (control +NGF) and compared with non-NGF-treated cells as negative control (control -NGF). 500 µg/ml BSA, glucose-BSA and fructose-BSA were added to PC12 cells in presence of NGF. Neurite outgrowth for each condition is expressed as percent compared to control +NGF. Bars represent standard derivation of mean values of three independent experiments carried out in triplicates.

4.4.7 Activation of signaling pathways by binding of RAGE

Since no neurite outgrowth was observed in the presence of glycated BSA, the activation of MAPK pathway was analyzed by Western blot. PC12 cells were cultured in the presence of 500 µg/ml control-BSA, glucose-BSA or fructose-BSA. Western blots for ERK1/2 and its activated form phosphoERK1/2 were performed (figure 27 A, B). ERK1/2 was detected in all PC12 cell cultures, but no phosphoERK1/2 could be detected. NGF-treated PC12 cells served as a positive control. It could be shown that glycated-BSA does not activate the MAPK pathway in PC12 cells. Furthermore, for confirmation that glycated BSA binds to endogenous RAGE on PC12 cells, we analyzed the NF-κB activation, which is described in a RAGE dependent manner [46]. Thus, Western blot analysis was performed for NF-κB activation (figure 27 C). An activation of NF-κB in presence of glucose-BSA or fructose-BSA was observed. In contrast, in addition of unglycated BSA we could not detect an activation for NF-κB in PC12.

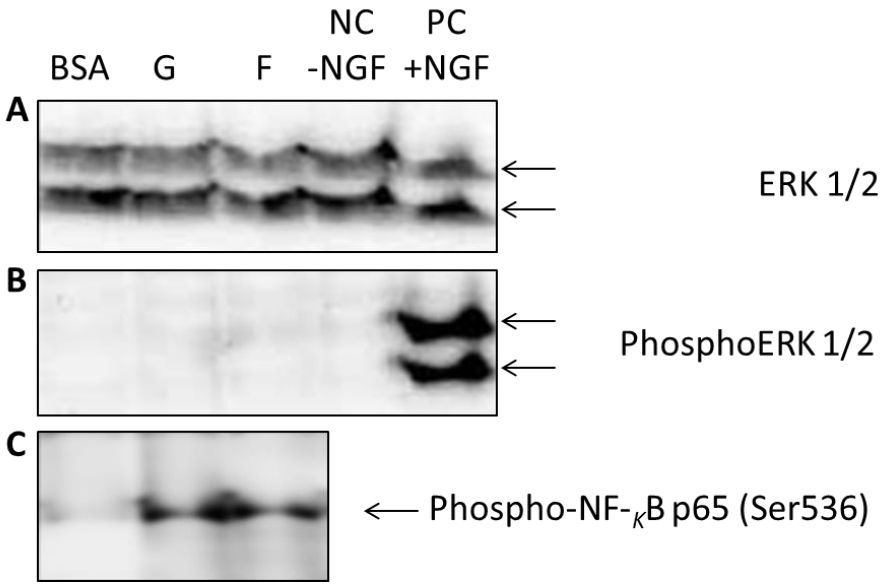

Figure 27: **ERK 1/2 activation of PC12 cells in presence of glycated BSA.**

PC12 cells were cultured in the presence of BSA, glucose-BSA (G) or fructose-BSA (F). 15 µg of a cell lysate of PC12 cells was subjected to 10% SDS gel electrophoresis and transferred to a nitrocellulose membrane.

A) ERK1/2 was detected by using monoclonal anti-ERK1/2 antibody.

B) phoshoERK1/2 was detected by using monoclonal anti-phoshoERK1/2 antibody. PC12 cells in absence of BSA were stimulated with 100 ng/ml NGF and serve as positive control (PC). PC12 cells in absence of BSA serve as negative control (NC). Predicted size of 42 or 45 kDa for ERK1/2 and phoshoERK1/2 could be confirmed. C) Phosho-NF-κB was detected by using monoclonal anti-NF-κBp65 antibody. Predicted size of 65 kDa for Phosho-NF-κB could be detected.

5 Discussion

5.1 Consequences of AGE-induced reduced neuronal plasticity

The human nervous system has a limited regenerative capability. The structural integrity and plasticity of neurons develop during adolescence. However, during ageing, the regenerative capacity of the nervous system is decreased with structural changes e.g. neurodegeneration and protein deposition visible in form of plaques such as in Alzheimer´s disease. AGE-accumulation as a prominent molecular event during ageing in all tissues affect also the neuronal tissue. Accumulation of AGEs has been identified in different compartments and regions in the human brain in age-related manner such as glycated proteins in β-amyloid plaques of patients, suffering from Alzheimer´s disease [139], [140]. AGE-precursors are highly reactive and physiological side-products of sugar metabolism. It is believed that this physiological pathway is used to eliminate an oversupply of sugars in the consequences of carbohydrate-rich nutrition or in disease-related manner such as diabetes mellitus. Moreover, several clinical and basic research showed a relation of increased risk to suffer from Alzheimer´s disease, if patients are handicapped by diabetes mellitus [141], [142].

Long-lived protein e.g. extracellular matrix proteins, caused of their long half-life time, serve as ideal substrate for glycation argents. Collagen modifications could be already detected in aged human skin by increased auto-fluorescence of the modified proteins as well in lenses during development and progression of cataract by crosslinking of lenses matrix proteins. In neuronal tissue, cell adhesion on extracellular matrix is an inalienable requirement for a high potential and functional nerve tissues. Therefore the effect of glycation on ECMs on cell adhesion is quantified. Methylglyoxal (MGO) was used as glycation argent. On the one side MGO is an endogenous physiological AGE-precursor, as a carbolite of the glucose metabolism and neurons are glucose-dependent. On the other side MGO is a high reactive intermediate of the Milliard reaction (glycation reaction) to shorten the glycation time from weeks to hours for reliable experimental conditions, thus MGO is the adequate reagent for stimulating glycation. Extracellular matrix, surrounding neuronal tissues in brain and along the basolateral site of endothelial cells on the blood brain barrier, is a mixture of several ECM proteins such as laminin, collagen IV and fibronectin. The model organism, PC12 cells were used, which adhere toughly on laminin and collagen IV. The formation of AGEs on laminin and collagen IV could be detected by AGE specific antibody recognition. Glycated laminin or collagen IV impairs the neuronal cell adhesion of PC12 cells about 80 up to 90%, indicating AGE-modifications or protein crosslinks on ECM proteins prevent interference with integrins,

the group of cell surface adhesion molecules, which are responsible for cell matrix interactions. The signal transduction mediated by integrins processes bi-directionally, this means into the cell corpus (outside-in), but also out of the cell (inside-out). This permits interlocking of the various functions of the receptors. Adhesion induces intracellular processes, which can lead to restructuring of the cytoskeleton and to the induction of signaling cascades, e.g. for neurite outgrowth. If regeneration capability of neurons or axons needs the contact to ECM proteins, it would explain also the reduced regeneration capacity in AGE-modified ECM environment. However, observing NGF–dependent neurite outgrowth on the border of glycated ECMs to unglycated ECMs, glycation has no effect on border-crossing neurites. Noticeable on glycated collagen IV the neurite are more branched than on glycated laminin. This could be an indication that signaling transduction, responsible for branching is affected by reduced cell adhesion. Such as described in literature, PC12 cells, cultured on laminin, also show branching at NGF-mediated neurite outgrowth [131]. The signaling mechanism of branching is still unknown only couple of computer assisted simulation are existing [143]. Thus, it is difficult to explain the different behavior of neurites on glycated laminin and collagen IV. However, one indication provides the model itself. PC12 cells express RAGE. RAGE is described as cell adhesion molecule of the group Ig CAMs. Although, involvement of integrin-related glycoproteins in attachment and process outgrowth is described [107], it is not excluded that RAGE also interferes in cell adhesion, just because collagen IV was recently identified as binding partner for RAGE. Adherence of RAGE could be an explanation of rescue branching at neurite outgrowth on collagen IV.

Cellular MGO concentration (free and bound) is supposed up to 300 µM [144]. There is no estimation for extracellular MGO concentration, but MGO is a membrane fluid compound (also other reactive AGE-precursors such as glyoxal). Therefore, cellular proteins and cell surface proteins serve as potential substrates for glycation reactions. MGO was added to the medium with several concentration of MGO, 0.1 mM, 0.3 mM or 1.0 mM MGO and monitored a concentration-dependent glycation effect by western blot analysis. The concentration of 1.0 mM is higher than physiological estimation but by adding MGO to medium an indefinite part of the compound captured by ingredients of the medium before MGO is up taken by cells and there exist no standardization per amount inserted cells. By default, 5,000 cells were used for glycation. The detection of glycation indicates that a wide range of intracellular proteins are glycated and that the glycation process is random and not selective. Interestingly, cells treated up to the concentration of 1.0 mM MGO have no decrease on cell viability. It appears cells can compensate AGE-induced oxidative stress until this concentration. Only at cell analysis of membrane integrity by flow cytometry a tendency to early apoptosis could be observed

with 1.0 mM MGO. Analysis of glycated PC12 cells by flow cytometry displayed besides intracellular glycation also extracellular glycation. The examination of cell adhesion of glycated cells to unglycated ECMs show the same drastic reduction compared to glycated ECMs to unglycated cells. It appears to be a sensitive binding, if one interaction partner is affected by glycation. Cell adhesion molecules, integrins, might as well be concerned by extracellular glycation and do not interfere anymore with the ECM proteins with the same consequence for functionality of neurons. Integrins are composed of different subunits, three original β subunits (β_1, β_2, and β_3) identified but now expanded to 8 and the number of α subunits includes at 17. These subunits interact non-covalently in a restricted manner to form more than 20 family members. $\alpha_1\beta_1$ and $\alpha_2\beta_1$- subunits of integrins, interact with collagen and laminin [145]. The binding site of these integrins is a lysine-, arginine- and tyrosine-rich amino acid sequence. These amino acids may interfere probably mainly with MGO and lead to AGEs on these structures and finally to reduced interference with ECM proteins. Binding of NGF to TrkA receptor activates the MAPK pathway or the phosphoinositide phospholipase Cγ/ proteinkinase C (PLCγ/ PKC) pathway, which inhibits cell proliferation and initiates neurite outgrowth. AGE-modified cells do not respond to NGF, while unglycated cells respond by activation of ERK1/2 and accordingly it leads to NGF-mediated neurite outgrowth. Interestingly, cells can compensate time-limited glycation. After recovering glycated cells respond to NGF and induce neurite outgrowth. Indicating glycated proteins, which are involved in signal pathway, are cleaved off probably by protein deposition and replaced by functional active proteins. Furthermore, AGE-precursor MGO can eliminate by glyoxalase system, which is up-regulated with increased cellular MGO concentration to reduce MGO on the non-toxic level [99], [101].

Moreover, receptors on the cell surface proteins can be also affected by glycation. Affected receptors may interrupt receptor mediated signaling pathway. Regarding to neurite outgrowth, the binding affinity of NGF to glycated TrkA receptor was investigated and the influence on its receptor-mediated signaling pathway. We quantified the binding by surface plasmon resonance (SPR) and found a reduced binding of NGF to its glycated receptor by a factor of three. The biological relevance was verified by reducing the NGF concentration by threefold in cell culture and measured NGF-induced neurite outgrowth. Glycation could influence the differentiation of neuronal cells dramatically by modification of the TrkA receptor. NGF has a half-life time of a couple of minutes [146], thus, glycation of NGF probably has no biological relevance. In contrast, TrkA receptor has a half-life time of several hours [146] and could be a possible target for glycation. In addition, during ageing NGF availability is decreasing and neuronal plasticity. One aspect could be the receptor glycation as well as the glycation of proNGF [120]. In contrast, a higher NGF

exposure on the cell surface of PC12 cells reduced the half-life time of the TrkA receptor [146]. NGF activates cell survival, differentiation and it is involved in inflammation and apoptotic processes [117]. Glycation of TrkA could influence its half-life time and consequently glycation of TrkA could be one aspect for reduced function of neuronal cells during ageing.

Resumed, glycation on ECM leads to reduced cell adhesion but the signaling pathway for neurite outgrowth appears to be unaffected. Such expected, cells, which do not adhere, do not show neurite outgrowth but already existing neurites grow and branch independently of glycated or unglycated ECM. It can be assumed that glycation on ECM proteins have minor effects of functionality of signal transduction pathways. In contrast cellular (intra- and extracellular) glycation interferes with signaling pathways, leads to an imbalance in the cellular signal transduction response and leads to impaired functions such as reduced neurite outgrowth. The result of decreased neurite outgrowth is a decreased cell-cell communication, a reduced cell adaption and finally in the destruction of nerve cells. The demonstrated effects of glycation on neuronal plasticity are shown in figure 28.

Neuronal tissues can only use glucose or a metabolite of glycolysis for energy gain to maintain cellular functions. Formation of AGE-precursors (e.g. glyoxal or methylglyoxal) is a physiological side process. AGE-precursors are physiological located intracellular and extracellular of nerve cells, amongst others cerebrospinal fluid levels of MGO is estimated from 10 up to 20 µM [100]. Regarding to these facts, there are several explanations in which way AGEs accumulate in the nerve tissue and lead to reduced neuronal plasticity. Firstly, with constant high glucose concentration during ageing the concentration of reactive precursors increased and accordingly the accumulation of AGE-modified proteins increased. AGE-modifications may modulate the protein properties by alteration of structure (conformation and folding), by reduced cleavage ability by proteasome and/ or alters consequently their activity. Starved neuronal cells such as altered brain tissue show low regeneration ability and cannot handle accumulation of modified intracellular proteins. An indication provides an age-related neurodegenerative disease, Alzheimer´s diseases, which accompanied with neuronal cell death and is simultaneously characterized by increased level of extracellular protein deposition as β-amyloid plaques, in which AGEs could be already detected. Secondly, during age-related diseases such as diabetes mellitus or Alzheimer´s diseases the blood brain barrier (BBB) is disrupted. High blood glucose concentration or high concentration of AGE-precursors such as MGO lead to BBB dysfunction [147]. On this point it has to be mentioned that diabetes patients have 6 fold increased MGO concentration in blood than in non-diabetic humans [148] and increased prevalence for Alzheimer´s disease

[141], [149]. Glucose transport is normally regulated by GLUT1 transporter. Disrupted BBB exhibits an increased permeability, therefrom glucose or AGE-precursors can directly pass into the brain without barrier. On the one hand, accumulation on extracellular matrix would be one consequence and this process is accompanied by reduced cell adhesion. On the other hand it results in an increased active glucose transport by GLUT 3 transporter from the extracellular matrix into the neurons, which promotes glycolysis and side ways to eliminate carbohydrate-induced stress and following these pathways promote AGE-accumulation.

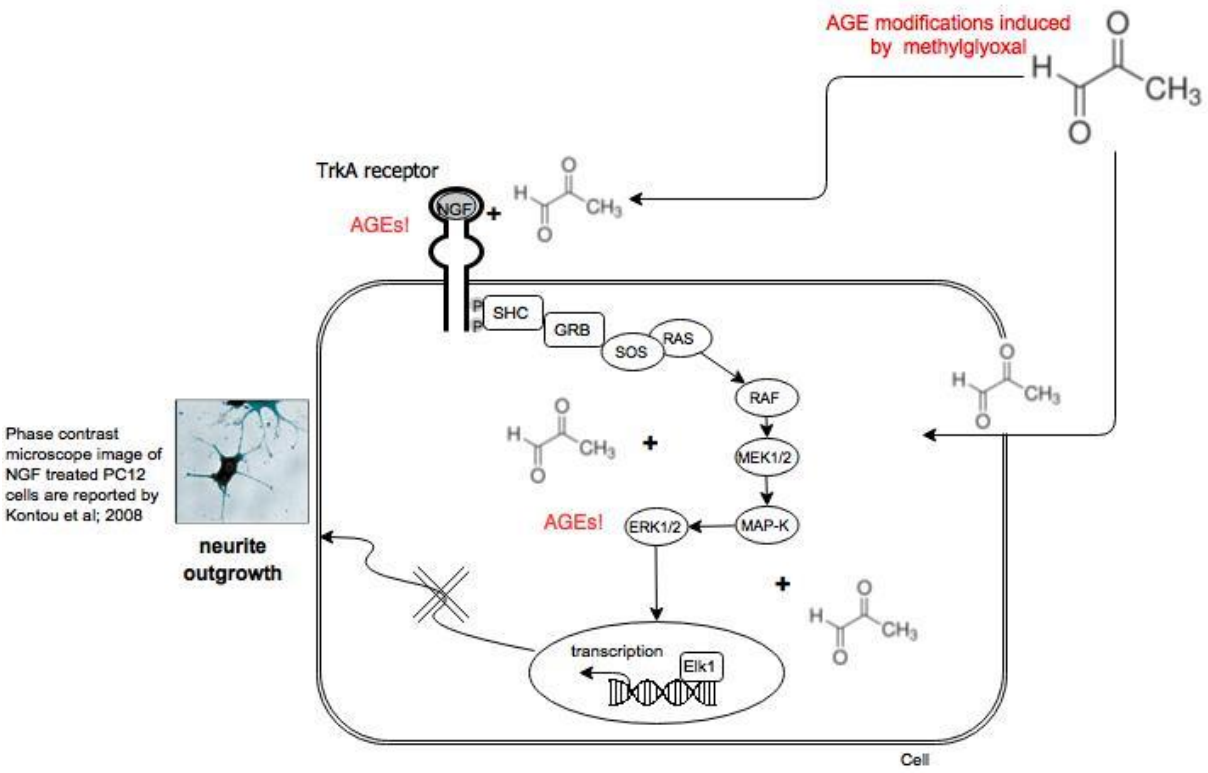

Figure 28**: Mechanistic overview of the influence of AGEs on neuronal plasticity.**
AGE-induced cellular effects on a neuronal plasticity. Receptor mediated pathway of neurite outgrowth is illustrated after addition of glycation argent Methylglyoxal. Neurite outgrowth is reduced after glycation. AGEs interfere with receptor mediated pathway. Intra-and extracellular proteins are concerned by glycation marked with AGEs!, in red AGEs = advanced glycation endproducts.

5.2 Influence of receptor glycation on substrate binding

Effects of receptor glycation has been already shown using NGF receptor TrkA which is accompanied by reduced substrate binding. This observation could be confirmed for RAGE. Both receptors show receptor glycation dependent reduction of substrate

binding. The conformable results can be discussed in a different way regarding their cell biological functions. NGF binding to glycated TrkA receptor has been investigated regarding the influence of glycation to activation of MAPK signal transduction pathway and interference with reduced neuronal plasticity. In this context, receptor glycation has strong negative influence on cellular functions. However, RAGE plays another cell biological role than TrkA. During development the RAGE-mediated pathways appear to play a significantly role, thus RAGE mRNA is expressed in all stages of development [9]. During ageing the RAGE-mediated pathways induced oxidative stress and inflammatory response. An involvement of RAGE in age-related diseases e.g. arteriosclerosis, diabetes mellitus or Alzheimer´s disease with an up-regulation of RAGE mRNA in endothelial cells and/or glia cells is also described. RAGE lacking mice shows resistance to arteriosclerosis and neurodegeneration [70]. AGEs as binding partner induced RAGE-mediated oxidative stress and inflammatory response but an involvement in cell growth and cell death is also described for RAGE. In this context of AGE-accumulation during ageing and age-related diseases ("AGEing") the inhibition of RAGE by modification (e.g. glycation) can be a therapeutic goal to inhibit RAGE-mediated cellular effects such as oxidative stress.

So far, no binding affinity of AGEs - RAGE binding has been determined, therefore in the frame of this thesis for the first time binding affinities of several glycated BSA were determined. Different glycated BSA were used, it means the glycated BSA differs in their glycation argent glucose (glucose-BSA), fructose (fructose-BSA) or ribose (ribose-BSA). Firstly, the equilibrium dissociation (K_D values) constant for binding of glycated BSA to RAGE could be described. For glucose-BSA a of K_D value of 13 µM and for fructose-BSA a K_D value of 19 µM. K_D value of ribose-BSA appears to be twofold higher than for glucose-BSA or fructose-BSA. Ribose-BSA displays a 70% reduced affinity to RAGE compared to glucose-BSA, thus a K_D value could not be determined. In contrast, unglycated BSA did not bind to RAGE as expected. The K_D values in µmolar range indicate low affinity binding. In comparison TrkA receptor is a high affinity receptor with K_D values in nmolar range for binding NGF (high sensitivity). Both are two different types of receptor proteins. TrkA receptor belongs to the neurotrophic tyrosine kinase receptor family and is involved in neuronal signaling. Members of this family display different binding affinities to certain types of neurotrophins. However, RAGE is a member of the immunoglobulin super family and such as demonstrated with low affinity ligands. In this group of immunoglobulin superfamily exist also high affinity receptors such as Fc-receptors for binding immunoglobuline E und G, which play an important role in the immune system [150]. This example shows that the biological relevance of receptor-mediated signaling pathways is closely linked with the binding affinity to its ligand. The

biological relevance of RAGE is still not completely known. On one side these both high and low affinity receptors differs in their cellular function and on the other side in their structure, which influence the binding affinity.

Glycation of the RAGE receptor leads to a reduced binding affinity of glucose-BSA about 5.7-fold with a K_D value of 76 µM for glucose-BSA and a complete inhibition of binding affinity for fructose-BSA. This effect is more drastically than for binding of NGF to glycated TrkA with threefold decrease of binding affinity independent of cellular effects. One reason could be that TrkA as a high affinity receptor can compensate couple of AGE-modifications in a better way than a low affinity receptor such as RAGE. The high affinity of the TrkA receptor results from greater intermolecular force than on a low affinity receptor, which is realized by a couple of interactions. TrkA receptor exists in a dynamic equilibrium between monomeric (low affinity) and dimeric (high affinity) structures [151]. Homodimerization is induced by binding of a NGF dimer [115]. Reduced binding affinity is dependent by modified exposed amino acids, which are involved in binding site (antigen). Basic amino acid and aromatic amino acid are glycation substrates. Binding of NGF (gen) is characterized by a plurality of electrostatic interactions, also hydrophobic interaction with an involvement of hydrophobic amino acids on binding site [152], which cannot primarily affected by glycation. This interaction does not disrupt by glycation. Nevertheless, threefold reduction of binding affinity of NGF to glycated TrkA induces already cellular decay of cell signaling and interferes in a physiological relevant manner. The strong reduction of binding affinity of glycated BSA to glycated RAGE caused probably in structure changes in the binding site of glycated RAGE. It has been shown that an amino acid exchange on arginine and/or lysine to alanine in the VC1 domains of RAGE prevents an interference with CML or CEL [153]. This result suggests an involvement of basic amino acid in the binding of AGEs, which represent also substrate for glycation. Consequently, RAGE may present sugar structures on the surface, which do not interfere with the sugar structures of glycated BSA or rather key-lock principal of the two complementary structures are steric mismatched after receptor glycation.

5.3 Technical aspects of interactions studies with glycated BSA

The differential binding behavior of the three used glycated BSA substrates presents another interesting aspect of receptor glycation of RAGE. Glycation of BSA was prepared under the same condition just the glycation reagent was changed. Under physiological conditions, the formation of AGEs and the binding to RAGE during various stages of ageing is an individual process depending on many environmental factors. The analysis of glycated BSA revealed many protein crosslinks for all glycated BSA preparations with

a smear on the Western blot at approximately 130 kDa and higher. Correlation between dose-dependent increase of glycation on albumin and higher molecular mass of proteins are already known [13]. The ratio of ribose-BSA between the high molecular smear and BSA band differs to glucose-BSA or fructose-BSA, indicating a high concentration of protein crosslinks for ribose–BSA than for the other ones. Glycation of BSA with ribose show a precise weak binding affinity to RAGE compared to glucose-BSA, indicating ribose, as a C5 sugar, induces different AGEs than glucose or fructose as C6 monosaccharides [154] and consequently supports other protein crosslinks. Ribose, a product of the pentose phosphate pathway, leads to AGEs such as CML and pentosidine [155]. Pentosidines are crosslinks between the amino acids arginine and lysine connected by carbonyl structures. Metabolism of glucose or fructose leads to the reactive dicarbonyl compound methylglyoxal, which induces the formation of AGEs such as CEL and pyrimidine [18], [95]. Comparing the binding affinity of the three glycated BSA ligands, the binding of glucose-BSA and fructose-BSA to RAGE is more efficient than the highly cross-linked ribose-BSA. This supports the theory that RAGE binds oligomer structures more efficient than high cross-linked structures [47]. In general C6 sugars appear to lead to reliable binding structures for RAGE, Mentioned glycation-mediated changes influence in the structure properties of albumin and their binding capacity [13]. A possibility for characterization glycated protein structures would be to use their enhanced auto- fluorescence compared to unglycated proteins. In the diagnostic, it is already used for measuring AGE-modified collagen in aged skin.

5.4 Influence on the neuronal plasticity of glycated BSA by binding to RAGE

Effect on the binding affinity of the substrates to RAGE after receptor glycation has been convincingly shown by SPR measurement. Not all AGEs bind with similar affinity to RAGE. Furthermore, in context of therapeutic approach glycation of RAGE could be a possibility for inhibition of AGE binding and following AGE-RAGE mediated oxidative stress or activation of NF-κB pathway. Anyway, RAGE may to be involved in cell growth and cell death, it represents an ambiguous role in cellular interactions. Regulation of RAGE expression will be an important issue to examine activation of signaling pathway. AGE-binding to RAGE has been shown to induce memory impairment, inflammation, arterial ageing and neurodegeneration by activation of inflammatory response by the NF-κB/JNK signaling pathway [156]–[159]. Further studies have shown that glycation induces the formation of β-sheet structures in β-amyloid protein, α-synuclein, transthyretin (TTR), copper-zinc superoxide dismutase 1 (Cu, Zn-SOD-1) or prion

proteins. Aggregation of β-sheets in each case creates fibrillar structures, probably causing Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, familial amyloid polyneuropathy or prion diseases [121]. In the context of Alzheimer ´s disease, Candela *et al.* showed that the transport of β-amyloid-protein through the blood brain barrier is mediated by RAGE. RAGE-deficiency in mice are viable and healthy and appear to protect AGE-induced neurodegeneration [70], [71]. Additionally, since AGEs by binding to RAGE activate MAPK pathway [138], [160], [161], the influence of RAGE on neuronal plasticity was investigated by binding of glycated-BSA to RAGE on the *in vitro* model PC12 cells. RAGE is expressed in cultured PC12 cells. First, cell viability of PC12 cells is not affected in presence of glycated BSA, even in presence of up to 500 µg/ml. Adding BSA, glucose-BSA, or fructose-BSA to PC12 cells do not show changes in proliferation or differentiation behavior in comparison to untreated PC12 cells. In PC12 cells, NGF-mediated activation of MAPK pathway induced neurite outgrowth [130]. Anyway, supplementing unglycated BSA, glucose-BSA or fructose-BSA in addition to NGF do not display neither an activation of MAPK pathway nor at least any enhancement in the neurite outgrowth of PC12 cells. For confirmation that glycated BSA binds to endogenous RAGE on PC12 cells, we analyzed the NF-κB activation, which is described in a RAGE dependent manner [46]. NF-κB is activated in presence of glucose-BSA or fructose-BSA in PC12 cells, suggesting glycated BSA binds to RAGE as expected and activates RAGE dependent pathways. However, MAPK pathway is not activated by glycated BSA binding to RAGE, application of BSA, glucose-BSA or fructose-BSA do not lead to ERK phosphorylation. Concluding, AGEs binding by RAGE do not interfere with neuronal plasticity by this pathway and following AGEs have no neuroprotective effect. However, the activation of NF-κB pathway could be demonstrated, suggesting RAGE induced a neuro-inflammatory response by expressing NF-κB regulated cytokines and recruiting leucocytes such as CD4+ or CD8+ [159]. RAGE-mediated neuro-inflammation would affect neuronal plasticity. The consequences of neuro-inflammation on regeneration capability and neuronal plasticity are especially precise manifested by the autoimmune disease multiple sclerosis or by the age-related injury by stoke [162]. Neuronal tissues has the possibility for adaption, but the regeneration capacity is limited, therefore the neuronal plasticity is really sensitive. Neurons appears to be really sensitive to exogenous influences such as toxins or peripheral transmitters. This is one reason wherefore the brain is protected by a fine-regulated blood brain barrier (BBB), which maintains an optimal chemical environment and insulate the brain from abrupt changes in the blood metabolism. The special structure of the BBB restricts the para-cellular pathway to small hydrophilic solutes and macromolecules, excludes amount of circulating leucocytes and uses transporter

systems for “feeding” the brain. In age-related diseases such as diabetes mellitus or Alzheimer´s disease the blood brain barrier is disrupted and the brain is exposed to destructive concentration of chemical compounds from the blood such as leucocytes without any regulation [163] or an increased glucose amount. AGEs stimulate a RAGE up-regulation in age-related manner and as demonstrated RAGE-mediated pathway activates NF-κB, which regulates recruiting more leucocytes and consequently, the inflammatory response caught in an upward spiral. RAGE activation by glycated BSA shows no neuroprotective effect but an activation of a pro-inflammatory transcription factor in neuronal cells. Following, RAGE activation by AGEs has a negative influence of neuronal plasticity.

Concluding, mental health during ageing is regarded as a major topic in the most societies of current research. Ageing is associated with an accumulation of advanced glycation endproducts (AGEs). AGEs accumulate in different regions of the human brain during age-related diseases. A strong destructive effect on the neuronal plasticity by AGEs could be demonstrated in the frame of this work. The demonstrated effect of glycation on neuronal plasticity highlights the demand to search for possibilities to prevent neuronal cells for AGE-induced implications. Inhibition of RAGE would be one possibility such as the reduced binding of AGEs to the glycated RAGE receptor demonstrated. Medical treatment with soluble RAGE or metalloproteinase ADAM 10 are also approaches for therapeutic gains. Soluble RAGE binds AGEs but does not activate the relevant signaling pathway. Metalloproteinase ADAM 10 cleaves off the transmembrane domain of RAGE [164] and thereby RAGE would react such as sRAGE. Furthermore, the function of the BBB including endothelial cells as well as glia cells (astrocytes and pericytes) has to be investigated more in detail. Human endothelial cells, which form the BBB, are under investigation by the working group of Prof. Horstkorte and these cells show a 24-fold higher resistance to MGO than neuronal cells. Therefore the responsible mechanism has to be identified and supported for higher resistance against AGEs and AGE-precursors. Interestingly, astrocytes exhibit a high glycolytic rate, produce accordingly MGO as side product, they play a central role in the blood brain barrier and interfere directly with the basolateral lying basal membrane. The basal membrane consists of ECM proteins mainly laminin, collagen IV and fibronectin, which serve as ideal substrate for AGEs. The uptake of β-amyloid proteins by RAGE receptor is transferred in astrocytes and there β-amyloid interfere with the glycolytic rate [61], [165]. Astrocytes capture a particular role and therefore, the influence of AGEs in astrocytes should be also analyzed in further studies.

6 Summary

Advanced glycation endproducts (AGEs) accumulate during ageing and in age-related diseases. The relevance for AGEs in context of vascular diseases or diabetes mellitus is already well-grounded as well for ECM protein-associated diseases such as cataract. The importance for neurodegenerative diseases such as Alzheimer's disease is precisely under investigation. Substantiated facts and molecular basics of the involvement of AGEs in neuronal tissue are missing. With this thesis the influence of AGEs on neuronal plasticity is comprehensively described and clarified. As marker for neuronal plasticity cell adhesion and especially neurite outgrowth was investigated. Furthermore the role of receptor glycation as well as the interference of RAGE on neuronal plasticity was investigated. For the investigations, PC12 cells were used as adequate *in vitro* model. Firstly, AGEs reduce cell adhesion by modifying ECM proteins and cell surface proteins such as cell adhesion molecules. Secondly, AGEs interact with intracellular and extracellular proteins leading to a radical reduction to the point of arrest of NGF-stimulated neurite outgrowth after glycation. It could be demonstrated that AGEs modify unselective intracellular and extracellular proteins. AGE-modified proteins prevent the NGF-induced neurite outgrowth extracellular signaling-regulated kinase (ERK) phosphorylation. Modifying of cell surface proteins by AGEs leads inevitably to the third point. Receptor glycation leads to strong reduced ligand binding affinity demonstrated on RAGE and TrkA. For the high affinity receptor TrkA a threefold reduction was determined and for the low affinity receptor RAGE a 5.7-fold reduction for glucose-BSA up to complete inhibition of fructose-BSA. In this context, for the first time the dissociation constants (K_D values) for glycated BSA to RAGE were proved. Nevertheless, the meaning of receptor glycation effect has to be interpreted in different ways. The glycation of the TrkA leads to a dramatic effect on impaired cell signaling with sweeping reduced neurite outgrowth, while the glycation of RAGE is an approach to inhibit AGEs induced cellular effects. In context of neuronal disorders, it means inhibition of reduced neuronal plasticity. Fourth and last but not least, the AGEs-dependent influence of RAGE on neuronal plasticity was investigated. Surprisingly, although an activation of MAPK pathway described for RAGE in several cell types, RAGE mediated no activation of MAPK pathway by binding AGEs (glycated-BSA) in PC12 cells, thus RAGE can no neuroprotective influence assigned. Furthermore, activation of nF-κB pathway by glycated BSA binding to RAGE could be demonstrated, suggesting RAGE induce neuro-inflammatory response. The results of this thesis show convincingly that AGEs are negatively prognostic marker for neuronal plasticity and build the foundation for further research in the neuronal aging field.

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8 Theses

1. Glycation of extracellular matrix (ECM) proteins leads to reduced cell adhesion but has minor negative effects on neurite outgrowth.

2. Glycation of PC12 cells (induction with ≤ 1.0 mM MGO) affects intracellular and extracellular proteins without interference of cell viability.

3. Glycation of PC12 cells interferes with cell adhesion and TrkA receptor-mediated MAPK signaling pathway for inducing neurite outgrowth.

4. Receptor glycation affects binding affinity. Glycated TrkA receptor displays a threefold reduction of binding affinity. Glycated RAGE showed dependent from the binding partner a 5.7-fold reduction for glucose-BSA and total inhibition of binding for fructose-BSA.

5. For the first time, binding affinity of RAGE to glycated BSA was determined by determination of the equilibrium dissociation constant (K_D). RAGE receptor binds glucose-BSA with a K_D value of $(1.39 \pm 0.7)*10^{-5}$ M and binds fructose-BSA with similar binding affinity of $(1.95 \pm 0.2)*10^{-5}$ M.

6. RAGE-mediated NF-κB signal pathway is activated by binding of glycated-BSA but RAGE-mediated activation in presence of glycated BSA does not activate MAPK pathway and consequently induces no neuroprotective effect.

Tabellarischer Lebenslauf

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Febr. 2009	Praktikum am Institut für Rechtsmedizin an der Martin-Luther Universität Halle-Wittenberg in der AG „Forensische Molekulargenetik“ (Dr.U.D. Immel)
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Okt. 2006 – Okt. 2007	Pharmazeutisch-technische Assistentin (Teilzeit) in der „Apotheke im Wal-Mart“ in Heidenau
März – Okt. 2006	Vollzeitbeschäftigung als Pharmazeutisch- technische Assistentin in der „Apotheke Kaufpark Nickern“ Dresden bis Studienbeginn
Okt. 2004	Praktikum im Pharma – und Kosmetikbetrieb LiiL GmbH, Dresden

Wissenschaftliche Methoden

Molekularbiologische Methoden:

- DNA-Analysen und zielgerichtete Mutagenese mittels Polymerasekettenreaktion (PCR)
- DNA- Quantifizierung mittels *Real-time* PCR
- rekombinante Genexpression mit T7-RNA-Polymerase Expressionssystem
- Plasmidpräparationen

Zellbiologische Methoden:

- Kultivierung von verschiedenen adhärenten und Suspensionszelllinien
- Zellviabilitätsanalysen mittels Färbemethoden, MTT- Test und Lichtmikroskopie
- Echtzeitzellanalyse mittels *Real-time cell analyzer* (RTCA)
- Durchflusszytometrie

Analytik:

- UV/VIS-Spektroskopie
- Oberflächenplasmonresonanzspektroskopie (SPR)
- Circulardichroismus (CD)
- Fluoreszenzemissionsspektroskopie
- MALDI-TOF Massenspektrometrie

Proteinbiochemische Methoden:

- Kationaustauscherchromatographie
- Ni-NTA Affinitätschromatographie
- Dialyse und Konzentrierung von Proteinlösungen
- Proteinanalyse mittels SDS-Polyacrylamidgelelektrophorese (SDS-PAGE) und Western Blot

Akademische Weiterbildung

24.März – 28.März 2014	FELASA-B Kurs „Tierschutz / Versuchstierkunde“ 2014
28.Juli - 01.August 2014	*Good manufacturing practice* (GMP)-Lehrgang

Ausbildung

Okt. 2006 - Okt. 2011	Studium im Fach Biochemie/Biotechnologie an der Martin-Luther Universität mit Abschluss Diplom (1,6) in Regelstudienzeit, Schwerpunkte: Proteinbiochemie und Medizinische Biochemie
Sept. 2003 – März 2006	Ausbildung zur Pharmazeutisch-technischen Assistentin am IFBE-Bildungszentrum Zwickau, Schwerpunkte der mündlichen Abschlussprüfung Arzneimittelgesetz (AMG), Betäubungsmittelgesetz (BtMG) und allgemeine Toxikologie
1995 – Mai 2003	Schiller-Gymnasium Bautzen mit Abschluss Abitur

Weitere Kenntnisse und Fähigkeiten

EDV-Kenntnisse	sicherer Umgang mit MS-Office, Sigma-Plot, Origin, Pymol, AIDA 2, Pharmatechnik und Promedisoft

Sprachen

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Spanisch	konversationssicher
Französisch	erweitere Grundkenntnisse

Engagement und Interessen

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Mai 2007 – Mai 2009	Allgemeine Sprecherin im STURA der Martin-Luther Universität Halle-Wittenberg
Legislaturperioden 2007/08 – 2009/10	Wahlleiterin der Hochschulwahlen an der Martin-Luther Universität Halle-Wittenberg
Ehrung	20. Mai 2014 in Magdeburg „feierliche Würdigung von Teilnehmern/-innen an Friedenseinsätzen“
2. Interessen	Leichtathletik, Tennis, Gerätetauchen, Standardtanz

Publikationsliste und Kongressbeiträge

1. Publikationen in Fachzeitschriften

1. **Bennmann D**, Weidemann W, Thate A, Kreuzmann D, Horstkorte R (2015) , *O-GlcNAcylation of M743T-variant of GNE interferes with stability and activity*, FEBS journal *under revision.*

2. Kreuzmann D, Horstkorte R, Kannicht C, Kohla G, **Bennmann D**, Bork K (2015), *A Transgenic Mous Model of the human Sialic Acid Biosynthesis Disorder Sialuria,* PLoS One., *submitted.*

2. **Bennmann D**, Kannicht C, Fisseau C, Jacobs K, Navarette-Santos A, Hofmann B, Horstkorte R. (2015), *Glycation of the high affinity NGF-receptor and RAGE leads to*

reduced ligand affinity, Mechanism of Ageing and Development., doi:10.1016 /j.mad.2015.07.003.

3. **Bennmann D**, Horstkorte R, Hofmann B, Jacobs K, Navarrete-Santos A, Simm A, Bork K, Gnanapragassam VS. (2014), *Advanced glycation endproducts interfere with adhesion and neurite outgrowth*. PLoS One., doi: 10.1371/journal.pone.0112115.

4. Weidemann W, Hering J, **Bennmann D**, Thate A, Horstkorte R. (2013*): The key enzyme of the sialic acid metabolism is involved in embryoid body formation and expression of marker genes of germ layer formation.* Int J Mol Sci., doi: 10.3390 /ijms141020555.

2. Kongressbeiträge

1. **Bennmann D**, Gnanapragassam VS, Kannicht C, Horstkorte R *Advanced glycation endproducts (AGEs) are negative prognostic markers for neuronal plasticity*, EMBO Symposium für *Mechanism of Neurodegeneration*, Juni 2015, Heidelberg, Poster-Präsentation

2. **Bennmann D**, *Posttranslational modifications (PTM´s) of GNE*, GNE myopathy consortium workshop Okt. 2014, Berlin, Fachvortrag

3. **Bennmann D**, Gnanapragassam VS, Horstkorte R, *Age-dependent Advanced Glycation Endproducts (AGEs) interfere with cell adhesion and neurite outgrowth*, „Stress und Altern – Chancen und Risiken" DGGG –Kongress, Halle, Sept. 2014, Poster-Präsentation

4. **Bennmann D**, Gnanapragassam VS, Horstkorte R, *Age-dependent Advanced Glycation Endproducts (AGEs) interfere with cell adhesion and neurite outgrowth*, 25th Joint Glcyobiology Meeting, Sept. 2014, Ghent, Poster-Präsentation

5. **Bennmann D**, *Age-dependent Advanced Glycation Endproducts (AGEs) interfere with cell adhesion and neurite outgrowth*, EMBO workshop Glycobiology & Glycochemistry Mai 2014, Lissabon, Fachvortrag

6. **Bennmann D**, Gnanapragassam VS, Horstkorte R., *Impact of Advanced Glycation Endproducts (AGEs) in PC12 cells „Dynamic nano- and glyco-sciences*" Spring Training School – COST CM1102 -MultiGlycoNano Joined meeting with the "Dynano" ITN network, Apr. 2014, Namur, Poster Pränsentation

7. **Bennmann D**, Gnanapragassam VS, Horstkorte R., *Impact of Advanced Glycation Endproducts (AGEs) in PC12 cells*, 24th Joint Glcyobiology Meeting, Nov. 2013, Wittenberg, Poster-Präsentation

Halle (Saale), den 29.08.2015

Dorit Bennmann

Selbstständigkeitserklärung

Ich erkläre hiermit, dass ich die vorliegende Dissertation selbstständig und nur mit Hilfe der im Anhang dieser Arbeit vermerkten Quellen angefertigt habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen wurden, sind als solche kenntlich gemacht. Bei den Untersuchungen, auf deren Ergebnissen diese Dissertation beruht, habe ich mich streng an die Satzung der Martin-Luther Universität zur „Sicherung guter wissenschaftlicher Praxis“ gehalten. Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Halle/Saale, den 29.08.2015

Dorit Bennmann

Erklärung über frühere Promotionsversuche

Hiermit erkläre ich, dass ich bisher keinen Promotionsversuch unternommen habe und dass kein Promotionsverfahren an einer anderen wissenschaftlichen Einrichtung läuft.

Halle/Saale, den 29.08.2015

Dorit Bennmann

Danksagung

Ich möchte mich bei allen, die an der Erstellung dieser Arbeit mitgewirkt haben, bedanken.

Mein besonderer Dank gilt zunächst Herrn Prof. Dr. Rüdiger Horstkorte, der mir diese interessante Aufgabe anvertraute, mir die Möglichkeit zur Durchführung dieser Untersuchungen am Institut für physiologische Chemie einräumte und mir jederzeit mit sachkundigem Rat zur Seite stand.

Ich danke Frau PD Dr. med. Britt Hofmann und Herrn Prof. Dr. Franz-Georg Hanisch für die Anfertigung der Gutachten zur dieser Arbeit.

Des Weiteren möchte ich mich bei allen Mitarbeitern der AG Horstkorte für die angenehme und motivierende Atmosphäre, die ein stressfreies und intensives Arbeiten ermöglichte sowie für die fachlichen Anregungen und den regen Gedankenaustausch bedanken. Es hat mir stets sehr viel Freude bereitet, in diesem Team zu arbeiten.

Ich danke auch den Mitarbeitern von Octapharma, die mir die Möglichkeit gaben, meine Oberflächenplasmonenresonanz-Messungen in ihren Räumlichkeiten durchzuführen und die mich mit interessanten Tipps und Kniffen unterstützten. Besonderer Dank hierbei gilt Herrn Dr. Christoph Kannicht, Frau Claudine Fisseau und Herrn Dr. Guido Kohla.

Vielen Dank auch der Arbeitsgruppe von Herrn Prof. Andreas Simm für die fachlichen Diskussionen im Rahmen des gemeinsamen Kooperationsprojektes. In diesem Zusammenhang möchte ich besonders Frau Kathleen Jacobs und Herrn Dr. Alexander Navarette–Santos danken für die Her- und Bereitstellung der glykierten BSA–Präparationen.

Ein großer Dank gilt besonders meinen lieben Eltern, die mir während meiner Promotionszeit immer zur Seite standen, mich jederzeit in meiner Motivation bestärkten und die es mir ermöglichten, mein Ziel wirklich zu realisieren.
Letztendlich danke ich meinen Freunden, die stets für mich da waren und mir jederzeit mit Rat und Tat zur Seite standen. Vielen Dank auch den zahlreichen unerwähnt gebliebenen Helfern.

Nochmals DANKE!!