Effect of distinct amyloid β1-42 assemblies on synaptic plasticity

Ph.D. Thesis

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„If the human brain were so simple that we could understand it,

we would be so simple that we couldn't."

Emerson M. Pugh

List of Publications

Full papers, directly related to the subject of the thesis

Integrin activation modulates NMDA and AMPA receptor function of CA1 cells in a dose-related fashion in vivo.
Juhász G, Vass G, Bozsó Z, Budai D, Penke B, Szegedi V.
Brain Res. 2008 Oct 3;1233:20-6. Epub 2008 May 21.
PMID: 18762286

An intraperitoneally administered pentapeptide protects against Abeta (1-42) induced neuronal excitation in vivo.
Juhász G, Márki A, Vass G, Fülöp L, Budai D, Penke B, Falkay G, Szegedi V.
J Alzheimers Dis. 2009 Jan;16(1):189-96.
PMID: 19158435

Full papers, not related to the subject of the thesis

Divergent effects of Abeta1-42 on ionotropic glutamate receptor-mediated responses in CA1 neurons in vivo.
Szegedi V, Juhász G, Budai D, Penke B.
Brain Res. 2005 Nov 16;1062(1-2):120-6. Epub 2005 Oct 24.
PMID: 16248989

Endomorphin-2, an endogenous tetrapeptide, protects against Abeta1-42 in vitro and in vivo.
Szegedi V, Juhász G, Rózsa E, Juhász-Vedres G, Datki Z, Fülöp L, Bozsó Z, Lakatos A, Laczkó I, Farkas T, Kis Z, Tóth G, Soós K, Zarándi M, Budai D, Toldi J, Penke B.
FASEB J. 2006 Jun;20(8):1191-3. Epub 2006 Apr 24.
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Abbreviations

AAI	Amyloid aggregation inhibitors
ACSF	Artificial cerebrospinal fluid
AD	Alzheimer's disease
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANOVA	Analysis of variance
AP	Action potential
APP	Amyloid precursor protein
APPsα	N-terminal soluble APP
Aβ	β-amyloid peptide
BACE-1	Acronym of a β-site APP cleaving enzime-1
BBB	Blood-brain barrier
BSB	β-sheet breakers
CA1	Cornu Ammonis 1
CaMKII	Ca^{2+}/calmoduline-dependent kinase II
CNS	Central nervous system
CRE	cAMP response element
CREB	CRE-binding protein
CSF	Cerebrospinal fluid
CTFα	α-carboxy terminal fragment
DG	Dentate gyrus
DLS	Dynamic light scattering
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
ESI-MS	Amino acid analysis and mass spectrometry
FAK	Focal adhesion kinase
fAβ1-42	Fibrillar Aβ1-42
fEPSPs	Field excitatory postsynaptic potentials
FRHDS	Phe-Arg-His-Asp-Ser
GABA	Gamma-aminobutyric acid
GGGGG	Pentaglycine
Glu	L-glutamate
GluR1	AMPA-type glutamate receptor 1
GRGDS	Gly-Arg-Gly-Asp-Ser
HFS	High-frequency stimulation
IDX	4'deoxy-4'iodorubicin derivates
I–O curve	Input–output curve
ip	Intraperitoneal
IP3	Ins(1,4,5)P3
KA	Kainic acid
KLVFFA	Lys-Leu-Val-Phe-Phe-Ala
KPI	Kunitz-type serine protease inhibitor
LFS	Low-frequency stimulation
LPFFD	Leu-Pro-Phe-Phe-Asp
LPYFDa	Leu-Pro-Tyr-Phe-Asp-amide
LTD	Long-term depression

(E)-LTP	Early-LTP
(L)-LTP	Late-LTP
LTP	Long-term potentiation
LVFFA	Leu-Val-Phe-Phe-Ala
MAPK	Mitogen-activated protein kinase
MEA	Multi-electrode array
mGluR	Metabotropic Glu receptors
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
NFTs	Neurofibrillary tangles
NMDA	N-methyl-D-aspartate
NSAIDs	Non-steroidal anti-inflammatory drugs
PI	Phosphoinositide
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PP2	4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine
PP3	4-Amino-7-phenylpyrazol[3,4-d]pyrimidine
PPF	Paired-pulse facilitation
PS1	Presenilin-1
PS2	Presenilin-2
PSB	Pontamine Sky Blue
PSD-95	PSD protein of 95 kDa
PSDs	Postsynaptic densities
RAGE	Receptor for advanced glycation end products
RGD	Arg-Gly-Asp
RHDS	Arg-His-Asp-Ser
SAP-102	Synapse-associated protein-102
SEM	Standard error of means
Src	Sarcoma
SRE	Serum response element
TBS	Theta-burst stimulation
TEM	Transmission electron microscopy
TM	Transmembrane domain
VDCCs	Voltage-dependent Ca^{2+} channels
vitamin E	α-tocopherol
τ	Tau

General introduction

More than a century ago, a German psychiatrist and neuropathologist, Alois Alzheimer presented the case of a female patient and described a neurodegenerative disorder which bears his name (Alzheimer, 1907, *1*). The main hallmarks of Alzheimer's disease (AD) are cognitive decline, neuronal and synaptic loss mainly in neocortex and hippocampus, extracellular senile plaques and intracellular neurofibrillary tangles.

Alzheimer's disease is an irreversible, progressive neurodegenerative disorder with an as yet unknown etiology, and despite intense research, there is no cure or effective therapy. It is the most common form of dementia among the elderly, affecting nearly 3% of the population over the age of 65, while 25-50% of people aged 85 suffer from AD and the great majority of them are females (Jorm, 1998, *2;* Forsyth, 1998, *3*). Age is the dominant risk factor in AD, and by the improvement in life expectancy in welfare states, plausibly the number of patients will be doubled by 2050. There are about 7-8 million demented patients in Europe, 4–5 million in the USA with an increase to 14 and 16 millions, respectively, by 2050. Rates of increase in developed countries are forecasted by 100% between 2001 and 2040, but by more than 300% in India, China and their Asian neighbours (Ferri, 2005, *4*). At the onset of the disease, patients show symptoms of mild cognitive impairment, but in the subsequent years, more severe memory loss occurs leading to confusion and lack of orientation. Eventually they often become institutionalized and bedridden, causing crescent expense to family members and society.

There are two forms of Alzheimer's disease, sporadic and familial, and they share common histological and clinical symptoms. The extracellular deposites, mainly composed of the so-called β-amyloid peptide (Aβ), wich is a cleavage product of a membrane spanning amyloid precursor protein (APP), are thought to be primordial and the intracellular neurofibrillary tangles are secondary phenomena. The extracellular accumulation and aggregation of Aβ peptides seem to be liable for Alzheimer's disease, but effective therapeutic intervention into this pathogenic process is still lacking.

Neuropathological features of Alzheimer's disease

Atrophy

The direct pathological examination of brain tissue by means of biopsy or autopsy is still the only way to establish a secure diagnosis of AD. Gross cortical athrophy can be observed macroscopically in AD brains leading to the loss of their volume by 10-25% compared to those of control patients (de la Monte, 1989, *5;* Hubbard, 1981, *6*). Synaptic loss accounts mostly for brain atrophy and not the decrease in the number of cell bodies (Terry, 2000, *7*). Synaptic dysfunction is likely to be the mechanism that causes memory loss (Small, 2001, *8*). Cellular degeneration and neuronal loss affect the outer three layers of temporal and frontal cortical regions primarily and later the parietal and occipital regions (Selkoe, 1997, *9*). The accelerated atrophy of the temporal cortical region is associated with symptomatic onset of AD, whereas hippocampal atrophy occurs one to two years earlier (Convit, 1997, *10;* Fox, 1996, *11*). Impairment in cholinergic transmission arise from the incipient failure of nucleus basalis of Meyert (Toledano, 2004, *12*).

Amyloid plaques

Extracellular deposites of about 50-100 μm in diameter surrounded by dystrophic axons and dendrites, reactive astrocytes and activated microglia can be found mainly in the amygdala, hippocampus and neocortex (Jellinger, 1998, *13*). The core of these neuritic plaques is composed predominantly of Aβ1-42 and Aβ1-40, proteolitical derivatives of the membrane spanning amyloid precursor protein (APP). Neuritic plaques are believed to arise from "diffuse plaques" found in large numbers in not typically AD related areas (e.g. cerebellum, striatum and thalamus) (Selkoe, 2001, *14*). It has been shown recently, that plaques appear extraordinary quickly in a mouse model of AD within 24 h, the overwhelming majority of plaques appear, and their size remain constant even for 1 week. (Meyer-Luehmann, 2008, *15*). The number of neuritic plaques does not correlate well with the severity of the dementia, not like the elevated levels of total Aβ in the brain with cognitive decline (Naslund, 2000, *16*). Five phases of Aβ-associated pathology can be established, based on the appearence of Aβ deposites. First is the neocortical phase, followed by the allocortical phase 2. In phase 3, the diencephalic nuclei, the striatum, and the cholinergic nuclei of the basal forebrain develop Aβ deposits, and in phase 4, several brain-stem nuclei

become additionally involved. Finally, phase 5 is characterized by cerebellar Aβ-deposition. These findings suggest that Aβ deposition expands anterogradely into regions that receive neuronal projections from regions already exhibiting Aβ (Thal, 2002, *17*).

The main component of plaques is a waxy substance called "amyloid" (from *amylum* or amylose) by Virchow (Virchow, 1854., *18*). It is a low molecural weight, ~4kDa polypeptide, wich was firstly isolated from vessels of an AD brain (Glenner, 1984, *19*) and soon after caracterized from plaques of AD and Down syndrome pateints' brains (Masters, 1985, *20*) Sequencing the amyloid led to the identification of its precursor protein (APP) (Kang, 1987, *21*).

Amyloid precursor protein

APP is a trasmembrane protein that undergoes post-translational modifications in the endoplasmatic reticulum through the secretory pathway meanwhile proteolytic cleavages may occur to release secreted derivatives into vesicles and the extracellular space. Its half-life is relatively short (<1 hour) (Weidemann, 1989, *22*).

The exact function of APP is poorly defined. APP is expressed throughout the body and has several isoforms, mainly $APP_{695,}$ APP_{751} and APP_{770}. Its distribution is highest in the brain and kidneys, while lower in the spleen, adrenal glands, lungs and liver (Tanzi, 1987, *23;* Golde, 1990, *24*). APP is also present in CSF (cerebrospinal fluid), where its level increases after traumatic brain injury (Olsson, 2004, *25*). APP_{695} isoform is mainly expressed in neurons (Selkoe, 2001, *14*). APP_{751} and APP_{770} contain a 56-residue insert in the middle of the ectodomain encoding a Kunitz-type serine protease inhibitor (KPI). The APP-KPI isoforms are mainly produced by glial cells (Abe, 1991, *26*) (Forloni, 1992, *27*), platelets (Van Nostrand, 1990, *28*)], and peripheral tissue (Tanzi, 1988, *29*) and are present in elevated levels in AD brains (Moir, 1998, *30*). APP is able to bind zinc, increasing its binding to heparin and has been shown to potentiate the inhibition of coagulation factor XIa by an APP isoform containing a Kunitz-type inhibitory domain (Bush, 1994, *31*). Treatment of cell cultures with APP results in neurite outgrowth (Koo, 1993, *32*) presumably via its N-terminal, heparin binding domain (Small, 1994, *33;* Mok, 1997, *34*). APP_{751} and APP_{770} account mainly for the neurite outgrowth activity, rather than APP_{695} (Qiu, 1995, *35*). APP is capable of complexing with and activating the trimeric G_0 protein, a major GTP binding protein in the brain, via its cytoplasmic and transmembrane domains (Nishimoto, 1993, *36;* Okamoto, 1995, *37*), thus APP may contribute to the neurodegeneration of AD where G-protein-associated

signaling pathways are altered. (Giambarella, 1997, *38;* Mahlapuu, 2003, *39;* Yamatsuji, 1996, *40*). Accumulating evidence suggest that it functions as a kinesin-1 cargo receptor targeting synaptic proteins to nerve terminals (Kang, 1987, *21*) and is essential in maintaining Cu and Fe homeostasis (Andrews, 2001, *41;* Barnham, 2004, *42*), by delivering Cu and Fe to metalloenzimes and proteins for instance superoxid dismutase 1 (SOD1) (Culotta, 1997, *43*) and the Cu ATPase (Waggoner, 1999, *44*). Soluble APP can protect cell cultures from glutamate or Aβ excitotoxicity and glucose deficiency (Oddo, 2003, *45;* Schubert, 1993, *46*). APP also plays role in the process of memory formation. (Mileusnic, 2000, *47;* Mileusnic, 2005, *48*).

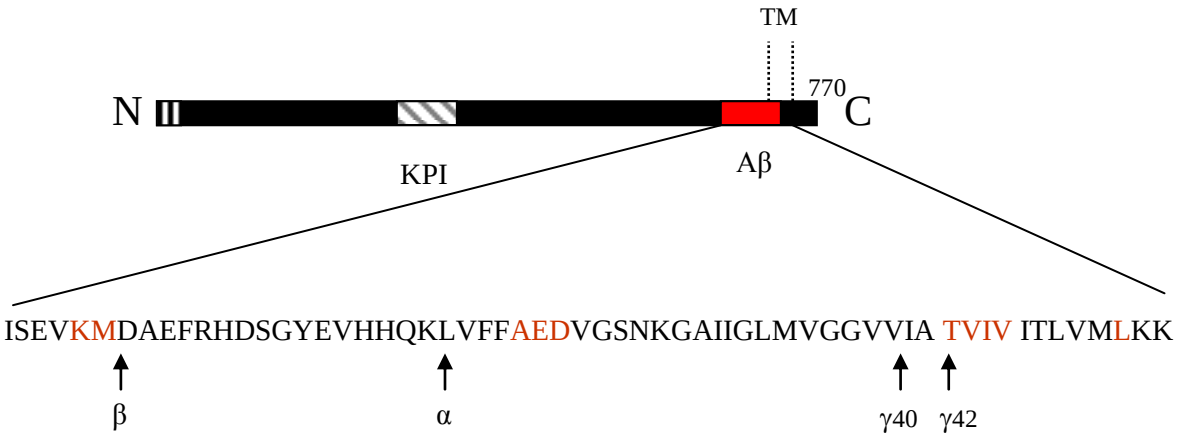

Fig. 1. Schematic diagram of APP. Regions of interest (at their correct relative position): the 17-residue signal peptide at the NH_2 terminus (box with vertical lines), two alternatively spliced exons of 56 and 19 amino acids inserted at residue 289 (the first is the KPI domain), the single transmembrane domain (TM) at amino acids 700-723 (vertical dotted black lines). Arrowheads indicate the cleavage sites of α-, β- and γ-secretase. Figure adapted from: (Selkoe, 2001, *14*).

Neurofibrillary Tangles

Intracellular deposits (in the entorhinal cortex, hippocampus, amygdala, frontal, temporal and parietal association cortices and in certain subcortical nuclei projecting to these regions) in AD brain are composed of hyperphosphorylated form of microtubule-associated protein tau (τ). These neurofibrillary tangles (NFTs) are present in dendrites, axons and in the pericaryon of neurons (Brion, 1998, *49*), and found extracellularly after cell death, hence they are persistent structures. NFTs are composed of pairs of approximately 10 nm filaments

wound into helices with a helical period of ~160 nm as revealed by electron microscopy (Yankner, 1991, *50*). The phosphorylation of tau reduces its ability to stabilize microtubules leading to disruption of neuronal transport and eventually to the death of affected neurons (Bhaskar, 2005, *51*). NFTs develop and spread in a predictable manner across the brain, making six distinguishable stages of disease progression (Thal, 2002, *17*): the transentorhinal stages I–II representing clinically silent cases; the limbic stages III–IV of incipient AD; and the neocortical stages V–VI of fully developed AD. The extent of cortical neurofibrillary tangles correlates positively with the severity of AD, however tangles are also found in other neurodegenerative diseases without amyloid deposites (Iqbal, 2005, *52*).

The "amyloid cascade" hypothesis

The most widely accepted theory is the amyloid cascade hypothesis claiming that the increased burden of Aβ in the brain is the primary intrinsic pathogenic event in AD (Hardy, 1992, *53*). Aβ is liable for the pathological changes observed in AD brains, resulting in synaptic loss, causing or enhancing NFT-pathology, activating inflammatory processes, eventually leading to neuronal death.

The processing of APP can drive into two pathways, a conventional pathway, that does not release Aβ and an alternative patway that leads to production of Aβ fragments that are prone to aggregate (Haass, 1993, *54*),(Fig.2.).

There are three proteases concerned in the processing of APP, namely the α-, β- and the γ-secretase. α-secretase is of the family of proteases that process other integral membrane proteins such as transforming growth factor-α and tumor necrosis factor-α (Blacker, 2002, *55*). It has a constitutive and a regulated component that can be activated via protein kinase C and other second messenger cascades. Several members of the disintegrin and metalloprotease family (ADAM) have been implicated in APP-processing: TNF-α converting enzyme (Buxbaum, 1998, *56*), ADAM10 and ADAM9 (Hotoda, 2002, *57*) and ADAM17 (Asai, 2003, *58*). The nonamyloidogenic pathway occures in the late Golgi compartment or in caveolae (plasma membrane invaginations) (LeBlanc, 1997, *59*). At first, the membrane associated α-secretase cleaves APP within the Aβ sequence between Lys16and Leu17 thereby destroying the sequence (Kang, 1987, *21*) and secreting the N-terminal soluble APP (APPsα) and leaving an 83 amino acid residue in the cell membrane. This way precludes the formation of Aβ (Selkoe, 1994, *60*). The APPsα might have biological functions in growth regulation and

neuroprotection, and in the case of isoforms containing the Kunitz proteinase inhibitor domain, in blood coagulation (Van Nostrand, 1990, *28*). The CTFα finally cleaved by the γ-secretase , resulting in a truncated 3-kDa Aβ fragment, called P3 (Haass, 1993, *54*).

In the amyloidogenic pathway, β-secretase cuts between residues 671 and 672 of the APP yielding the N-terminus of Aβ, releasing a large soluble compartment of the ectodomain, termed sAPP-β, and leaving the membrane associated CTF-β fragment, which contains the intact Aβ sequence (Haass, 1993, *54;* Busciglio, 1993, *61*). β-secretase is an aspartyl protease, called BACE-1 (acronym of a β-site APP cleaving enzime-1) (Vassar, 1999, *62*). BACE-1 is expressed in the brain and upregulated after an ischemia, while its close homolog, BACE-2 is mainly found in peripheral tissues and its expression is unaffected after ischemia, such event in which the upregulation of APP expression is apparent. (Abe, 1991, *26;* Bennett, 2000, *63;* Wen, 2004, *64*). The CTF-β fragment is cleaved at several positions between amino acid 39 and 43 of the Aβ sequence by a multiprotein enzime complex, termed γ-secretase, releasing Aβ (Golde, 1990, *24*). This complex is composed of presenilin-1 (PS1), presenilin-2 (PS2), nicastrin, Aph1 and PEN2 (rewieved in: (Haass, 2004, *65*)). Presenilins are expressed in the brain, primarily in neurons, contain multiple transmmbrane domains, with both amino and carboxy terminus as well as a large hydrophilic loop. PS1 is involved in neurogenesis and formation of axial skeleton, as well as in γ-secretase activity. PS1 has two transmembrane aspartate residues necessary to Aβ production suggesting that PS1 is an essential cofactor for γ-secretase or maybe the secretare itself (Kimberly, 2000, *66*). The two transmembrane residues of PS2 are also critical for γ-secretase activity. Mutations in PS genes selectively enhance the formation of Aβ1-42, cousing an increase in Aβ1-42/ Aβ1-40 ratio, enhancing oligomer formation of amyloidogenic Aβ1-42. Gamma-secretase is also able to process other integral membrane proteins, like Notch-1 (Song, 1999, *67;* Nakajima, 2000, *68;* Moehlmann, 2002, *69*), which participates in gene transcription and cell differentiation, ephrinB2 (Georgakopoulos, 2006, *70*) and N-cadherin (Marambaud, 2003, *71*).

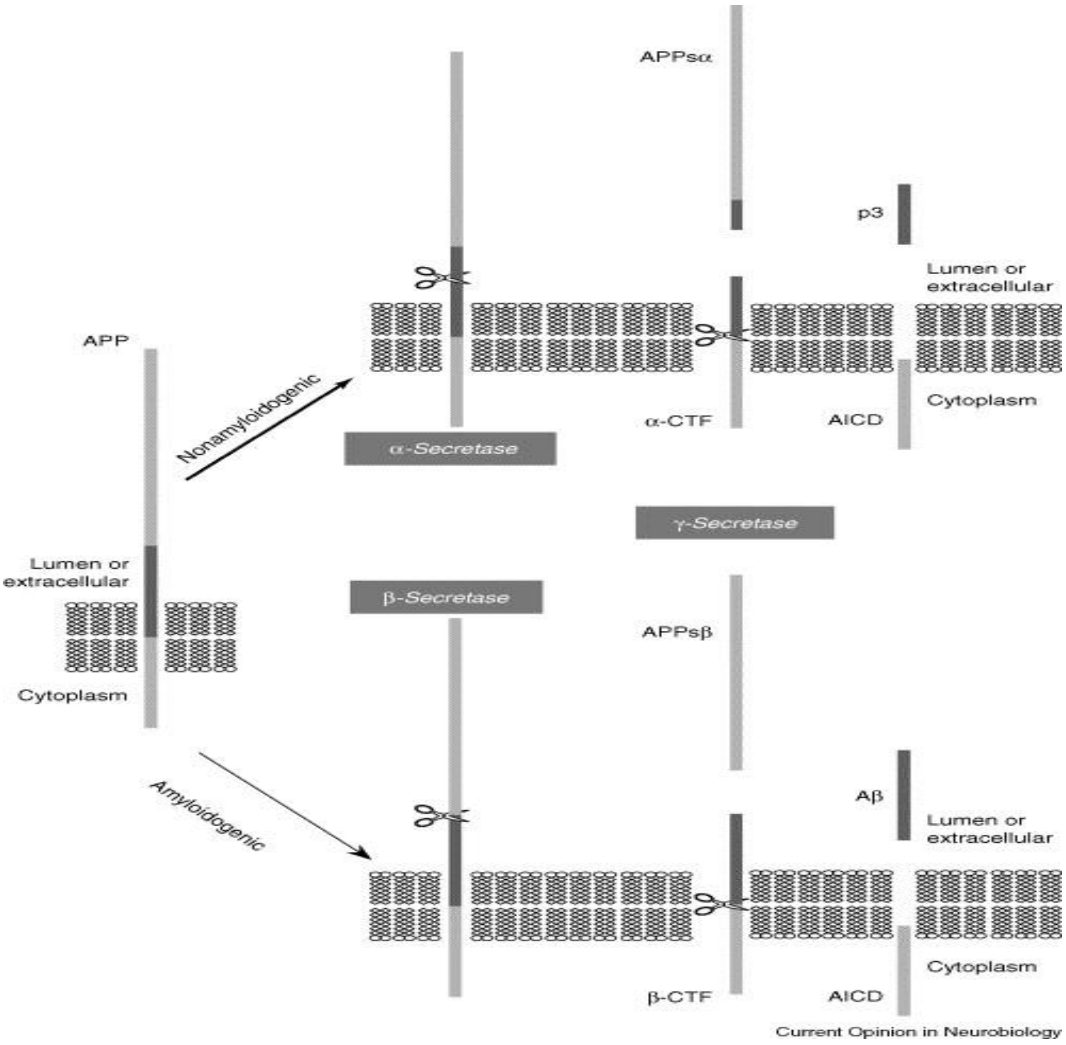

Fig. 2.: Proteolytic processing of APP by the secretases. The majority of APP is processed in the nonamyloidogenic pathway (thick arrow); APP is first cleaved by α-secretase within the Aβ domain (darker shaded region), leading to APPsα secretion and precluding Aβ generation. α-carboxy terminal fragment (CTF) is then cleaved by γ-secretase within the membrane, releasing the p3 peptide and AICD. Alternatively, amyloidogenesis (thin arrow) takes place when AβPP is first cleaved by β-secretase, producing APPsβ. Aβ and AICD are generated upon cleavage by γ-secretase of the β-CTF fragment retained in the membrane. Scissors indicate the cleavage sites of α-, β- and γ–secretase. Figure from (Wilquet, 2004, *72*).

As a result of the non-amyloidogenic pathway, the most commonly formed Aβ contains 40-amino-acids (Aβ1-40), but a minor amount of 42-amino-acid containing form (Aβ1-42) is also produced. Aβ1-42 aggregates into amyloid fibrils much more readily than Aβ1-40 (Jarrett, 1993, *73*). Initially, the fibrillar Aβ1-42 form was assumed to be liable for the induction of the pathological and clinical symptoms of AD. Nowadays it is less clear which aggregational state of Aβ1-42 has the major impact in the pathogenesis of AD. Throughout the aggregational process, distinct forms can be observed from small oligomeric assemblies to

larger protofibrillar species (Isaacs, 2006, *74;* Hilbich, 1992, *75;* Burdick, 1992, *76;* Snyder, 2005, *77*). Amyloid assemblies may have different biological actions in distinct aggregational states (Meyer-Luehmann, 2008, *15;* Dahlgren, 2002, *78*). Small oligomeric Aβ1-42 assemblies, also termed as Aβ-derived diffusible ligands (Klein, 2002, *79*), furthermore intracellular accumultion of Aβ1-42 (Gouras, 2000, *80*) may also be responsible for neurotoxicity. An alternative aggregation pathway resulting in stable, globular oligomeric species was also proposed (Barghorn, 2005, *81*) while a 56 kDa 12-mer Aβ1-42 aggregate, termed Aβ*, was described as the ultimate cause for cognitive decline (Lesne, 2006, *82*). The aggregation process leads to the formation of fibrillar deposits, known as senile plaques, which are potential reservoir of oligomeric Aβ. The halo of oligomeric Aβ surrounding plaques within 50 μm is synaptotoxic and synapse loss correlates well with cognitive decline in Alzheimer's disease (Koffie, 2009, *83*).

The high lipid content and high oxigen consumption make the brain susceptible to oxidative stress (Reiter, 1995, *84*). In vitro, Aβ can cause oxidative stress, mitochondrial dysfunction, disturbances in calcium homeostasis, and microglial activation (Small, 2001, *8*). Microglial cells and astrocites are involved in the cronic inflammatory responses in AD via the upregulated expression of phospholipase A2, subsequent arachidonic acid / prostaglandin inflammatory pathway activity by secreting interleukin-1 (Griffin, 1989, *85*), activation of complement pathways (Rogers, 1992, *86*), and by producing several potentially neurotoxic compound, including superoxides, glutamate, and NO (Grosshans DR, 2001, *87;* Colton, 1994, *88*). Aβ interacts with a number of different membrane components including lipids, carbohydrates, ion channels, and receptors. Aβ can interact strongly with the lipid bilayer (Terzi, 1997, *89;* Subasinghe, 2003, *90*). Lipid rafts containing a ganglioside cluster catalysate Aβ oligomerization after binding (Kakio, 2003, *91*). The toxic effects of Aβ might be mediated by its binding to or alteration of cell surface receptors and several neuronal membrane proteins (such as the α7-nicotine acetylcholine receptor, the receptor for advanced glycation end products (RAGE), and the p75 neurotrophin receptor, insulin receptor, serpine complex receptor, integrin β1, N-methyl-D-aspartate receptor, APP, collagen-like Alzheimer amyloid plaque component precursor) (Verdier, 2004, *92*). While the internalized Aβ has been shown to bind to numerous vital housekeeping enzymes (Verdier, 2005, *93*).

Therapeutic approaches

Cholinesterase inhibitors (Donepezil (Aricept®), galantamine (Reminyl®) and rivastigmine (Exelon®)) are widely used in the treatment of patients with mild to moderate AD. These drugs lead to an increase of acetylcholine in the brain and stabilize the memory decline for 6 to12 months (Cutler, 2001, *94*).

Depression and anxiety are often concomitant phenomena of AD, hence anti-depressants and anxiolytics may also aid patients. Some of the atypical antipsychotics proved to be effective in the treatment of psychosis and agitation in patients with AD (Street, 2001, *95*). The lipophilic free radical scavenger α-tocopherol (vitamin E) showed some encouraging results especially when combined with ascorbic acid (Bano, 1997, *96*). Estrogens have been shown to modulate neurotransmission, act as free radical scavengers, activate nuclear estrogen receptor in intracellular signaling (Behl, 1999, *97*), prevent Aβ formation by favouring the non-amyloidogenic α-secretase pathway (Xu, 1998, *98*) and in high doses, improve cognition in postmenopausal female AD patients (Asthana, 2001, *99*). Polyphenolic antioxidants seem to modulate AD phenotypes beneficially through multiple Aβ-related mechanisms (Ho, 2009, *100*). Antioxidant therapies were succesful in preclinical studies, but less in human intervention studies or clinical trials (Kamat, 2008, *101*).

The noncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist memantine is a safe and effective symptomatic treatment of AD. It binds to postsynaptic NMDA receptors, preventing glutamate excitotoxicity which leads to massive calcium influx and eventually to cell death (Wilkinson, 2001, *102*), but does not prevent physiological activation of the receptors.

Certain non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen, indomethacin, and fluriprofen may have Aβ-lowering properties in cell cultures and transgenic models of AD (Townsend, 2005, *103*). The selective Aβ-lowering property of NSAIDs arises from an allosteric effect on γ-secretase without affecting their other targets (Beher, 2004, *104;* Lleo, 2004, *105*). These drugs inhibit COX-1 and COX-2, which are involved in the first steps of the synthesis of prostaglandins from the substrate arachidonic acid (Vane, 1998, *106*).

All of the aforementioned approaches represent only symptomatic treatment and do not aim the central cause of the disease, namely the accumulation, misfolding, clearence and aggregation of Aβ. Succesful studies were carried out by immunizing transgenic AD mice with Aβ1-42 leading to the generation of an immune response (Nicoll, 2003, *107*). Passive

immunization with anti-amyloid antibodies resulted in decreased amyloid level in the brain and improved cognitive performance compared to non treated animals. Unfortunately, when active immunization with Aβ was utilized in clinical trials in humans, some of the patients developed a severe meningoencephalitis and the study had to be halted (Hock, 2003, *108*).

The apparent contribution of β- and γ-secretase to the formation of Aβ motivated researchers to aim to block them. BACE1/BACE2 double knockout mice do not show any phenotypic problem (for review, see (Pietrzik, 2005, *109*)) nowadays, some oral administered BACE1 inhibitors appeared to be effective in lowering Aβ in transgenic AD mice and in nonhuman primates (Machauer, 2009, *110;* Sankaranarayanan, 2009, *111*). In contrast, models knocking out γ-secretase have been more problematic behaviorally due to the importance of PS1 in the γ-secretase protein complex and Notch signaling (Kobayashi, 2005, *112*). Notch signaling is of great importance in embryogenesis and in adult life as it regulates hematopoiesis (Suzuki, 2005, *113*) and neurite outgrowth and maintenance (Berezovska, 1999, *114*). Clinical trials of AD patients with LY450139, a γ-secretase inhibitor, showed promising results in animal models, however failed to show a marked reduction in CSF Aβ1-42 (Siemers, 2005, *115*). In the development of specific and effective secretase inhibitors, the BACE1 rout seems to be safer.

The proclaimed culprit molecule in AD, Aβ1-42 undergoes an aggregation and misfolding process. In the course of this process, a conformational change occurs from the soluble α-helical / random coiled structure to a self aggregation-proned β-hairpin structure. Interfaring with this conformational change, whether stabilizing the α-helical structure or destabilizing the β-sheet form, is a promising strategy. It is achievable either by non-peptide and peptide-based compounds, termed β-sheet breakers or amyloid aggregation inhibitors (BSB, AAI (Talaga, 2001, *116*)). The non-peptide BSB's are generally planar molecules with a hydrophobic bi-or triciclic scaffold. There are such natural molecules, as melatonin (Pappolla, 1998, *117*), nicotine (Salomon, 1996, *118*) and curcumin (Ono, 2004, *119;* Yang, 2005, *120*). The artificial ones are 4'deoxy-4'iodorubicin derivates (IDX, (George, 1999, *121*), benzofuran derivates (Howlett, 1999, *122*) and the antibiotic rifampicin (Tomiyama, 1994, *123*). It has been reported, that a sequence in Aβ, (KLVFFA, Aβ16-21) has major role in the self-aggregation process (Hilbich, 1992, *75;* Tjernberg, 1999, *124*). This fragment of Aβ sequence serves as an AAI (Tjernberg, 1996, *125*). The modification of the LVFFA sequence to LPFFD resulted in the interference with the aggregation of Aβ1-42 (Soto, 1996, *126;* Soto, 1998, *127*). The Aβ31-35 fragment also has an important role in the toxicity of Aβ1-42 (Yan, 1999, *128*), while an analogue of Aβ30-34 protects against the Aβ1-42 induced

intracellular Ca^{2+} increasing effect in vitro (Laskay, 1997, *129*). However, non-toxic aggregates exist in mammals, hence these compounds have to be selective to the toxic amyloid assemblies (Fowler, 2006, *130*).

Glutamate receptors

The major excitatory neurotransmitter in the central nervous system (CNS) is the amino acid L-glutamate (Glu). It is a non essential amino acid that is unable to cross the blood-brain barrier (BBB). Glu arises directly from α-ketoglutarate either through transamination of aspartate or by conversion from glutamine. The synthesis takes place in the mitocondrial compartment of Glu-erg nerve terminals (for review see: (Tapiero, 2002, *131*)). Glu is then packaged into synaptic vesicles and may release into the inter-synaptic cleft by highly Ca^{2+}-dependent mechanisms following an action potential (AP). The Glu-flood may activate two large families of receptors: ionotropic and metabotropic Glu receptors (mGluR). The G-protein coupled mGluRs are implicated in synaptic plasticity, excitability and neuronal connectivity (Conn, 1997, *132*). Finally, Glu transporters, termed excitatory amino acid carriers (EEAT's) terminate the neurotransmission by Glu uptake into neurons or glial cells (Shigeri, 2004, *133*).

Ionotropic glutamate receptors pharmacologically diverge from each other by specific binding of antagonist *N*-methyl-D-aspartate (NMDA), kainic acid (KA), and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA). These receptors gate voltage-dependent and voltage-independent currents of Na^+, K^+ and, in some cases, Ca^{2+}.

NMDA receptors are multimeric assemblies of NR1, NR2 and NR3 subunits containing a large amino (N)-terminal extracellular domain, three membrane-spanning domains, a hairpin loop that forms the pore-lining region and an intracelular carboxy (C)-terminal domain. NMDA receptors are higly permeable to Ca^{2+} (in addition to Na^+ and K^+), thus are essential for synaptogenesis, experience-dependent synaptic remodeling and long lasting changes in synaptic plasticity, such as long-term potentiation (LTP) and long-term depression (LTD), while the overactivation of these receptors appears to cause excitotoxiciy (Collingridge, 2004, *134*). Synaptic NMDA receptors are localized in postsynaptic densities (PSDs). A large macromolecular signaling complex of synaptic scaffolding and adaptor proteins links the receptors to kinases, phosphatases and other downstream signaling proteins and to group I mGluRs. PSD protein of 95 kDa (PSD-95) and synapse-associated protein-102

(SAP-102) are synaptic scaffolding proteins that anchor NMDA receptors in the PSD (for review see (Kim, 2004, *135*)). NMDA receptors are blocked by relatively low concentrations of Mg^{2+} via a voltage-dependent manner, thus function as a coincidence detector of presynaptic and postsynaptic firing, and as the trigger of LTP and LTD. The activation of NMDA receptors also requires the concomitant binding of glycine to a specific glycine binding site. The depolarization of the postsynaptic membrane and relief of Mg^{2+} block induce NMDAR-mediated postsynaptic Ca^{2+} influx, that activates kinases, notably Ca^{2+}/calmoduline-dependent kinase II (CaMKII), protein kinase A (PKA), protein kinase C (PKC) and mitogen-activated protein kinase (MAPK), and protein phosphatases. Activated CaMKII phosphorylates the AMPA-type glutamate receptor 1 (GluR1) subunit, which, in turn, promotes synaptic incorporation of GluR1-containing AMPARs, thereby increasing AMPAR number and channel conductance (Collingridge, 2004, *134;* Hsia, 1999, *136*). By contrast, LTD and depotentiation involve dephosphorylation of GluR1 and retrieval of AMPARs from synaptic sites.

KA and AMPA receptors belong to the non-NMDA subclass of ionotropic glutamate receptors, they mediate fast excitatory neurotransmission and are associated primarily with voltage-independent channels that gate a depolarizing current primarily carried by an influx of Na^+ ions (for review see (Monaghan, 1989, *137*)).

AMPA receptor channels comprise of one or any two of four subunits: GluR1-GluR4 forming a nonselective cation channel permeable to Na^+, K^+, and in the case of GluR2 subunit containing receptors, to Ca^{2+} ions (Jonas, 1994, *138*). AMPA receptor mediated currents exhibit fast kinetics with rapid onset, offset and desensitization and considered to be major mechanism for fast excitatory signaling in the brain (Wisden, 1993, *139*). These receptors are widely distributed in the brain, particularly in the hippocampus and olfactory tubercle (Petralia, 1992, *140*).

The KA subclass of receptors are tetrameric, formed from five subunits (GluR5-GluR7 and KA-1-KA-2) and are similar to AMPA receptors regarding ion gating and kinetics (Petralia, 1994, *141*). They present pre-and postsynaptically, have a role in synaptic plasticity and in epilepsy through the strategic control of network excitability (Lerma, 2006, *142*).

Neuronal plasticity

Chemical synapses are functional connections enabling neurons to communicate with each other and to form circuits within the central nervous system. A single neuron can form

synapses up to a number of several thousands and regarding the part of neurons forming the synapse the connection can be termed as axo-dendritic, axo-somatic, axo-axonic or dendro-dendritic synapse. The magnitude of synaptic transmission depends on the strength of the connection which can be altered by the frequency by which the synapses are stimulated in the appropriate temporal window, and by neurotrophic factors and neuromodulators. The ability of synapses to change their strength is known as synaptic plasticity, which may be the cellular basis of learning and memory (Hsia, 1999, *136*).

There are shorter- and longer-lasting forms of synaptic plasticity. For instance, when two action potentials arrive at the same synaptic bouton within several tens or hundreds of milliseconds, the second AP arrives before the intracellular Ca^{2+} level could return to its baseline value. As the release of synaptic vesicules requires Ca^{2+} influx, the second AP triggers more neurotransmitter release. This phenomenon is a short form of synaptic plasticity, termed as paired-pulse facilitation (Steidl, 2006, *143*).

The longer-lasting forms of synaptic plasticity, termed as long term potentiation (LTP) and long-term depression (LTD), are the most studied forms of long-term synaptic plasticity in mammals (Hsia, 1999, *136*). Most of the work in this field has been conducted in the hippocampus, a structure required for memory consolidation. Initially, it was observed that repeated delivery of conditioning stimuli in the dentate gyrus (DG) of hippocampus resulted in synaptic potentiation of greater magnitude and persistence and led to the original description of LTP (Bliss, 1973, *144*). Multiple repetitions of a specific induction paradigm resulted in a protein synthesis-dependent type of LTP [late (L)-LTP], that lasts hours *in vitro* and weeks or month *in vivo*, while single presentation of an induction stimulus eventuated a more decremental and protein synthesis-independent early (E)-LTP (Raymond, 2003, *145;* Nguyen, 2003, *146*). Deep analisys of LTP decay rates and molecular mechanisms revealed three discrete forms of LTP (LTP1, 2 and 3) in the hipposcampus (Raymond, 2003, *145;* Reymann, 2007, *147*). In this nomenclature, LTP1 is identical with (E)-LTP, it is rapidly decaying, independent of protein synthesis and requires post-translational modifications (primarily phosphorylation) of synaptic proteins. LTP2 is considered to be the early phase of L-LTP, depends on protein synthesis but not on gene transcription. However, LTP3 represents the durable, protein synthesis- and gene transcription-dependent form of L-LTP.

The serine/threonine kinases (CaMKII) and (PKC) are implicated in LTP (Chapman, 1999, *148*). CaMKII has a role in both early and late phases of NMDA receptor-dependent LTP (Lisman, 2002, *149*) and seems to be the primary effector for LTP1, while PKC seems to be involved in activating longer-term maintenance mechanisms of LTP (Reymann, 2007,

147). The extracellular signal-regulated kinase (ERK)–mitogen-activated protein kinase (MAPK) pathway also seems to be important for longer-lasting LTP (Winder, 1999, *150;* Rosenblum, 2002, *151*). Phosphoinositide (PI) 3-kinase is also involved in the induction of LTP2 or LTP3 but not in the induction of LTP1 (Raymond, 2003, *145;* Karpova, 2006, *152;* Sanna, 2002, *153*). Albeit, inhibition of PI 3-kinase can reversibly inhibit pre-established LTP1, it does not block induction if applied during the tetanus (Sanna, 2002, *153*)

In the case of LTP2, pre-existing messenger RNA (mRNA) in the dendrites is required for the translation in LTP of intermediate duration (Sutton, 2005, *154*). Protein synthesis-dependent form of LTP has been reported in slices, where the dendrites and somata were phisically isolated (Cracco, 2005, *155;* Tsokas, 2005, *156;* Vickers, 2005, *157*). A translation-dependent form of LTP can be achived in the DG *in vivo* by utilizing a moderate tetanization protocol (Petralia, 1994, *158*). Group I mGlu receptors function through phospholipase C (PLC) to generate Ins(1,4,5)P3 (IP3) and diacylglycerol and are required for LTP2 (Raymond, 2003, *145*). IP3 receptor-mediated Ca^{2+} release is also reported to be necessary for this type of LTP (Raymond, 2003, *145*). Furthermore, activation of mGlu receptors is a major trigger for dendritic protein synthesis (Sutton, 2005, *154*). The increase of Ca^{2+} levels mediated by NMDA receptors or IP3 receptors can activate PKC in discrete dendritic microdomains by working synergistically with diacylglycerol generated by activation of mGlu receptors (Codazzi, 2006, *159*). The previously mentioned ERK-MAPK pathway serves as a downstream target of PKC and is crutial for the regulation of translation in dendrites (Sutton, 2005, *154;* Kelleher, 2004, *160*). By contrast, the PKA-dependent ERK–MAPK stream was important for LTP induced with co-activation of β-adrenoceptors (Shalin, 2006, *161*), indicating the complexity of mechanisms underlying LTP. Similar to the ERK-MAPK pathway, the PI 3-kinase- mTOR (mammalian target of rapamycin) pathway regulates the initial step of translation (Sutton, 2005, *154;* Kelleher, 2004, *160*). One of the main targets of mTOR is p70 S6 kinase, which increases the general translational capacity. Its phosphorylation in dendrites occurs by utilizing multiple tetanization protocol, and this can be inhibited by blocking either PI 3-kinase, mTOR or NMDA receptors (Karpova, 2006, *152;* Cammalleri, 2003, *162*). The trafficking of CaMKII mRNA into synaptodendritic compartments and also its local translation have been reported to be important for the maintenance of LTP triggered by multiple tatanization (Qiu, 1995, *35;* Havik, 2003, *163;* Ouyang, 1999, *164*).

LTP3 requires robust induction protocols (e.g. three or more 100 Hz trains, six to eight TBS (theta-burst stimulation) trains or multiple, brief 200 Hz trains) (Raymond, 2003, *145;* Nguyen, 2003, *146*). In the regulation of neuronal gene transcription, which occurs in LTP3, rise in intracellular Ca^{2+} level is an important signal (Chawla, 2002, *165*). Gene transcription is sensitive to temporal aspects of Ca^{2+} signals and is consistent with the requirement for repetitive HFS (high-frequency stimulation) in the induction of LTP3 (Fields, 2005, *166*). The proper functon of L-type voltage-dependent Ca^{2+} channels (VDCCs) is sufficient for translation- and transcription-dependent forms of LTP (Impey, 1996, *167;* Moosmang, 2005, *168*) and they have role in the activation of cAMP response element (CRE)-mediated transcription (Bading, 1993, *169*). A NMDA receptor- and a L-type VDCC-dependent component of LTP3 seem to exist (Raymond, 2003, *145;* Morgan, 2001, *170*). NMDA receptors can mediate the phosphorylation of CRE-binding protein (CREB), but it is not sufficient to activate transcription (Chawla, 2002, *165*), however NMDA receptors might contribute to transcription via serum response element (SRE) (Bading, 1993, *169*) by activating the ERK–MAPK pathway (Naslund, 2000, *16*). In addition to Ca^{2+}, PKA is another important factor in CRE-mediated gene transcription related to LTP3 (Nguyen, 2003, *146*). Dopamine D_1 and D_5 receptors and β-adrenoreceptors stimulate cAMP production, and hence PKA activation, in the hippocampus and reports indicate the involvement of D_1 and D_5 receptors in the induction of transcription-dependent LTP in CA1 (Reymann, 2007, *147*).

LTD is a persistent, activity dependent decrease of the efficiency in synaptic transmission (Hsia, 1999, *136;* Parent, 1999, *171*) and complementary mechanism of LTP. It can be induced in the CA1 region of the hippocampus by utilizing 1-3 Hz stimulation paradigms (Dudek, 1992, *172*). LTD is dependent upon NMDA receptor activation, modest rise in Ca^{2+} and activation of phosphatases (Dudek, 1993, *173;* Mulkey, 1993, *174*). The participation of $GABA_B$ receptors in young and $GABA_A$ receptors in adult animals in the induction of LTD is also apparent (Wagner, 1995, *175*). Recent studies indicate close relationship between activity-dependent synaptic plasticity (such as LTP and LTD) and strusctural plasticity. Induction of LTP in the hippocampus results in the growth of new spines (Engert, 1999, *176;* Toni, 1999, *177*), eventually to the formation of new synapses (Nagerl, 2007, *178*). In contrast, the induction of LTD leads to retraction of spines on CA1 pyramidal neurons (Nagerl, 2004, *179*).

Aims

In the course of my Ph.D. work, we would have liked to reveal the answers for the following questions from an electrophysiological point of view.

1. Have the different aggregational states of Aβ1-42 distinct effects on rat CA1 postsynaptic ionotropic glutamate receptors?
2. Amyloid β1-42 has an RHDS (Arg-His-Asp-Ser) within its sequence which resembles to RGD (Arg-Gly-Asp) motif of integrin ligands. Thus, are the effects of Aβ1-42 on AMPA and NMDA receptors due to the activation of integrin signaling?
3. If yes, is it possible to interfere in the Aβ1-42-induced activation of integrin signaling?
4. Is an N-protected pentapeptide able to penetrate the blood-brain barrier and to keep its protective effect after intraperitoneal administration?
5. What effect has oligomer Aβ1-42 on synaptic plasticity?

Materials and Methods

Compounds

Aβ1-42, LPYFDa, GRGDS and pentaglycine were synthetized in-house by a solid-phase procedure involving the use of Merrifield resin and Boc chemistry. Purity control and structure proof were carried out by amino acid analysis and mass spectrometry (ESI-MS) (Zarandi, 2007, *180*). The aggregation state of the Aβ1-42 used was verified by transmission electron microscopy (TEM) and dynamic light scattering (DLS) studies. Other compounds used in this study were either purchased from Sigma-Aldrich Corporation (NMDA, AMPA hydrobromide, Pontamine Sky Blue (PSB) and chloral hydrate, (St. Louis, MO, USA)), from Calbiochem (4-Amino -5-(4-chlorophenyl) -7-(t-butyl) pyrazolo [3,4-d] pyrimidine (InSolution™ PP2), 4-Amino-7-phenylpyrazol [3,4-d] pyrimidine (PP3)), or from Chemicon (anti-β1 integrin, MAB1987Z; anti-α2 integrin, MAB1950Z).

In vivo single-unit recordings and iontophoresis

The head of chloral hydrate-anesthetized male Wistar rats weighing 280-330 g was mounted in a stereotaxic frame, the skull was opened above the hippocampus (antero-posterior coordinates: -2.8 to -3.8 from bregma; lateral: 2 mm on either side from the midline), and the dura mater was carefully removed. The location of the electrode was verified by iontophoretic Pontamine Sky Blue ejection (-5 μA for 15 min) followed by conventional histology. The principles of laboratory animal care (NIH publication No. 85-23) and the protocol for animal care approved by the Hungarian Health Committee (1998) and the European Communities Council Directive of 24 November 1986 (86/609/EEC) were followed.

Single-unit activity was recorded extracellularly by means of a low-impedance (< 1 MΩ) 7 μm carbon fiber-containing microelectrode (Kation Scientific, Minneapolis, MN; Fig.3. (Szegedi, 2005, *181*)). The action potentials were amplified (Szegedi, 2005, *181;* Szegedi, 2005, *182*), filtered, and then monitored with an oscilloscope. The filter bandpass frequencies were 300 to 8000 Hz. A window discriminator (WD-2, DAGAN, Minneapolis) was used for spike discrimination on the basis of spike amplitude and duration. The amplified signals were sampled and digitalized at 50 kHz. The number of action potentials per second was counted by the computer and peristimulus time histograms were calculated, displayed in line and digitally stored for off-line analysis (DataWave SciWorks Version 5.1). Iontophoretic

drug delivery and experimental data collection were performed by a multifunction instrument control and data acquisition board PCI-1200 (National Instruments, Austin, Texas, USA) placed in a computer, programmed in LabVIEW 6, and by iontophoretic pumps (Minion-16 and BAB-350, Kation Scientific).

The drug barrels of the combined recording/iontophoresis electrode contained one of the following freshly made solutions: 100 mM NMDA Na salt (pH 8.0, Sigma), 10 mM AMPA hydrobromide (pH 8.0), the integrin ligand pentapeptide Gly-Arg-Gly-Asp-Ser (GRGDS) or the negative control pentaglycine (GGGGG, pH 6.4) either in the concentration of 5 mM or 50 mM; 0.1 mg/ml 4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (InSolution™ PP2, Calbiochem), 0.1 mg/ml 4-Amino-7-phenylpyrazol[3,4-d]pyrimidine (PP3, Calbiochem), 0.1 mg/ml anti-integrin antibodies (anti-β1 integrin, MAB1987Z; anti-α2 integrin, MAB1950Z; pH 9, Chemicon) and 50 μM Aβ1-42 sample (pH 6.4) either in fibrillar or oligomer form and to mark the position of the electrode, 4% Pontamine Sky Blue (PSB). Aβ1-42 containing samples were sonicated (Merck Eurolab 120 W apparatus) for 15 min prior to use.

NMDA and AMPA were ejected with negative iontophoretic currents ranging from 10 to 100 nA. Retaining currents in the interval 2–16 nA of opposite direction were used. The current levels were selected during recording the control so that the spiking rate was to reach ~30-80 spike/sec. In that way, the rate of amyloid or GRGDS induced excitation was not dependent of the initial firing rate. Following a stable control sequence, Aβ1-42 was ejected at -380 nA for 60 sec, GRGDS or GGGGG was ejected for 3 min with +100 nA, while PP2 and PP3 were ejected at +100 nA for 2 mins and the anti-integrin antibodies were ejected at -100 nA for 2 mins. Cells were excited by alternating repetitive ejection of NMDA and AMPA and the interval between two excitation epochs was 120 s. In a set of experiments, in order to minimize the possible interaction between NMDA and AMPA receptor function, cells were excited by the ejection of either NMDA or AMPA alone. At this case, NMDA by itself was repetitively ejected every minute, whereas AMPA alone was repetitively applied in every 90 sec.

Recording sites were marked by the iontophoretic ejection of PSB at a negative current of 5 μA for 10 min. At the end of each experiment, the animals were euthanized with an overdose of chloral hydrate. The brain was quickly removed and fixed in 4% paraformaldehyde. 50 μm thick brain sections were counterstained with Neutral Red, and the PSB localization was verified according to the stereotaxic atlas of Paxinos and Watson (1986).

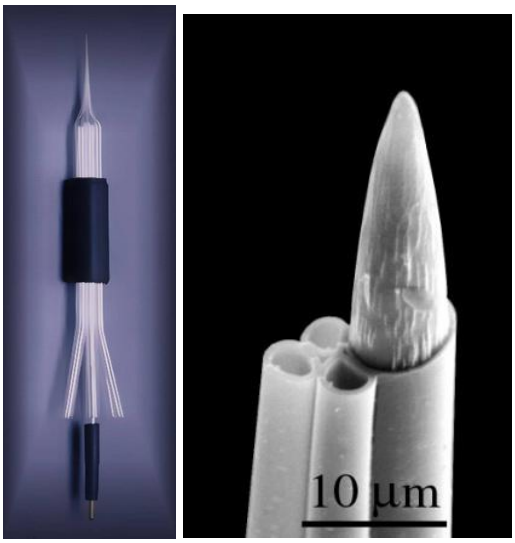

Fig. 3. The four barrel Carbostar 4 carbon fiber combination electrodes are for extracellular recording and microiontophoresis. One barrel contains the carbon fiber as the recording element, the other three barrels are for microiontophoresis. Ultrastructures of a Carbostar 4 electrode as revealed by scanning electron microscopy. Courtesy of Kation Scientific.

Ex vivo Electrophysiological recordings and stimulation protocols

Using standard procedures, 350 µm thick transverse acute hippocampal slices were prepared from the brain of 6 months old mice using a McIlwain tissue chopper (Campden Instruments, Loughborough, UK). Slices were incubated in carbogenated preparation solution at ambient temperature for 60 min, and then transferred to carbogenated standard ACSF (pH 7.4) that contained the followings in mM: NaCl, 130; KCl, 3.5; $CaCl_2$, 2; $MgCl_2$, 2; NaH_2PO_4, 0.96; $NaHCO_3$, 24; D-glucose, 10. Individual slices were transferred to a 3D-MEA chip with 60 tip-shaped electrodes (30 µm in diameter and 25 - 35 µm in height, spaced by 100 µm; purchased from Ayanda Biosystems, S.A., Lausanne, Switzerland; Fig. 4.). The surrounding solution was removed quickly and the slice was immobilized by a grid. The slice was continuously perfused with carbogenated standard artificial cerebrospinal fluid (ACSF) (1.5 ml/min at 34 °C) during the whole recording session. Data were recorded by a standard, commercially available MEA (multi-electrode array) setup (Multi Channel Systems MCS GmbH, Reutlingen, Germany).

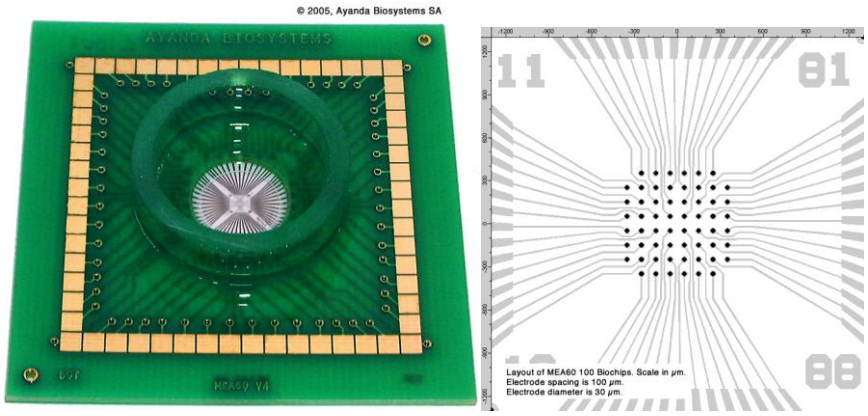

Fig. 4. A MEA60 100 3D biochip and its shematic electrode array.
Ayanda Biosystems SA

The Schaffer-collateral was stimulated by injecting a biphasic current waveform (± 100 µs) through one selected electrode at 0.033 Hz. The positioning of the stimulating electrodes and that of the regions in the slices, compared to each other, were constantly synchronized during the various investigations. The peak-to-peak amplitudes of field excitatory postsynaptic potentials (fEPSPs) at the distal and proximal part of stratum radiatum of CA1 were analyzed. After a 30 min incubation period, the threshold and the maximum of stimulation intensity for evoke responses was determined. For evoking responses, 30 % of the maximal stimulation intensity was used. When stable evoked fEPSPs were detected (for at least 20 min), the perfusion system was set to recycled and 1 µM oligomer amyloid was added into ACSF, than the wash-in period of amyloid was followed for an hour. Than a stimulus strength–evoked response curve (i.e. input–output, I–O curve) was recorded by gradually increasing stimulus intensity until the maximal stimulus strength was reached. The stimulus intensity was continuously increased from 0 to 100 µA with 10 µA steps. Stronger stimulation led to large Faradic effects on the electrodes causing artifacts. 3 data sets were recorded at each stimulation intensity. After I-O curve recordings, a paired-pulse facilitation (PPF) protocol was applied by administering two identical biphasic current waveform with 50 ms interval, repeated three times at 0.033 Hz. Following a stable 15-min control sequence after PPF protocol, LTP was induced, using a theta-burst stimulation (TBS) protocol applied at the maximum stimulation intensity. TBS comprised of 15 trains administered at 5 Hz, the individual trains contained 4 pulses separated by 10 ms. LTP was followed for 180 min. Finally, the depotentiation ability of the synapses was determined applying 3 Hz stimulation intensity (low-frequency stimulation, LFS) for five min, than fEPSPs were recorded for half an hour.

Data analysis

Statistical evaluation was performed by using the total number of spikes evoked during each epoch of excitation by the iontophoretic application of NMDA. Differences in magnitude between the different response epochs of a single cell were confirmed by one-factor analysis of variance (ANOVA, with the Bonferroni test for *post hoc* analysis) by comparing the total numbers of spikes per excitation period. The mean of NMDA- or AMPA-evoked responses before the application of any of the compounds mentioned before was taken as 100% in every experiment. The maximum response rate after application was given as percentage of the control. A p value <0.05 was considered significant.

Recordings obtained after iontraperitoneal (ip) application of Leu-Pro-Tyr-Phe-Asp-amide (LPYFDa) and GGGGG were divided into 9 groups based on the time elapsed between Aβ1-42 application and administration of the pentapeptides. Data of 40-min intervals were pooled and means ±SEM (standard error of means) of percentage values were calculated. p values <0.05 were considered significant in all cases. Only cells showing no or only a very low basal activity (a few spikes/s at most) were selected for recording. The pre-Aβ1-42 control evoked firing rate was set between ~30-80 spike/sec. In that way, the rate of amyloid induced excitation was not dependent of the initial firing rate. The pre Aβ1-42 firing values were considered as 100% at each recording, and the maximum post Aβ1-42 firing rates in % value were used to statistical evaluations.

For the statistical analysis of *ex vivo* recordings, the peak-to-peak amplitude of evoked fEPSPs recorded from the proximal part of stratum radiatum was calculated. For PPF recordnigs, the ratio of the $2^{nd}/1^{st}$ fEPSPs was calcualted, evoked by two symmetric bipolar currents following each other by 50 ms. The level of LTP and depotentiation were compared to the average of the last 10 peak-to-peak amplitude of evoked fEPSPs before inducing TBS or LFS, respectively. Data from Aβ1-42-treated and control slices were compared using Two Sample independent t-Tests and p values <0.05 were considered significant in all cases.

Results

In vivo single-unit recordings

Effects of fibrillar and oligomeric Aβ1-42 on NMDA and AMPA elicited firing *in vivo*

Data of 55 cells was obtained from 32 anaesthetized rats to study the effects of different aggregational states of Aβ1-42 on NMDA and AMPA induced firing. Hippocampal CA1 neurons were excited by alternating repetitive ejection of NMDA or AMPA. Following a stable control sequence, Aβ was ejected for 1 min with -0.38 μA (Fig. 5.A and B and Fig. 6.).

Iontophoresis of fibrillar Aβ1-42 onto CA1 neurons gradually enhanced the NMDA-elicited responses up to a level of 260 ± 28% (n = 12). The enhancement was significant after 5-7 minutes of Aβ application and endured throughout the time frame of the experiment. Nevertheless, the level of AMPA evoked firing decreased in all of the recorded cells. The elicited responses continuously decreased, then virtually disappeared, reaching only 9.5 ± 5.5% of the control level (n = 12). A rapid fade out of the AMPA-responses was observed in the case of 3 cells while the escorting NMDA-response enhancement remained, clearly demonstrating the excitability of the cell. It should be noted, that 3 cells did not show any change in the NMDA induced activation after fibrillar Aβ1-42 ejection, although a slight decrease in the AMPA triggered firing rate emerged (data not shown).

The oligomeric form of Aβ1-42 was tested on 23 cells recorded from 10 rats. The ejection of oligomer Aβ1-42 containing solution caused similar increase in NMDA elicited neuronal firing as the fibrillar one, reaching a level of 207 ± 14% (n = 8). The enhancement followed the same temporal pattern, and was proved to be permanent as in the case of fibrillar Aβ1-42 application. Unexpectedly, the AMPA-elicited responses also showed a less pronounced, yet significant increase compared to control level (182 ± 10%; n = 8). Two cells did not display any significant change either in NMDA or AMPA induced responses after oligomer Aβ1-42 ejection.

In order to exclude the possible interaction between NMDA and AMPA receptor activation, a second set of experiments were conducted by using only either NMDA or AMPA alone. NMDA by itself was repetitively ejected in every minute for 5 sec, and AMPA alone was repetitively applied every 90 sec for 5 sec. Under these circumstances, the effects of Aβ1-42 in either small n- or fibrillar aggregation states did not differ from the effects described

above. Either fibrils or oligomers enhanced NMDA-responses (254 ± 16%, n = 8 ; 231 ± 18%, n = 7 respectively), meanwhile the AMPA-induced firing significantly attenuated (fibril application, 11 ± 5%, n = 9), or increased (oligomer application, 193 ± 23%, n = 6 , data not shown). The changes in firing rates followed analogous temporal pattern as was seen previously.

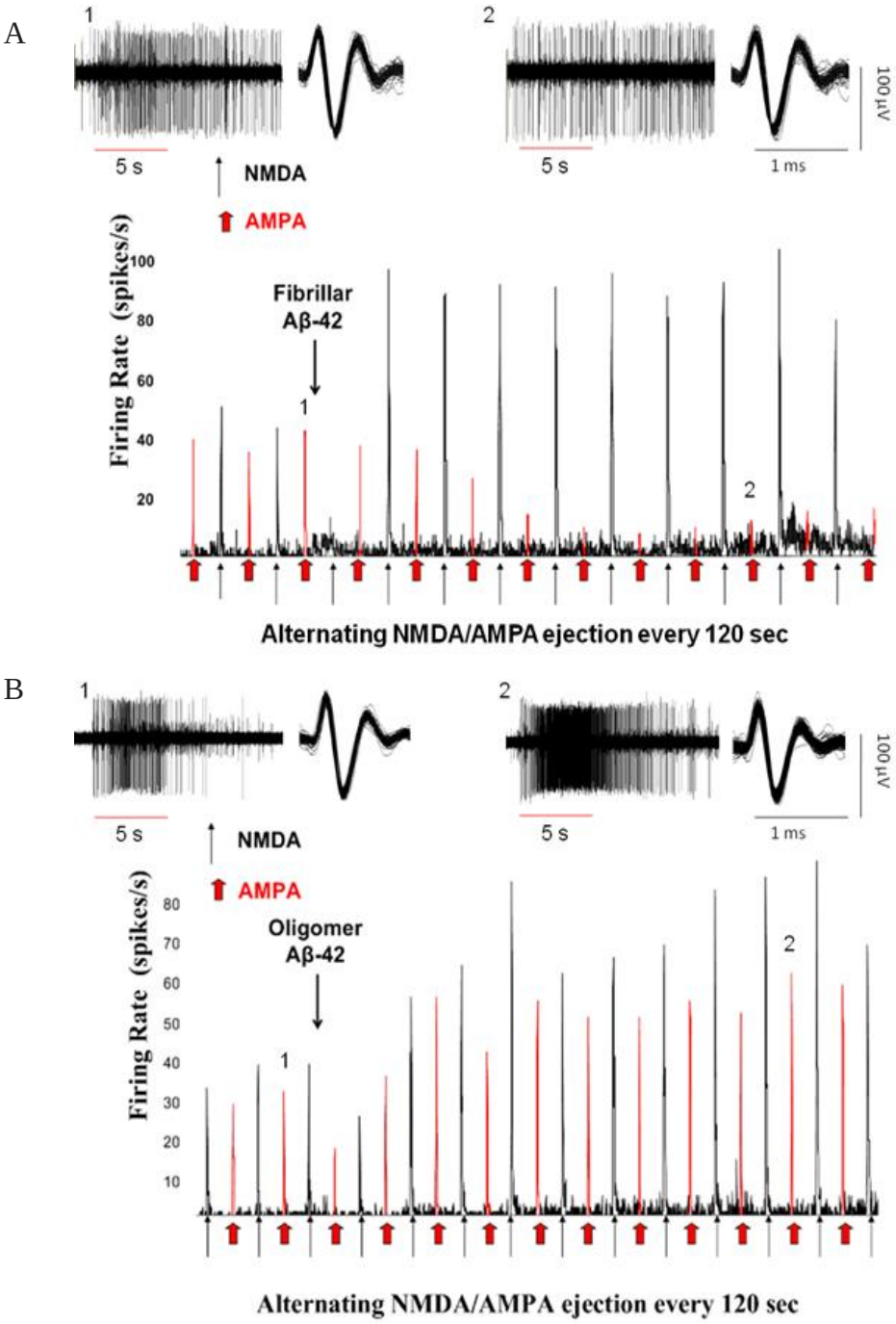

Fig. 5.: Representative peristimulus time histograms displaying the effects of A fibrillar and B oligomeric Aβ1-42 on NMDA and AMPA elicited neuronal firing *in vivo* in the hippocampal CA1 region. NMDA and AMPA were ejected for 5 sec at -54 nA and -67 nA (Panel A) or at -62 nA and -47 nA (Panel B) respectively. Aβ1-42 samples were applied for 1 min at 0.38

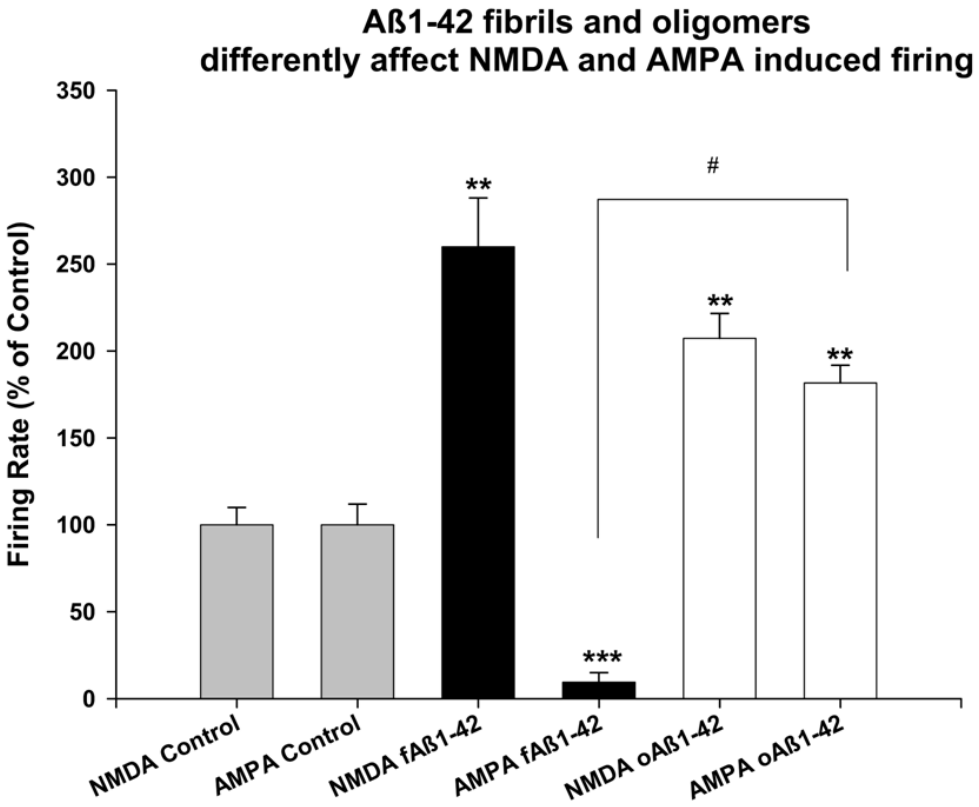

Fig. 6.: Summary of the effects of fibrillar and oligomer Aβ1-42 on NMDA and AMPA-evoked responses measured by *in vivo* single-unit electrophysiology, normalized by the control data. Asterisks indicate significant differences from control (ANOVA $P < 0.05$). n = 12, 9, 8, 8 respectively.

Effects of GRGDS on NMDA and AMPA-induced neuronal firing

Data of 52 CA1 cells from 30 anaesthetized rats were used to study the effects of high and low concentration of GRGDS and GGGGG peptides on NMDA and AMPA induced firing. Only cells showing no or only a very low basal activity (a few spikes/s at the most) were selected for recording. The applications of the peptide-containing samples were carried out after setting a stable control response for both NMDA and AMPA application.

Following the ejection of 5 mM GRGDS, NMDA responses gradually enhanced up to a level of 268 ± 36% ($P \leq 0.05$; n = 11; Fig. 7.). The enhancement was significant after 6- 8 minutes of peptide application, and proved to be permanent within the time frame of the experiments. In contrast, the level of AMPA evoked firing significantly attenuated in all of the recorded cells. Characteristically, the responses continuously decreased, reaching only 19 ± 6% of the control level ($P \leq 0.05$; n = 11; Fig. 7.).

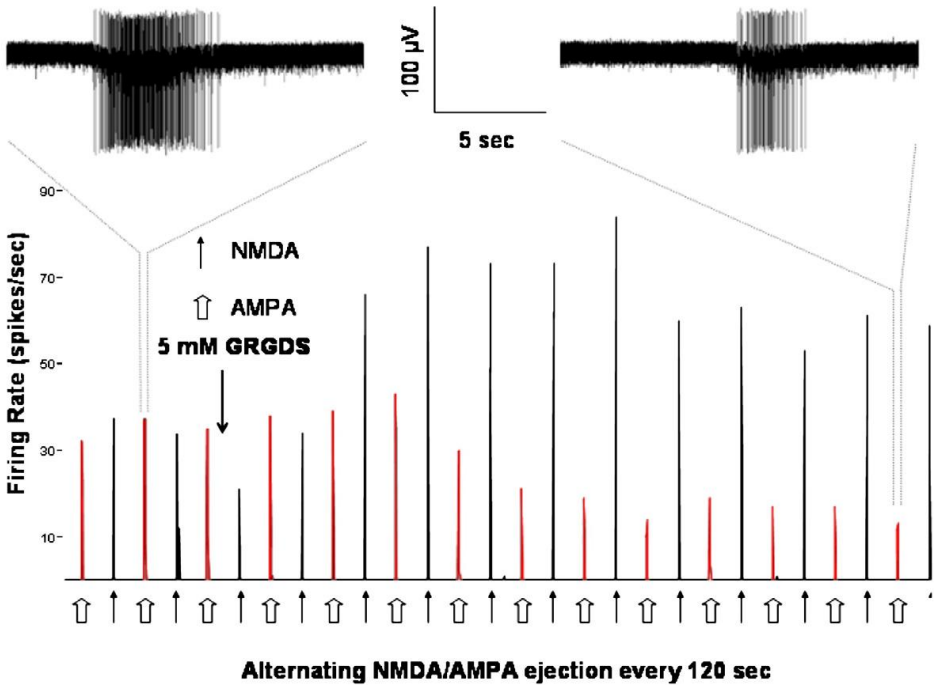

Fig. 7.: A representative peristimulus time histogram showing the effects of low (5 mM) concentration GRGDS peptide on NMDA and AMPA induced neuronal firing of a CA1 neuron. NMDA was ejected at -24 nA, while AMPA was applied with -83 nA.
Insets show the spikes elicited by AMPA ejection before and after 5 mM GRGDS application.

High GRGDS concentration (50 mM) was tested on 8 cells recorded from 4 rats. The change in NMDA elicited neuronal firing was similar to the effect of low GRGDS induced one. Responses were gradually increased after the application of high GRGDS concentration, reaching a level of 301 ± 41% ($P \leq 0.05$; n = 8; Fig. 8.). The enhancement followed the same temporal pattern, and was proved to be permanent as in the case of the application of low GRGDS concentration. Unexpectedly, AMPA responses also increased, although, the increment was less pronounced, yet significant compared to control level (226 ± 59%; $P \leq 0.05$; n = 8; Fig. 8.). In order to exclude the possible interaction between NMDA and AMPA receptor activation, a second set of experiments were conducted by using only either NMDA or AMPA alone. NMDA by itself was repetitively ejected in every minute for 5 sec, and AMPA alone was repetitively applied every 90 sec for 5 sec. Under these circumstances, the effects of GRGDS in either low or high concentration did not differ from the effects described above. Both 5 mM and 50 mM concentration of GRGDS enhanced NMDA-responses (284 ± 16%, $P \leq 0.05$, n = 5; 231 ± 18%, $P \leq 0.05$, n = 5 respectively), however, AMPAinduced firing was significantly attenuated (5 mM GRGDS, 23 ± 9%; $P \leq 0.05$; n = 5), or increased

(50 mM GRGDS, 231 ± 44%; P ≤ 0.05; n = 5, data not shown). All changes followed the same temporal pattern as was seen previously.

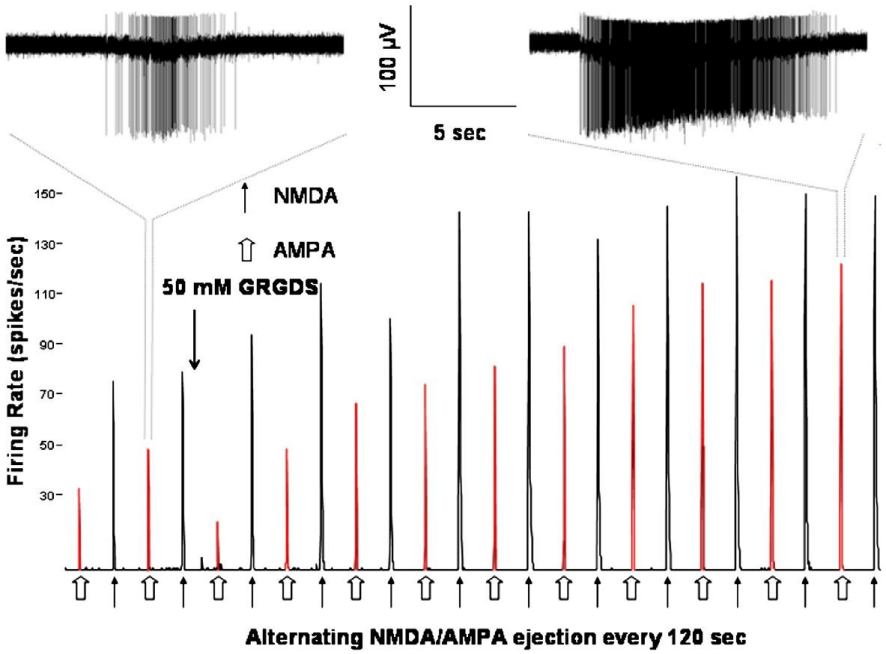

Fig. 8.: A representative peristimulus time histogram showing the effects of high (50 mM) concentration GRGDS peptide on NMDA and AMPA induced neuronal firing of a CA1 neuron. NMDA was ejected at -62 nA, while AMPA was applied with -59 nA.
Insets show the spikes elicited by AMPA ejection before and after 50 mM GRGDS application.

Effects of GGGGG on NMDA and AMPA-induced neuronal firing

We utilized pentaglycin solutions as negative control. Neither the low concentration (5 mM) nor the high concentration (50 mM) GGGGG sample modulated the evoked firing rate (Fig. 9.). Following the ejection of 5 mM pentaglycin, both the NMDA- and AMPA-evoked firing rate remained at control level (95 ± 13%, 106 ± 8%, n = 6). The application of 50 mM pentaglycine did not alter the rate of evoked responses (106 ± 14% for NMDA, and 95 ± 16% for AMPA; n = 7).

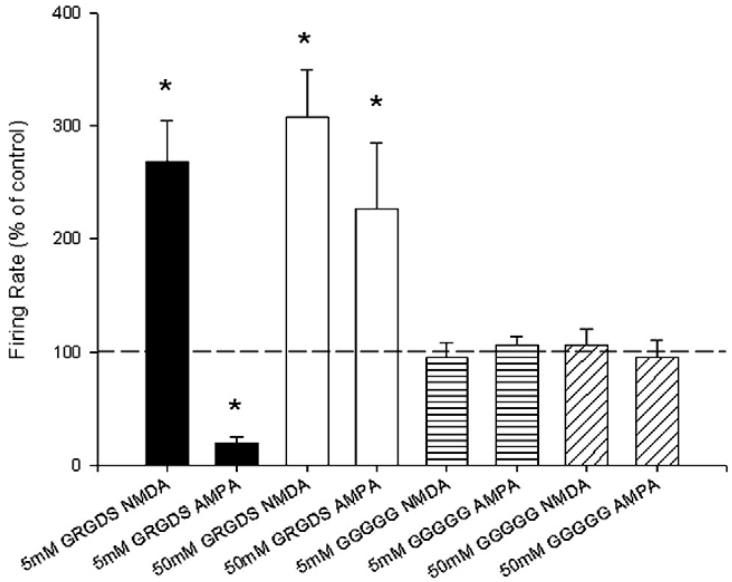

Fig. 9.: Summary of the effects of low and high concentration of GRGDS and GGGGG peptide on NMDA and AMPA triggered neuronal firing. * denotes significant difference from control (100%). $P \leq 0.05$, ANOVA.

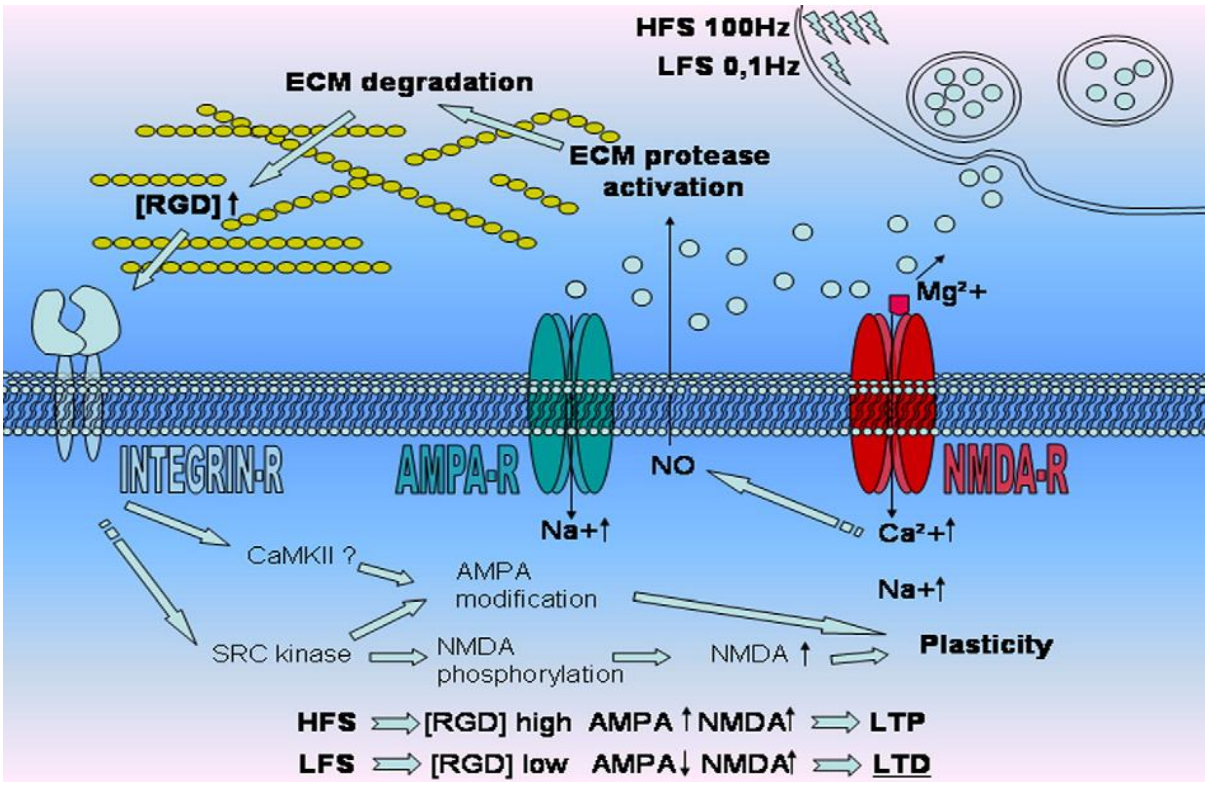

Fig. 10.: A proposed hypothesis of LTP and LTD stabilization by the integrin pathway. LTP is commonly induced by high frequency stimulation (HFS), which leads to massive Ca^{2+} influx to the postsynapse, and consequently highly increased NO production. On the other hand, the common LTD inducing protocol uses low frequency stimulation (LFS) of the presynapse, which is accompanied by slow intracellular Ca^{2+} fluxes, and much less NO production. NO can activate extracellular proteases, which partially degrade extracellular matrix (ECM) molecules, liberating RGD containing sequences. These RGD boxes may bind to integrins, activating the downstream signaling pathways, affecting NMDA and AMPA receptor efficiency depending on the scale of integrin activation.

Aβ1-42 enhances NMDA receptor sensitivity by activating the integrin signaling pathway

It was described above, that aggregated Aβ1-42 enhances NMDA evoked neuronal firing in the CA1 region *in vivo*. Indeed, after the application of fibrillar Aβ1-42 (fAβ1-42), NMDA elicited neuronal firing increased reaching the zenith at 248 ± 21% in 10 recordings out of 13. However, ejection of the anti-β1 integrin antibody before fAβ1-42 application eliminated the NMDA response enhancement-affect of fAβ1-42. Responses remained at the control level, reaching a maximum of 108 ± 11% (n = 10). Contrary to anti-β1 integrin antibody, application of the anti-α2 one was unable to prevent the increase in NMDA triggered firing in 7 out of 8 recordings (maximum of 246 ± 25%, n = 7). Figs 11. and 12. show two representative recordings. The A panels (perievent histograms of spiking) clearly show the slowly emerging fAβ1-42 induced NMDA response enhancement, on which α2-integrin antibody did not have any effect, while β1-integrin antibody prevented this phenomenon. The presented spike trains indicate that single-units were recorded. The color coded peri-event time histogram with 100 ms bin (Panel B) and the average of the firing rate of 5 successive trials before and 30 min after fAβ1-42 application shows that the responses gradually became wider and higher, suggesting that the peptide affects NMDA receptor kinetics. Interestingly, the onset of spiking did not change. The red line represents the onset of 5 sec NMDA ejection. Again, β1-integrin antibody prevented this slowly emerging increase in spiking response. Panels C display the average spiking rates of 5 NMDA ejection before and 20 min after amyloid-beta application. Both the widening and the spike rate enhancement after amyloid-beta ejection are clearly seen at the α2-integrin antibody treated recording. On the other hand, β1-integrin antibody treatment protected against these effects of fAβ1-42.

It was also examined, whether the Src tyrosine kinase family, activated by integrins, contributes to the fAβ1-42 excitatory effect on NMDA receptors. On behalf of this, a speific cell permeable inhibitor of the Src kinase family, PP2 was ejected before fAβ1-42 ejection. Responses reached a maximum of 98 ± 4% (n = 9). The EGFR tyrosine kinase inhibitor, PP3 was used as control and it did not modify the rate of NMDA response enhancement after fAβ1-42 ejection in 6 out of 8 recordings (a maximum of 267 ± 31%, n = 6). These compounds alone had no effect on the NMDA evoked responses within the time frame of the experiments: ejecting either anti β1- (108 ± 11%, n = 5) or anti α2-integrin antibody (98 ±

10%, n = 5). Similarly, neither the Src tyrosine-kinase inhibitor PP2 (94 ± 8%, n = 6), nor the EGFR tyrosine kinase inhibitor PP3 (109 ± 8%, n = 6) affected NMDA responses.

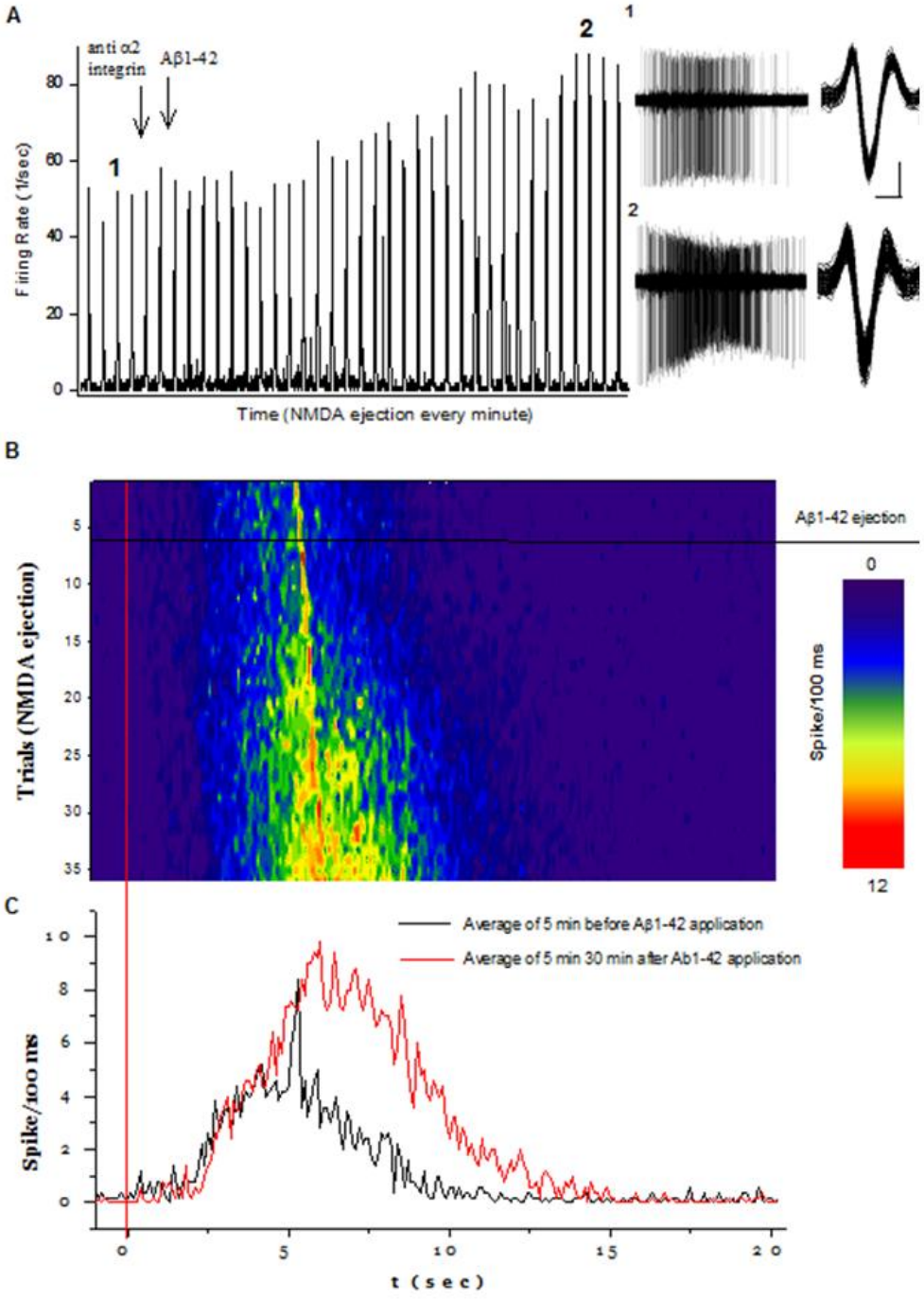

Fig. 11.: Representative data of the effects of anti-α2 integrin pretreatment on Aβ1-42 induced enhancement of NMDA-evoked spiking activity. Peristimulus histogram (Panel A) shows the sluggish, but significant increase of evoked spiking rate of a CA1 neuron after Aβ1-42 application. The color-coded perievent histogram with 100 ms bins (Panel B) displays the widening and amplitude-increase of evoked responses. The averaged perievent histogram of 5 NMDA application (Panel C) shows remarkable difference between the spiking distribution of the first 5 min (pre-amyloid recording) and 30 min after Aβ1-42 treatment. Calibrations 60 μV and 0.8 ms. The displayed spike trains are 15 sec long. Red line denotes the onset of 5-sec NMDA ejection.

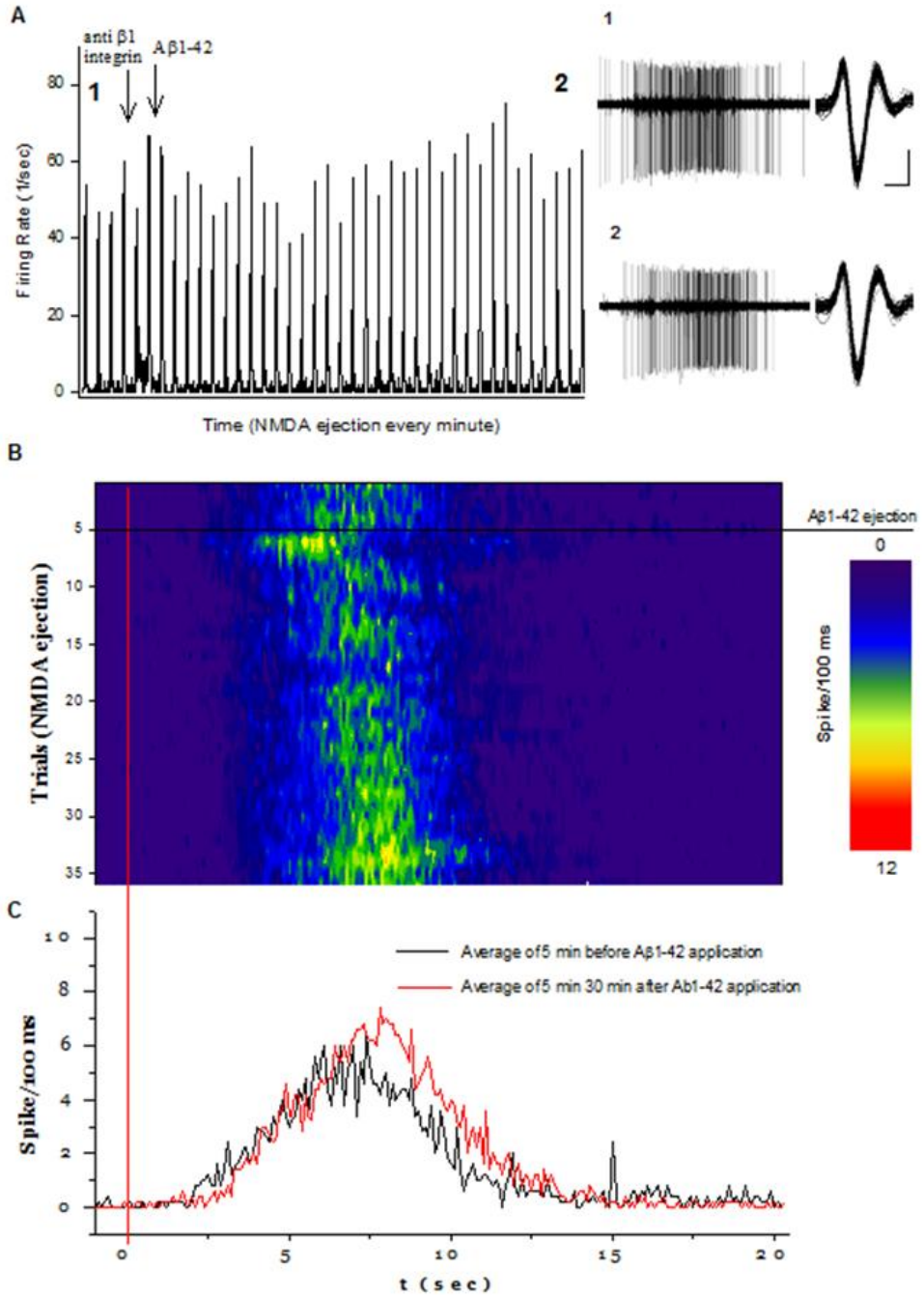

Fig. 12.: Representative data of the effects of anti-β1 integrin pretreatment on Aβ1-42 induced enhancement of NMDA-evoked spiking activity. Peristimulus histogram (Panel A) shows no significant increase of evoked spiking rate of a CA1 neuron after Aβ1-42 application. The color-coded perievent histogram with 100 ms bins (Panel B) displays the unchanged pattern of evoked responses. The averaged perievent histogram of 5 NMDA application (Panel C) shows no significant difference between the spiking distribution of the first 5 min (pre-amyloid recording) and 30 min after Aβ1-42 treatment. Calibrations 60 μV and 0.8 ms. The displayed spike trains are 15 sec long. Red line denotes the onset of 5-sec NMDA ejection.

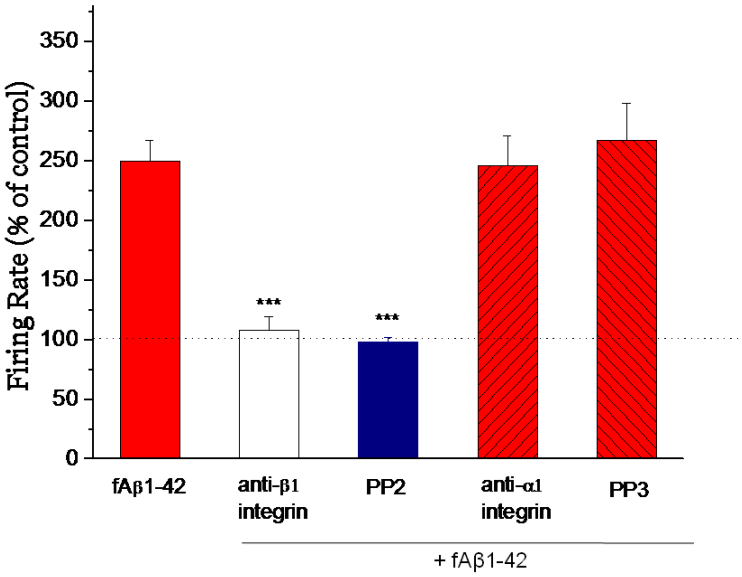

Fig. 13.: Summary of the maxima of NMDA evoked spiking rate. Anti-β1 integrin antibody and PP2 pretreatment prevented the excitatory effect of Aβ1-42. Compounds used for control purposes (anti-α1 integrin antibody and PP3) were not effective in interfering with Aβ1-42 induced effect. *** denotes significant difference compared to fAβ1-42 data at $P \leq 0.001$ (Student's paired t test).

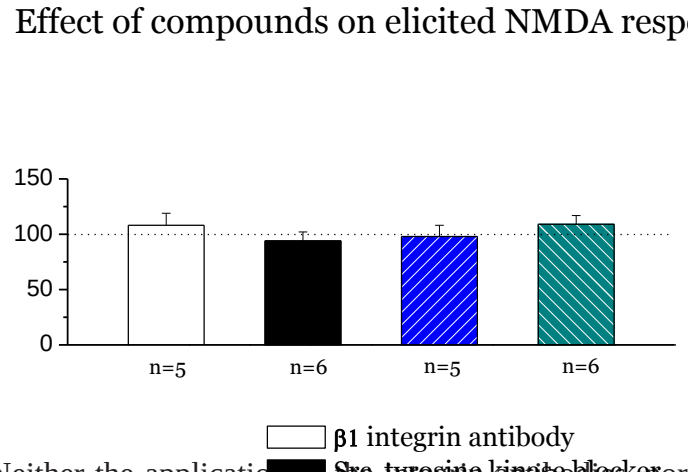

Fig. 14.: Neither the application of the integrin antibodies, nor the tyrosine kinase inhibitors have any significant effect on elicited NMDA responses.

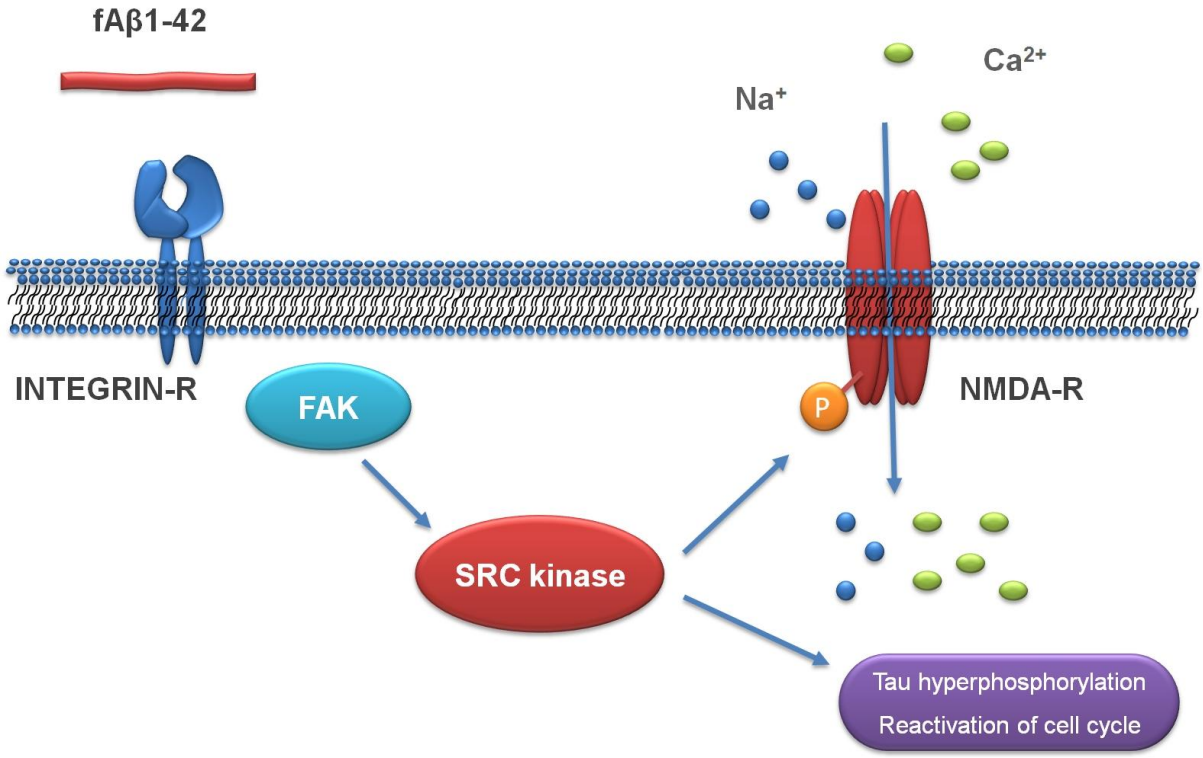

Fig.15.: Proposed mechanism of fAβ1-42 induced NMDA receptor enhancement. Fibrillar amyloid-beta is recognized as an extracellular matrix component by the β1 subunit containing integrins. Upon binding, the integrin activated molecular cascade initiates, which involves focal adhesion kinase (FAK), and subsequent src kinase activation, leading to the phosphorylation of NMDA receptors. Increased phosphorylation of NMDA receptors results in enhanced Ca^{2+} influx and eventually neuronal death. Overactivation of Src kinase may cause tau hyperphosphorylation and reactivation of cell cycle.

Intraperitoneal administration of LPYFDa and *in vivo* single-unit recordings

Extracellular single-unit recordings were obtained from 72 CA1 neurons from a total of 32 anesthetized rats. The recordings made between 0 and 40 min after the ip administration of LPYFDa revealed a robust elevation of the NMDA-evoked firing frequencies, which peaked at 210 ± 16% (n = 4) following fAβ1-42 administration as compared with the pre-fAβ1-42 control. At 40-80 min after LPYFDa administration, the NMDA-evoked peaks were 190.9 ± 9% (n = 4) relative to the control level (Fig. 16.A1). In contrast, there was not a significant elevation at 80-120, 120-160, 160-200 or at 200-240 min following ip LPYFDa administration (136.7 ± 11%; 120 ± 14%; 120.6 ± 12%; and 125.4 ± 11%, respectively; n = 4 for all data; Fig. 16.A2). The recordings at 240-280, 280-320 and > 320 min after ip administration again demonstrated the excitatory effect of fAβ1-42, with changes of 200 ± 15%; 165 ± 14% and 169.2 ± 16%, respectively (n = 4 for all data; Fig. 16.A3).

The recordings from GGGGG-treated animals exhibited a pronounced enhancement in each time of these time intervals (216 ± 11%; 200 ± 15%; 224 ± 8%; 206 ± 14%; 195 ± 17%; 216 ± 9%; 208 ± 16%; 186 ± 8% and 176 ± 13%, respectively; n = 4 for all data; Fig 16.B1, B2, B3).

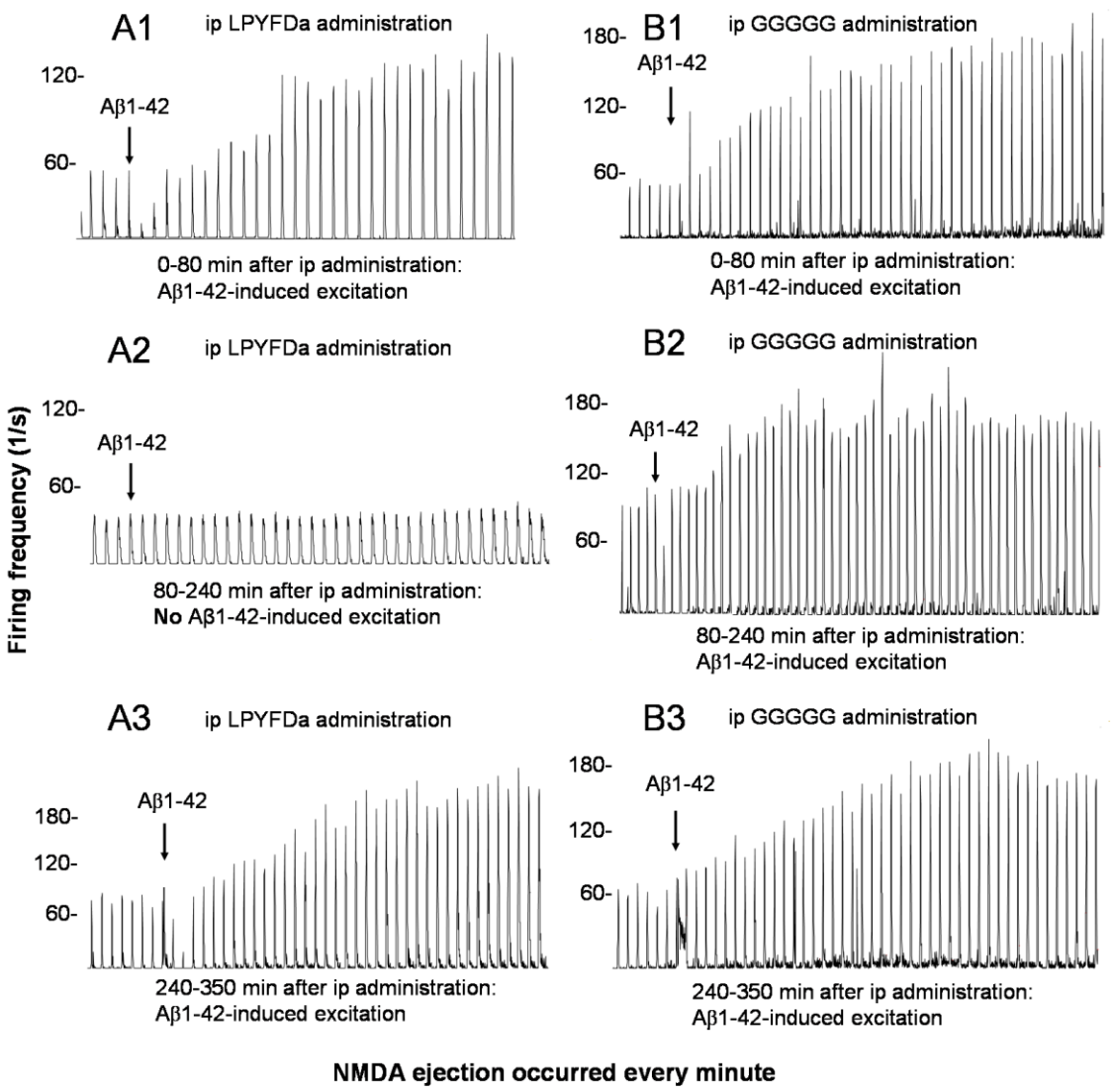

Fig. 16.: Representative peristimulus histograms of hippocampal CA1 neurons recorded after ip administration of either LPYFDa (column A) or GGGGG (column B). Arrows denote the ejection of fAβ1-42. The three different time intervals are denoted 1, 2 and 3, respectively. Interval 1 means 0-80 min after ip administration, where Aβ1-42 induced excitation could be observed. Interval 2 means 80-240 min after ip administration, where LPYFDa prevented Aβ1-42 induced NMDA response enhancement, while the control peptide, GGGGG did not protect. Interval 3 means 240-350 min after ip administration, where Aβ1-42 induced excitation could be observed again. One single-unit recording lasted ~35-45 min. The control evoked responses were set between ~30-80 spikes/sec. In that way, the rate of excitatory effect of Aβ1-42 was independent of the initial value of control firing rate. The pre Aβ1-42 firing values were considered as 100% at each recording, and the maximum post Aβ1-42 firing rates in % value were used to statistical evaluations.

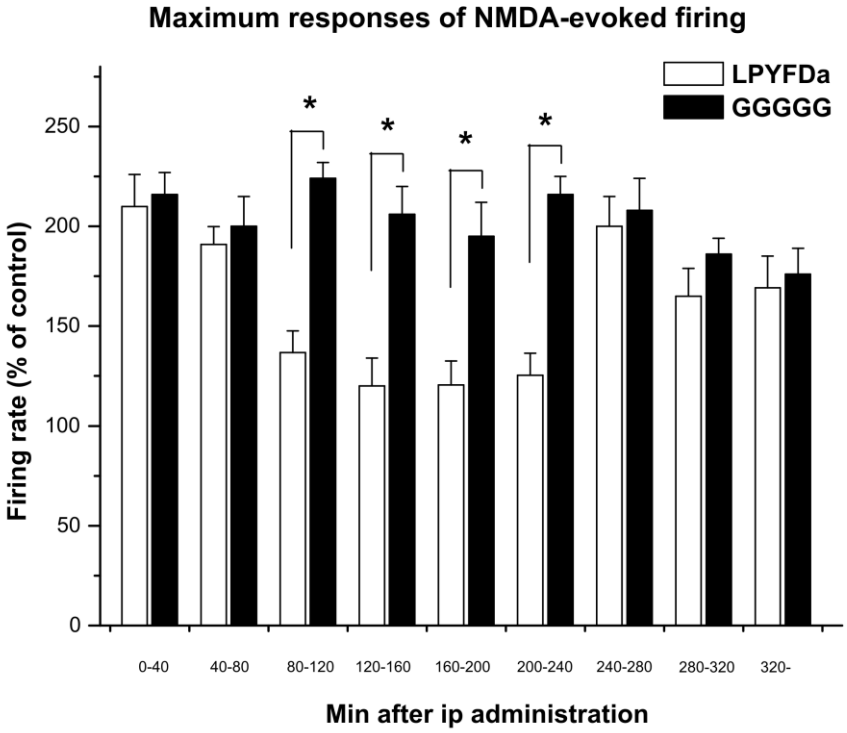

Fig. 17.: Mean of maximum NMDA-evoked responses, normalized by the control data (the total spike number during each excitation epoch before Aβ1-42) ± SEM. LPYFDa protected against the NMDA response-enhancing effect of Aβ1-42 between 80 and 240 min after ip administration of the pentapeptide. The control pentapeptide, GGGGG, did not interfere with the Aβ1-42-induced excitation. Asterisks indicate significant differences (Student's *t* test $p < 0.05$). n = 4 for all data.

Ex vivo electrophisiologycal recordings with multi-electrode array (MEA)

We used MEA electrodes to record evoked fEPSPs from CA1 hippocampal acute mice slices by choosing one of them as a stimulating electrode, while the rest of them could be used as recording electrodes. The peak-to-peak amplitudes of fEPSPs were analyzed from the proximal part of stratum radiatum.

At first, we performed I-O curve recordings to determine the excitability of neurons because we were not able to avoid the increase of fEPSP amplitudes in the wash-in period even in the case of control measurements (134 ± 6%, 139 ± 6%, data not shown), which was presumably the effect of the closing of perfusion. Comparing the data from amyloid-treated slices (n = 5) with non-treated ones (n = 6) showed pronounced and significant increase in the peak-to-peak amplitude of fEPSPs when at least 20 μA current strength was used. Data of 30-

30 channels were compared in both cases showing the increases as follows: 247 ± 21%, 258 ± 16%, 219 ± 17%, 206 ± 17%, 195 ± 16%, 153 ± 15% (Fig. 18.).

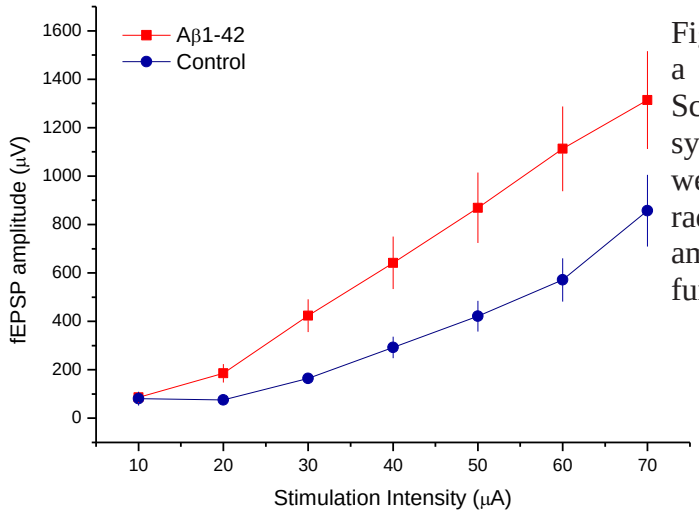

Fig. 18.: Amplitude of evoked fEPSPs as a function of stimulus intensity. The Schaffer collateral was stimulated with symmetric bipolar current and fEPSPs were recorded in the proximal stratum radiatum. Mean values ± SEM of fEPSP amplitudes (in μA) were plotted as a function of the stimulation intensity.

In the case of 4 amyloid- and 6 non-treated slices, a PPF protocol was used to evaluate the effect of oligomer Aβ assemblies on presynaptic function. The ratio of the peak-to-peak amplitudes of the subsequent fEPSPs elicited by the PPF protocol was compared, and a notable decrease of paired-pulse facilitation can be observed in the amyloid-treated slices. There were 46 and 61 channel recordings from amyloid- and non-treated slices showing 1.25 ± 0.03 and 1.4 ± 0.03 fold increase of fEPSP ratio respectively (Fig. 19.).

After one hour wash-in period, LTP was elicited by applying a theta-burst stimulation protocol and followed for 3 hours. The average of the peak-to-peak amplitudes of fEPSPs before the LTP induction was taken as 100%. The difference between the level of LTPs was significant after 2-3 hours (Fig. 20.). It is notable, that within this time frame, fEPSP amplitudes returned to control level (97 ± 5%) when amyloid was washed-in before TBS (n = 19 channels from 4 slices), while remained at an elevated level without its presence (121 ± 8%, n = 28 channels from 6 slices).

Following the LTP recording, the depotentiation ability of synapses was measured by applying low frequency stimulation (3 Hz for 5 minutes, LFS) and followed for half an hour. The last 10 minutes of LTP was taken as 100%. The peak-to-peak amplitudes of fEPSPs from amyloid- and non-treated slices were compared to each other at the end of these recordings and indicated a higher depotentiation ability of amyloid treated synapses (86 ± 2%, n = 19 for channels from 4 slices; 102 ± 5%, n = 28 channels from 6 slices; Fig. 21.).

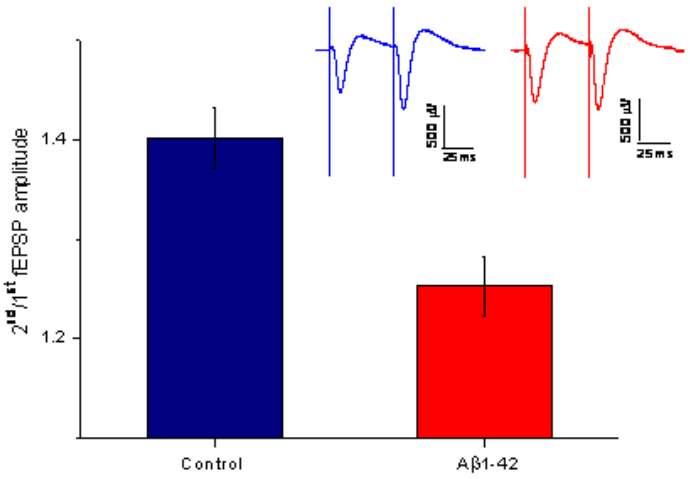

Fig. 19. shows the ratio of the $2^{nd}/1^{st}$ fEPSPs evoked by two symmetric bipolar currents following each other by 50 ms. Error bars represent SEM. Insets show representative fEPSPs for each cases.

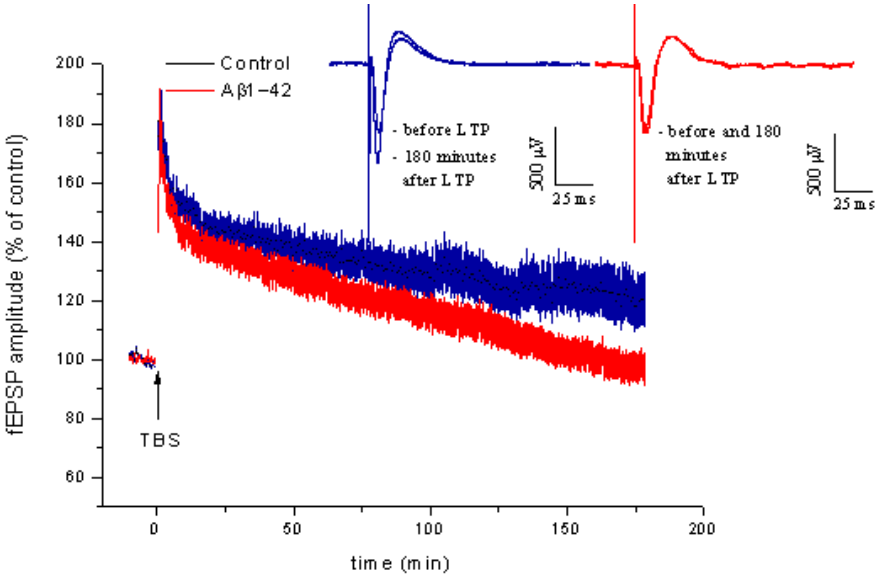

Fig. 20. shows the amplitude of fEPSPs normalized to preLTP control. Insets show representative fEPSPs recorded before and 180 minutes after LTP induction from the proximal stratum radiatum of CA1. Note the impaired LTP in amyloid treated slices. There is no significant difference between the evoked fEPSPs before and 180 min after LTP induction of Aβ1-42 treated slices.

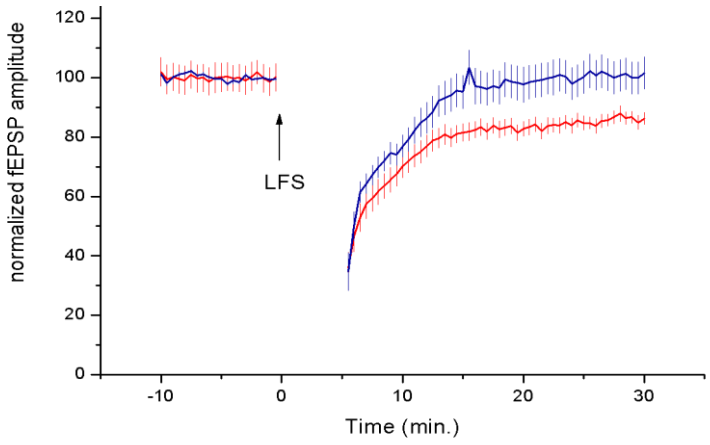

Fig.21. indicates the level of depotentiation after LTP. The amplitude of fEPSPs after LFS were normalized to fEPSPs recorded in the last 10 minutes of LTP. The depotentiation was more robust in Aβ1-42-treated slices.

Discussion

As a result of the misfolding and aggregation, different Aβ species may exist and may have distinct effects on the pathomechanism of AD. We have examined two well characterized forms of Aβ in our *in vivo* experiments and found that they similarly affect NMDA, but not AMPA receptors. Both the low n-aggregates and the highly aggregated forms of Aβ1-42 elevated the NMDA-evoked responses, while they have adverse effect on AMPA receptors, causing similar increase in NMDA-elicited responses in the case of oligomeric Aβ1-42, but a robust decrease after the application of fibrils.

There are contradictory results in the literature about the impaired function of NMDA and AMPA receptors in AD models. The hypo- (Raymond, 2003, *145;* Chen, 2002, *183*) and the hyperfunction (Carette, 1993, *184;* Wu, 1995, *185*) of NMDA receptors are also reported. Furthermore, synthetic and naturally secreted Aβ equally proved to promote endocytosis of NMDA receptor subunits (Snyder, 2005, *77*). Aβ1-42 is reported either to potentiate (Tozaki, 2002, *186*) or inhibit (Shemer, 2006, *187*) AMPA receptors and the GluR2 containing, Ca^{2+}-permeable AMPA channels may have central role in AD (Blanchard, 2004, *188*). Because of the interconnected function of AMPA and NMDA receptors, the Aβ induced chronic increase in the activity of glutamate receptors may lead to a down-regulation of NMDA receptors over time (Oster, 1993, *189;* Resink, 1995, *190*), and the hypofunction of AMPA receptors may lead to loss of dendritic spines, and consecutively decreased NMDA receptor responses (Hsieh, 2006, *191*).

The experiments of Tjernberg and Soto (Tjernberg, 1996, *125;* Soto, 1996, *126*) demonstrated that fragments of the Aβ sequence may be able to interfere with the process of Aβ aggregation. Based on the same concept, a protective pentapeptide, Phe-Arg-His-Asp-Ser (FRHDS), which is supposed to bind to integrin receptors was also reported (Szegedi, 2005, *182*). Peptides containing the Arg-Gly-Asp (RGD) recognition motif of the integrins enhance the fast AMPA receptor-dependent post-synaptic responses (Kramar, 2003, *192*) and modulate NMDA receptor function and subunit phosphorylation (Bernard-Trifilo, 2005, *193;* Lin, 2003, *194*).

Based on these data, we have thought to explore the short time effects of integrin activation by an RGD peptide (GRGDS) on the function of NMDA and AMPA receptors. In this set of experiments, GRGDS was used both in low and in high concentration. The application of RGD containing peptide in low concentration enhanced NMDA-elicited responses, but attenuated AMPA-evoked neuronal firing, while high doses of GRGDS

increased the responses of both NMDA and AMPA receptors. The control compound GGGGG did not affect the neuronal firing elicited neither by NMDA nor by AMPA.

The underlying mechanisms of integrin activation are relatively well described, namely RGD induced integrin activation (Giancotti, 1999, *195*) leads to activation of associated protein kinases like FAK and Pyk2, phosphorylation of CaMKII, the GluR1 subunit of AMPA receptor (Kramar, 2003, *192*), Src, and the NR2A and NR2B subunits of NMDA receptors (Bernard-Trifilo, 2005, *193;* Hisatsune, 1999, *196*). The main candidate for mediating the signaling between integrin and NMDA/AMPA receptors is Src kinase, since Src inhibitors block the GRGDSP effects on NMDA and AMPA receptor transmission (Kramar, 2003, *192;* Bernard-Trifilo, 2005, *193;* Lin, 2003, *194*) and the NMDA receptor-dependent ERK1/2 phosphorylation (Watson, 2007, *197*). However, the inhibitory action of low scale integrin activation has not been reported yet. As far as we know, only Wildering et al. (Fig. 5. C. and D. in (Wildering, 2002, *198*)) showed similar results in molluscan neurons, but the authors did not discuss the phenomenon.

Thus we aimed to get some data that Aβ, containing Arg-His-Asp-Ser (RHDS) within its sequence, which is somewhat similar to RGD and is required to activate integrins, acts troughout this pathway (Ghiso, 1992, *199;* Sabo, 1995, *200*). We hipothetised that blocking either the integrin-Aβ binding or the activation of Src results in the lack of increase in the NMDA-evoked neuronal firing after the application of fibrillar Aβ. To achieve this aim, we used antibodies against α2 (almost lacking in the hippocampus (Pinkstaff, 1999, *201*)) and β1 integrin subunits, which are most prominent in the hippocampus and involved in the formation of synaptic plasticity (Chan, 2006, *202;* Staubli, 1999, *203*), and we also used the Src tyrosine kinase inhibitor PP2 and as its control, the EGFR tyrosine kinase inhibitor PP3. When we ejected β1 integrin antibody onto the recorded neuron just before the fAβ1-42 application, Aβ failed to increase the NMDA-elicited responses. Src tyrosine kinase is involved in the phosphorylation of NMDA subunits (Grosshans DR, 2001, *87;* Hisatsune, 1999, *196*), and its blockade by the application of PP2 actually eliminated the NMDA-response enhancing effect of Aβ. In contrast, neither the α2 integrin antibody, nor the EGFR tyrosine kinase inhibitor was able to block the effect of Aβ on NMDA receptors. None of these compounds had any effect on basal NMDA-elicited neuronal firing alone.

A number of data indicate the participation of integrin activation and its downstream pathways in AD. It was shown recently, that α2β1 and αVβ1 integrin-antibodies could prevent Aβ-induced neurotoxicity (Wright, 2007, *204*). Integrins modulate glycogen synthase kinase-3 β activity (Ishii, 2003, *205;* Huang, 2009, *206;* Ho, 2008, *207*), participating in the

hyperphosphorilation of tau (for review see (Muyllaert, 2008, *208*)). The activation of Src family kinases may also take part in the formation of this key hallmark of AD (Bhaskar, 2005, *51;* Lee, 2004, *209;* Lesort, 1999, *210*). Furthermore, neurons in AD brains are found with multiple chromosome number (Mosch, 2007, *211;* Yang, 2001, *212*), which is lethal to these terminally differentiated cells leading to their apoptosis. It may be the result of integrin mediated, Aβ-induced cell-cycle reactivation via Src family kinases (Frasca, 2008, *213*). According to the supposed role of this family of kinases in the pathogenesis of AD, a member of these kinases, the Fyn kinase has elevated level in AD brains (Shirazi, 1993, *214*) and corresponds to synapse loss and memory impairment in transgenic AD mice (Chin, 2005, *215*). Integrins also have role in the modulation of synaptic plasticity and memory, they may affect NMDA receptor efficiency in the adult brain (Lin, 2003, *194*), moreover Src activation is reported to be essential to the induction of LTP presumably by increasing NMDA receptor currents (Lu, 1998, *216*).

Integrins are membrane spanning heterodimeric (α and β subunit) adhesion receptors integrating the extracellular matrix (ECM) with the cytoskeleton, hence corresponding to cell adhesion, motility, proliferation, apoptosis, induction of gene transcription and differentiation (Hynes, 1992, *217*). Extracellular matrix components e.g. collagen, fibronectin and laminin form fibrilloid structure just as the Aβ1-42. Integrins may not only bind these extracellular components, but also fAβ1-42, recognised as a fake member of ECM. Aβ1-42 may mimic ECM molecules because of its misfolding and aggregation, and may trigger apoptosis as it was reported about the naturally globular albumin when it gained fibrillar form (Huang, 2009, *206*). This abnormal activation of integrins by fAβ1-42 may result in the reactivation of cell-cycle in CNS cells, overactivation of NMDA receptors via Src family kinases, leading to increased Ca^{2+} influx, eventually to cell death. Either interfering with the direct amyloid-integrin binding, or the subtle modulation of downstream fyn kinase pathway may represent promising objectives for drug development for the treatment of AD.

Aβ1-42 derived pentapeptides are reported to be protective against the neuromodulatory and neurotoxic effects of fAβ1-42 both *in vitro* and *in vivo* (Szegedi, 2005, *182*). In a series of experiments, to further characterize the most effective one, LPYFDa was tested whether it penetrates the blood brain barrier and keeps its protective effect. This N-protected pentapeptide was administered intraperitoneally and proved to be protective against the fAβ1-42, applied onto CA1 neurons by microiontophoresis. Recordings obtained less than 80 or more than 240 minutes after ip administration, showed no favourable effect of the peptide. However, between the time frame of 80-240 mins, LPYFDa eliminated the NMDA

response enhancing effect of fAβ1-42. The control compound, GGGGG was unable to elicit such effect in any of the observed time periods. Thus, it seems that LPYFDa and/or its metabolite may pass through the BBB within 80 minutes, and conserves its beneficial effect against fAβ1-42 for almost three hours. After reaching an adequate concentration, it quickly intercepts iontophorised fAβ1-42, preventing the subsequent binding of the aggregated peptide to the cell surface. Further structure optimization of LPYFDa may enhance the efficiency of this lead pentapeptide and it may be exploited as a putative drug compound against AD.

LTP of synaptic transmission is regarded as a primary experimental model of memory formation and is often described as a Hebbian learning mechanism. There are controversial reports about Aβ1-42 effect on long-term memory indicating either decrease (Oddo, 2003, *45;* Chapman, 1999, *148;* Gureviciene, 2004, *218;* Trinchese, 2004, *219;* Jacobsen, 2006, *220*), unchanged, or even enhanced synaptic plasticity (Hsia, 1999, *136;* Parent, 1999, *171;* Wu, 1995, *221;* Larson, 1999, *222;* Fitzjohn, 2001, *223;* Jolas, 2002, *224;* Roder, 2003, *225*). This may be because of the different aggregational states of Aβ1-42 used in those studies and the majority of them have not examined the actual Aβ form, thus the interpretation and comparison of data is rather difficult.

The Aβ1-42 that was used in our experiments have been extensively studied and characterized by various physico-chemical methods, such as transmission electronmicroscopy (TEM) and dynamic light scattering (DLS). Considering, that low-n oligomeric assemblies may have greater possibility to diffuse into the deeper cell layers of a slice, we have studied the electrophysiological effect of oligomer Aβ1-42 on acute hippocampal slices of adult mice using MEAs. It was found, that Aβ1-42 added into the perfused ACSF in 1 μM concentration, had no significant effect on basal elicited fEPSPs within the 1 hour wash-in period compared to controls. Because an increase of fEPSP amplitudes occured even in the case of control measurements, we performed I-O curve recordings after the Aβ1-42 wash-in. These recordings showed increased excitability and it is presumably the result of increased ionotropic glutamate receptor function, supporting our *in vivo* experiments.

It was found that *in vivo* application of oligomer Aβ1-42 enhances both the AMPA- and NMDA-elicited spiking rate. The AMPA and NMDA receptor hyperfunction may lead to the saturation of the net excitability, therefore impede further increase of the synaptic strength (Vaillend, 2004, *226*). AMPA receptors presumably contribute to stabilization of spines (Passafaro, 2003, *227*) and their removal could lead to spine elimination. Overactivation of

the functionally interconnected AMPA and NMDA receptors may result in their endocytosis respectively (Hsieh, 2006, *191*).

The increased NMDA (and GluR2 containing, Ca^{2+} permeable AMPA) currents and the VDCCs may easily alter the normal Ca^{2+} homeostasis. Thus, a PPF protocol was performed after the Aβ1-42 wash-in to evaluate the effect of the peptide on presynaptic function, which is higly dependent on proper Ca^{2+} homeostasis (Steidl, 2006, *143*). These results indicate impaired synaptic vesicle release and perturbation of Ca^{2+} regulation after wash-in compared to controls.

In order to investigate the effect of Aβ1-42 on LTP mechanisms, a strong LTP protocol was applied, which is eligible to induce all three forms of LTP. The results showed impairment mainly in the late, transcription-dependent phase and it was most prominent 2-3 h after the induction of LTP. The depotentiation ability of synapses was also evaluated by applying LFS 3 hours after LTP induction. Slices treated with Aβ1-42 showed more robust depotentiation compared to untreated ones. The promotion of depotentiation was also reported about Aβ1-42 or its fragments by Shankar and Cheng, recently (Shankar, 2008, *228;* Cheng, 2009, *229*).

At first, we have found that Aβ1-42 impaired presynaptic function and increased excitability. Either the presynaptic vesicle release or the induction of LTP3 or LTD are related to proper Ca^{2+} influx. Our *in vivo* results indicated increased AMPA and NMDA currents after oligomer Aβ1-42 application. The proper function of NMDA receptors and L-type VDCCs is required to LTP3 induction (Raymond, 2003, *145;* Morgan, 2001, *170*). LTP3 is a transcription-dependent form of long term potentiation, and gene transcription is sensitive to temporal aspects of Ca^{2+} signals which is consistent with the requirement for repetitive HFS in the induction of LTP3 (Fields, 2005, *166*). Results from the acute slice recordings suggest that oligomer Aβ1-42 impairs mainly the transcription-dependent form of LTP, possibly via blocking the formation of e.g. new PSD proteins or proteins required for maintenance of extant spines.

These results represent that both low-n aggregates and fibrils of Aβ1-42 have rapid and significant effects on synaptic plasticity, and taken the data of Aβ1-42-induced suppression of LTP3 together with the promotion of depotentiation, these may lead to the impairment of cognitive function seen in the early phase of AD.

Summary

First, we have evaluated the effects of different well characterized assemblies of Aβ1-42 on AMPA and NMDA receptors *in vivo*. The low-n aggregates augmented both the NMDA and the AMPA elicited firing of CA1 neurons while fibrillar Aβ1-42 affected adversely these receptors, elevating the currents of NMDA receptors, but waning or almost eliminating AMPA elicited firing.

Second, we have tested whether Aβ1-42 exerts its effect via activating the integrin pathway. It seemed to be possible, that the RHDS within the sequence of Aβ1-42 may represent a ligand for integrin binding. Thus, we applied GRGDS onto CA1 neurons in low and high concentration to imitate the difference in the degree of possible binding surfaces of oligomer and fibrillar Aβ1-42. The integrin ligand GRGDS showed similar effect in low concentration as fAβ1-42, and in high concentration as the oligomer aggregates.

Third, we have further analyzed the integrin-activation by fAβ1-42. We aimed to interfere in this pathway at integrin-fAβ1-42 binding and at Src kinase activation. Thus we used antibodies against integrins and inhibitors against kinases. Beta1 integrin antibody prevented the induction of this pathway while α2 not. The inhibitor of Src kinases, PP2 also blocked NMDA hyperfunction in contrast with EGFR kinase inhibitor PP3.

Fourth, we have tested the BBB penetrating ability of LPYFDa by intraperitoneal administration. We found that this pentapeptide / or its metabolite is able to cross the BBB and still keeps its protective effect against the increase of fAβ1-42-induced NMDA elicited firing whithin a time frame of about 80-200 minutes after ip delivery.

Fifth, we have examined the impact of oligomer Aβ1-42 on synaptic plasticity using acute hippocampal mouse slices. Oligomer Aβ1-42 caused impairement of presynaptic function, increased the excitability of neurons, damaged the transcription-dependent phase of LTP, and enhanced the depotentiation after LTP induction.

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