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Abeta(1-42) enhances neuronal excitability and disrupts synaptic plasticity by altering glutamate recycling

Ph.D. Thesis

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“Life is not easy for any of us. But what of that? We must have perseverance and above all confidence in ourselves. We must believe that we are gifted for something, and that this thing, at whatever cost, must be attained.”

/Marie Curie/

LIST OF PUBLICATIONS

RELEVANT PUBLICATIONS

I. Edina Varga, Gábor Juhász, Zsolt Bozsó, Botond Penke, Lívia Fülöp, Viktor Szegedi: **Amyloid-β1-42 disrupts synaptic plasticity by altering glutamate recycling at the synapse.**
JOURNAL OF ALZHEIMER'S DISEASE. 2015 Jan; 45(2):449-56.
IF_{2013}=3.612

II. Edina Varga, Gábor Juhász, Zsolt Bozsó, Botond Penke, Lívia Fülöp, Viktor Szegedi: **Abeta(1-42) enhances neuronal excitability in the CA1 via NR2B subunit-containing NMDA receptors.**
NEURAL PLASTICITY. 2014:584314.
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OTHER PUBLICATION

III. Melinda E. Tóth, Viktor Szegedi, Edina Varga, Gábor Juhász, János Horváth, Emőke Borbély, Balázs Csibrány, Róbert Alföldi, Nikolett Lénárt, Botond Penke, Miklós Sántha: **Overexpression of Hsp27 ameliorates symptoms of Alzheimer's Disease in APP/PS1 mice.**
CELL STRESS AND CHAPERONES. 2013 Nov; 18(6):759-71.
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I. TABLE OF CONTENTS

II. ABBREVIATIONS

Abeta(1-42)	amyloid-beta(1-42)
ACh	acetylcholine
ACSF	artificial cerebrospinal fluid
AD	Alzheimer's disease
AICD	amyloid precursor protein intracellular domain
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA	analysis of variance
APP	amyloid precursor protein
Apo	apolipoprotein
BDNF	activity-dependent brain derived neurotrophic factor
CaMKII	calcium/calmodulin-dependent protein kinase II
cAMP	cyclic adenosine monophosphate
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CREB	cyclic adenosine monophosphate response element binding protein
CTF	C-terminal fragment
EAATs	excitatory amino-acid transporters
E-LTP	early phase of long-term potentiation
EPSC	excitatory postsynaptic current
ERK	extracellular signal-regulated kinase
esyn	extrasynaptic
FAD	familiar Alzheimer's disease
fEPSP	field excitatory postsynaptic potential
GABA	gamma-aminobutyric acid
Glu	glutamate
GluR	glutamate receptor
GOT	glutamic-oxaloacetate transminase
GPT	glutamic-pyruvic transaminase
GSK3β	glycogen synthase kinase 3β
ifenprodil	α-(4-Hydroxyphenyl)-β-methyl-4-benzyl-1-piperidine-ethanol-(+)-tartrate
iGluR	ionotropic glutamate receptor
iNOS	nitric-oxide synthase
IP3	inositol triphosphate
Low Mg^{2+} ACSF	low magnesium concentration-containing artificial cerebrospinal fluid
LTD	long-term depression
L-LTP	late phase of long-term potentiation
LTP	long-term potentiation
MAPK	mitogen-activated protein kinase
MEA	multi-electrode array

mGluR	metabolic glutamate receptor
MK801	(+)-MK801 hydrogen maleate
NFT	neurofibrillary tangles
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
Oxa	oxaloacetate
PKA	protein kinase A
PKC	protein kinase C
PSEN	presenilin
PPF	paired-pulse ratio
Pyr	sodium pyruvate
SAD	sporadic Alzheimer’s disease
SRE	serum response element
Syn	synaptic
TBOA	DL-threo-β-benzyloxyaspartate
VGlut	vesicular glutamate transporter
WB	Western Blot

III. INTRODUCTION

3.1. GENERAL DESCRIPTION OF ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is the most prevalent form of dementia affecting mainly the elderly population. Estimates suggest that 36 million people were diagnosed with AD worldwide, and this number will double every 20 years (Lundkvist, Halldin et al. 2014). Not just the patients themselves, but their families and the society are also affected. The total costs of dementia were estimated to be $600 billion worldwide (Menting and Claassen 2014).

AD was first described in a female patient by a German psychiatrist and neuropathologist, Alois Alzheimer in 1907 and the disease was named after him (Alzheimer, Stelzmann et al. 1995). The main hallmarks of AD are the progressive cognitive impairment, behavioral changes and permanent loss of memory (De-Paula, Radanovic et al. 2012). Although AD is in the focus of intense research, no effective treatment is currently available.

The disease is associated with early onset and progressive impairment in several brain areas having critical role in episodic memory, language and behavior (Weiner, Neubecker et al. 2008). It is estimated that the pathological process in AD brain begins at least 10-15 years before the first cognitive symptoms appear (Perrin, Fagan et al. 2009), including short-term memory loss and logopedic progressive aphasia (Magnin, Sylvestre et al. 2013). As the disease progresses, more severe memory loss and behavioral disturbances leading to confusion and lack of orientation occur (Mintzer, Mirski D et al., 2000). Two forms of AD can be distinguished, sporadic (SAD) and familiar (FAD), however they share common histological and clinical symptoms (Sassi, Guerreiro et al. 2014), and presumably common pathomechanisms. Two main neuropathological brain lesions are described in the brain tissues of AD patients: the extracellular accumulation of amyloid-beta (Abeta) and the intraneural neurofibrillary tangles (NFT) formed by hyperphosphorylated tau protein (Grundke-Iqbal, Iqbal et al. 1986).

The heterogeneity of AD is evident in their clinical, anatomical and biological characteristics. Fisher and coworkers demonstrated neuropsychologically defined AD subtypes using CERAD for their work (Consortium to Establish a Registry for Alzheimer's Disease) along with PET studies and classified the disease into five neuropsychological AD syndromes: typical AD, temporal (pure amnestic) variant AD, left (language) variant AD, right (visuoperceptive) AD and frontal (executive) variant AD (Fisher, Rourke et al. 1996, Fisher, Rourke et al. 1997, Lam, Masellis et al. 2013).

3.2. FORMATION OF AMYLOID-BETA PLAQUES

3.2.1. THE AMYLOID CASCADE HYPOTHESIS

Several factors can contribute to the pathogenesis of AD, however the exact mechanism is unknown. Soon after the discovery of AD-associated mutations in the amyloid precursor protein (APP) gene, the Abeta cascade hypothesis was postulated by John Hardy in 1992 that became the dominating hypothesis in the field (Hardy and Higgins 1992). The Abeta cascade hypothesis proposes that the deposition of Abeta in the brain is a crucial step in the development of AD. The hypothesis links histopathological and genetic properties that lead to development of Abeta depositions and AD dementia.

Amyloid depositions are formed by Abeta peptides that derive from the much larger APP. APP is a widely expressed transmembrane glycoprotein and its gene is located on human chromosome 21 and expressed in several tissues (Lemmin, Dimitrov et al. 2014). It exists at least in ten isoforms, which are generated by differential splicing of exons 7 and 8, mainly APP_{695}, APP_{751} and APP_{770} in the brain. In human cortex, the ratio of different APP isoforms is approximately $APP_{695}/APP_{751}/APP_{770}$ = 20:10:1, however this ratio could change in different regions (Nalivaeva and Turner 2013). APP_{695} is the exon 7-skipped isoforms, and is most abundant in the neurons (LeBlanc, Chen et al. 1991), while APP_{751} and APP_{770} are expressed in glial cells (Kitazume, Yoshihisa et al. 2012). It was shown that Abeta was produced from APP_{695} isoform via the cleavage of the APP intracellular domain by the action of β-secretase (Belyaev, Kellett et al. 2010), while another group suggests that Abeta could be released from APP_{770} by the actions of both α- and β-secretases in the endothelial cells of the brain (Kitazume, Tachida et al. 2010). APP plays a major role in the regulation of several important signaling pathways especially in the nervous system, such as synaptogenesis (Wang, Yang et al. 2005), axon elongation (Chasseigneaux, Dinc et al. 2011) or iron export (Duce, Tsatsanis et al. 2010), however its basic physiological function is not fully clear.

APP can be processed by secretases in two main pathways depending on the secretase that actually cleave it (reviewed by Vingtdeux, Sergeant et al. 2012). In the non-amyloidogenic pathway (**Figure 1, left**), α-secretase cuts APP within the Abeta sequence, releasing soluble secreted form of APP (sAPPα) and the membrane-associated C-terminal fragment (CTFα), which in turn is further processed by γ-secretase resulting in the release of the P3 peptide and amyloid precursor protein intracellular domain (AICD).

In the amyloidogenic pathway (**Figure 1, right**), APP is initially cleaved by β-secretase to generate a large soluble secreted form of APP (sAPPβ) and leaving the membrane-anchored C-terminal fragment comprised of 99 amino-acid (CTFβ or C99). The intracellular sorting of β-secretase is a critical step in APP processing (Chia, Toh et al. 2013). The subsequent action of CTFβ by γ-secretase releases Abeta peptide on the extracellular side, which produces Abeta species of 39-43 amino acids and the APP intracellular domain (AICD or C51) in the cytoplasm. It is noteworthy that AICD may have a potential gene expression regulatory function (Mattson 2003). Furthermore, lysosomal processing of APP is a likely site of Abeta production (Tam, Seah et al. 2014). The γ-secretase complex is composed of presenilin (PSEN), nicastrin (NCT), γ-secretase activating protein (GSAP), Pen-2 and Aph-1 proteins.

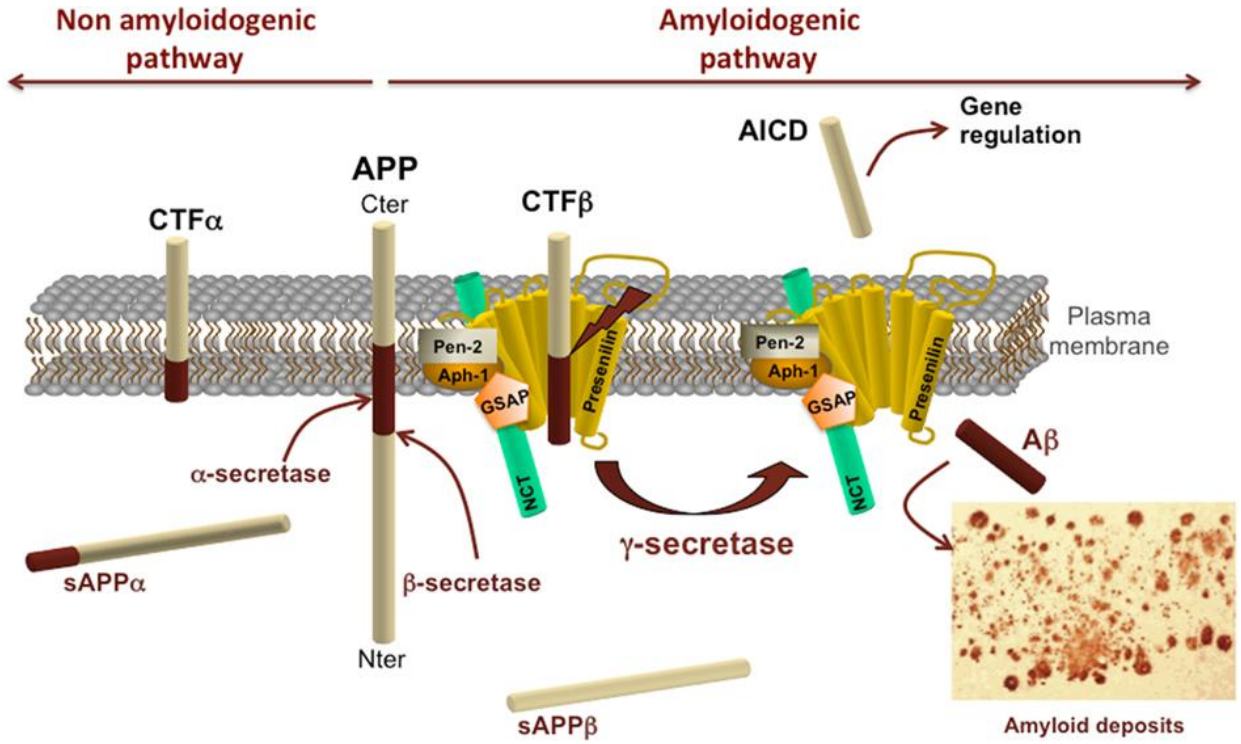


Figure 1. Schematic representation of the two APP proteolytic pathways. APP may be cleaved in two distinct processes: non-amyloidogenic (left) and amyloidogenic (right). Alternative cleavage of APP by the α-secretase enzyme produces soluble, non-toxic compounds such as membrane-associated CTFα and sAPPα. In the amyloidogenic pathway, Abeta is released from the APP through the action of the β- and γ-secretases. Abeta and AICD are produced by the γ-secretase cleavage of the CTFβ, which is then retained in the cytoplasmic membrane. Abbreviations: APP (amyloid precursor protein), CTF (C-terminal fragment), sAPP (soluble secreted form of amyloid precursor protein), Abeta (amyloid-beta) and AICD (amyloid precursor protein intracellular domain). Figure from Vingtdeux, Sergeant *et al.* 2012.

The hypothesis, that AD could be inherited in an autosomal dominant manner is further supported by research data. The disease-associated mutation that was first described is in the gene coding APP. More AD-associated mutations have been identified, such as the autosomal dominant mutations in presenilin 1 (PSEN1) and presenilin 2 (PSEN2), which induce increased activity of γ-secretase, causing a shifted balance towards amyloidogenic Abeta(1-42) versus shorter Abeta(1-40) or Abeta(1-38) species (Wanngren, Lara et al. 2014), and also disrupt the endoplasmic reticulum Ca^{2+} homeostasis (Schneider, Reverse et al. 2001). PSEN1 mutation is found in approximately 80% of cases of FAD (Sepulveda-Falla, Barrera-Ocampo et al. 2014).

The apolipoprotein E (apoE) gene, which plays a role in the clearance of Abeta from the brain, was also shown as the major genetic risk factor for familial and sporadic AD. Three common apoE alleles have been described: apoE2, apoE3 and apoE4. The apoE4 allel has been consistently associated with AD, and this allel causes dysfunction of the lipid transport system and lipid homeostasis in the brain of AD patient (Guerreiro, Gustafson et al. 2012).

3.2.2. DIFFERENT AGGREGATION FORMS OF AMYLOID-BETA

Abeta, a misfolded protein, is widely regarded as a central player in the in the pathogenesis of AD. The accumulation of soluble Abeta(1-42) in the brain of patients and animal models of AD is associated with impairments of cognition and memory (Walsh, Klyubin et al. 2002). Progressive neuronal degeneration was also aggravated by membrane-associated oxidative stress induced by aggregation of Abeta (Mattson 2003).

Abeta aggregation is a complex process, involving the misfolding of Abeta into soluble and insoluble forms. Monomeric Abeta is mainly composed of α-helical conformation, whereas the misfolded polymers are rich in β-sheet structures (Zhao, Chiu et al. 2012). It was shown that the soluble pool of Abeta may exist both at the extracellular and at the intracellular space (LaFerla, Green et al. 2007). Several studies have shown that misfolding of monomeric Abeta leads to the formation of oligomers, which then rapidly grow to larger polymers or fibrils (Ni, Shi et al. 2011, Zhao, Chiu et al. 2012).

Two forms of amyloid plaques are distinguished in the AD brain: diffuse and senile, or neuritic plaques, which were originally described by Alois Alzheimer. Diffuse plaques are amorphous in their shape and are not surrounded by dying or damaged neurons (Joachim, Mori et al. 1989), however these deposits may be the precursors of fibrillogenesis (Pappolla, Omar et al. 1992). Neuritic plaques have a diameter of around 50-200 μm; they contain a central core riched

in Abeta. It is often associated with axonal and dendritic injury and surrounding reactive astrocytes (McGeer, Itagaki et al. 1987). The major components of amyloid deposits are the 39-43 amino acid-containing residues of Abeta peptide, which derive from the sequential cleavages of its precursor protein.

The aggregation and deposition of Abeta in the brain is believed to be the link to the neuronal cell death and loss of cognitive function seen in AD. Although the fibrillated form of Abeta containing plaques is large, insoluble and more compact than the oligomers, the most toxic forms of Abeta are generally believed to be the soluble oligomers. The size of oligomer populations ranges from 8-22 KDa (low molecular weight) to 123-908 KDa (high molecular weight) (Fulop, Mandity et al. 2012). Evidence has been reported that both synthetic and brain-derived human oligomer Abeta(1-42) are synaptotoxic, playing an essential pathogenic role in decreased spine density, damage certain forms of synaptic plasticity (Shankar, Bloodgood et al. 2007, Selkoe 2008, Li, Hong et al. 2009, Fulop, Mandity et al. 2012). The oligomer Abeta-induced long-term potentiation (LTP) inhibition can be rescued by the application of anti-Abeta antibodies *in vivo* (Walsh, Klyubin et al. 2002), suggesting that indeed Abeta is the primal synaptotoxic agent. The number of plaques does not correlate well with the severity of dementia (reviewed by Shankar and Walsh 2009), which suggest that the highly aggregated form of the peptide is nontoxic. On the other hand, the aggregated insoluble forms of Abeta may lead to later-onset and more severe memory loss, associated with inflammatory reactions induced by fibrillar Abeta (Bamberger, Harris et al. 2003). Moreover, so-called “reverse oligomers” may dissociate from the plaques forming a halo of highly toxic Abeta forms around the plaque-core (Martins, Kuperstein et al. 2008, Koffie, Meyer-Luehmann et al. 2009).

3.3. NEUROFIBRILLARY TANGLES

Although the amyloid cascade hypothesis is the most widely accepted hypothesis that explains the development of AD, it does not consider the interaction of Abeta peptide with tau. Tau plays an essential role in regulating microtubule dynamics, axonal transport and neurite outgrowth (Biernat and Mandelkow 1999). These functions are modulated by site-specific phosphorylation of the tau protein (Hamdane, Sambo et al. 2003).

It was reported that physiological assembly of the microtubule system in AD brain is disturbed by abnormally hyperphosphorylated forms of microtubule-associated tau protein forming intracellular NFTs. NFTs are formed by paired, helical ~10 nm filaments especially in

the hippocampal formation, amygdala, cortical and subcortical areas (reviewed by Selkoe 2011). The phosphorylated tau proteins are no longer able to stabilize the microtubules, which leads to disruption of neuronal transport and cause cell death (Baumann, Mandelkow et al. 1993).

3.4. EXCITATORY SYNAPTIC TRANSMISSION IN THE CA1

3.4.1. GLUTAMATERGIC SYNAPTIC TRANSMISSION

The non-essential amino-acid, L-glutamate (Glu) is the major excitatory neurotransmitter in the mammalian central nervous system, and plays principal role in neural activation, especially in the cortical and hippocampal regions, which are associated with learning and memory (Dale and Kandel 1993, Staubli, Rogers et al. 1994). There are two large families of glutamate receptors (GluRs) located on the membrane of the neurons: ionotropic GluRs (iGluRs) and metabotropic GluRs (mGluRs), respectively. All of these receptors are comprised of 4 subunits forming a tetrameric structure (Willard and Koochekpour 2013).

α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) is an ionotropic transmembrane receptor, which has a lower affinity for Glu than the N-methyl-D-aspartate receptor (NMDARs) (Patneau and Mayer 1990). AMPARs are the major mediators of Glu-mediated fast excitatory synaptic transmission, are critical for the mediation of field potentials often used as an experimental readout for neuronal excitability (Bashir, Alford et al. 1991). The functional properties of the AMPARs are highly dependent on its subunit combinations. AMPARs lacking GluR2 subunit are permeable to Ca^{2+} (Wright and Vissel 2012).

NMDARs are both ligand-gated and voltage-dependent, and their activation requires the simultaneous binding of Glu and either D-serine or glycine (Kleckner and Dingledine 1988). NMDAR activation also requires membrane depolarization, because either Mg^{2+} or Zn^{2+} ion blocks the pore of the channel under basal circumstances, and this blockade is voltage-sensitive (Low, Zheng et al. 2000). This type of receptor is a multimeric assembly of glycine- or D-serine binding NR1 and NR3A-B, as well as Glu-binding NR2A-D subunits and is predominantly permeable to Ca^{2+}, in addition to Na^{+} and K^{+} (Laube, Kuhse et al. 1998, Awobuluyi, Yang et al. 2007). Ca^{2+} influx via NMDARs triggers several cascade pathways that may alter synaptic efficacy and neuronal morphology. Of particular interest here, NR2A subunit-containing NMDARs localized mainly at the synaptic domain are antiapoptotic and participate in the induction of LTP in the CA1. In contrast, extrasynaptic NMDARs (esyn NMDARs) containing

mainly NR2B subunit (on pyramidal cells) were shown to activate apoptotic pathways and to promote synaptic depression (Hardingham, Fukunaga et al. 2002).

Following presynaptic depolarization, vesicles may release their contents of Glu into the synaptic cleft by a highly Ca^{2+}-dependent mechanisms. Glu can bind to GluRs of the post-synaptic cell, initiating cation-flow, which depolarizes the post-synaptic neuron (Llinas, Steinberg et al. 1981). After Glu is released into the synaptic cleft, the unwanted Glu is cleared off by the Glu transporter systems. The released Glu requires extremely large number of Glu transporters. There are two Glu transporter systems in the brain: the vesicular Glu transporters (VGLUT) and the excitatory amino-acid Glu transporters (EAATs), both are abundantly expressed both in neuronal and glial cells in many regions of brain (Bar-Peled, Ben-Hur et al. 1997).

To prevent over-stimulation, EAATs bind Glu from the extracellular space and then undergo a conformational change which allows transportation of the Glu through the plasma membrane (Focke, Wang et al. 2013). The EAAT subtypes are expressed in different regions of the brain. EAAT1 and the most abundant transporter EAAT2 are expressed on glial cells of the hippocampus and cerebral cortex and responsible for 80-90% of all Glu transport (Rothstein, Dykes-Hoberg et al. 1996). Recent data also shows that the Glu uptake capacity of astrocytes is much higher than that of neurons (Proper, Hoogland et al. 2002). Furthermore, EAAT2 is also present on presynaptic side of the neuron (Yang, Gozen et al. 2009). EAAT3 is also highly expressed in these regions, most probably on post-synaptic side in neuronal cells (Popoli, Yan et al. 2012). EAATs-mediated Glu transport is Na^+-dependent and is highly efficient even against a 1000-fold concentration gradient (Attwell and Mobbs 1994). One Glu molecule is transported together with two or three Na^+ ions and one H^+ exchanges for one K^+ ion. These transporters are regulated by protein kinases, growth factors and second messengers (Focke, Wang et al. 2013).

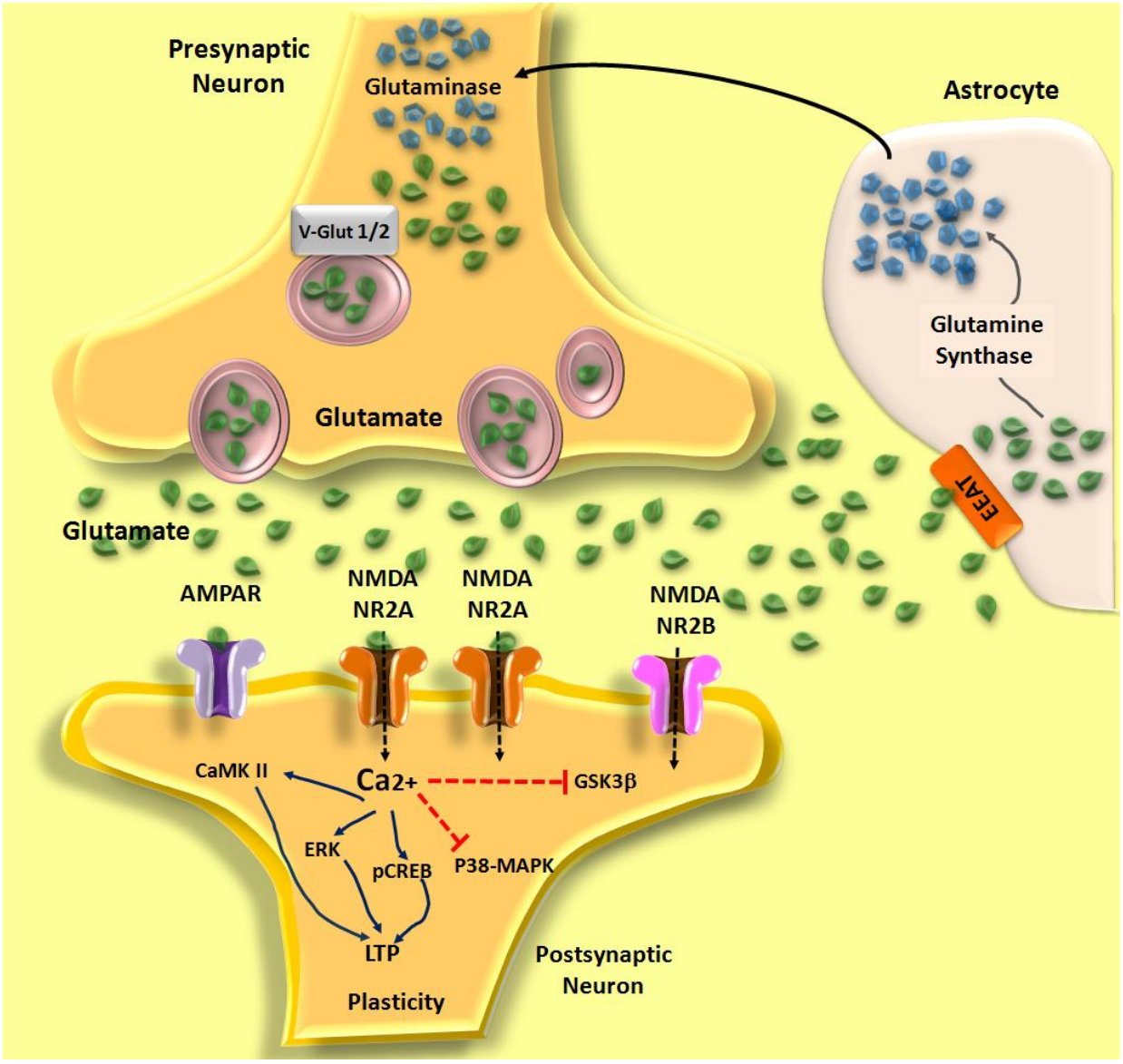


Figure 2. Glutamatergic synaptic transmission. During excitatory synaptic transmission, Glu is briefly released into the synaptic cleft, and then it binds to GluRs present mainly on the postsynaptic cell. The Ca^{2+} influx through NMDARs or AMPARs lacking GluR2, leads to activation of several molecular pathways such as CaMKII and ERK-MAPK stream which are critical to CREB phosphorylation and induction of LTP. However, other intracellular signaling can also be activated, which may lead to tau hyperphosporylation (GSK3β) and LTD (p38-MAPK). The normal physiological level of Glu is mediated by both neuronal and glial EAATs. Glu is transported via EAATs and transformed to glutamine by the glutamine synthase, then released into the extracellular space. The glutamine is taken into the presynaptic terminals and is converted into Glu by glutaminase. The produced Glu is packaged into vesicles via VGLUT. Abbreviations: Glu (glutamate), GluRs (glutamate receptors), NMDARs (N-methyl-D-aspartate receptors), AMPARs (α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic receptors), CaMKII (Ca^{2+}/calmodulin-dependent protein kinase II), ERK (extracellular signal-regulated kinase), p38-MAPK (p38-mitogen-activated protein kinase), CREB (cAMP response element binding protein), LTP (long-term potentiation), GSK3β (glycogen synthase kinase 3β), LTD (long-term depression), EAATs (excitatory amino-acid glutamate transporters) and VGLUT (vesicular glutamate transporters). Figure from Campos-Pena V and Meraz-Rios MA, Neurochemistry, 2014.

Without glial cells' contribution, normal synaptic function of glutamatergic neurons is not possible involving development of learning and memory (Hertz and Zielke 2004). Recent data shows that the Glu uptake capacity of astrocytes is much higher than that of neurons (Proper, Hoogland et al. 2002). In addition, glial EAATs have high diffusion rate in the membrane: unbound transporters further away from the synapse can replace Glu-bounded closer to the synapse (Edwards 2015). Glial cells take up Glu through highly efficient EAATs and convert it to glutamine via the glutamine synthase pathway, then release glutamine into the extracellular space (see above, **Figure 2**).

This mechanism is an essential contributor to normal Glu release from the neuron (Rothstein and Tabakoff 1984). The glutamine is taken up into the presynaptic terminals and metabolized into Glu by the mitochondrial phosphate-activated glutaminase enzyme. The produced Glu is packaged into synaptic vesicles by VGLUT. This process is highly dependent on the tight cooperation of synaptic terminals and glial cells (Campos-Pena and Meraz-Rios 2014).

3.4.2. NEUROPLASTICITY

The ability of synapses to change their efficiency is also known as synaptic plasticity. This mechanism is dependent on the pattern by which synapses are stimulated and by several neurotropic factors and modulators. LTP is the most studied form of long-term synaptic plasticity, which widely regarded as one of the major mechanism that might be the cellular basis of learning and memory (Teyler and Discenna 1984). LTP was first described in the rabbit dentate gyrus of the hippocampus by Terje Lømo and Timothy Bliss in 1973 (Teyler and Discenna 1984). Since its original observation, it has been also described in other brain structures, such as cerebral cortex, cerebellum and amygdala (Kimura, Nishigori et al. 1989, Clugnet and LeDoux 1990, Crepel and Jaillard 1991). In particular, NMDAR-dependent LTP in the CA1 is the most widely studied type of LTP, and there is evidence showing that this form of neuroplasticity is impaired in AD (reviewed by Selkoe 2008). In my thesis, I am focusing on the NMDAR-dependent LTP at the Schaffer-collateral pathway in the CA1.

Two forms of LTP can be distinguished: early-phase of LTP (E-LTP) and late-phase of LTP (L-LTP). A weak, high frequency stimulation (single train of 100 pulses at 100 Hz) can trigger an increase in synaptic efficacy that lasts for about 1-2 hours, while triggering the L-LTP requires repeated, strong high frequency stimulation (multiple trains of 100 pulses at 100 Hz) that lasts for over 3 hours or even days (**Figure 3**, reviewed by Lu, Christian et al. 2008). However, Villers A

and her coworkers found that a single train of high frequency stimulation (100 Hz, 1 sec) also induces L-LTP (8 h) in mice hippocampal slices (Villers, Godaux et al. 2012). It is generally evidenced that the induction of E-LTP and L-LTP is very different, thus their biochemical pathways can lead to distinct changes in the synapses. E-LTP requires modification of various proteins and their trafficking at synapses, but is independent from gene transcription and protein synthesis, while L-LTP requires changes in gene expression and protein synthesis (Lu, Christian et al. 2008). However, it is also shown that L-LTP induced by a single train is independent from protein synthesis (Villers, Godaux et al. 2012).

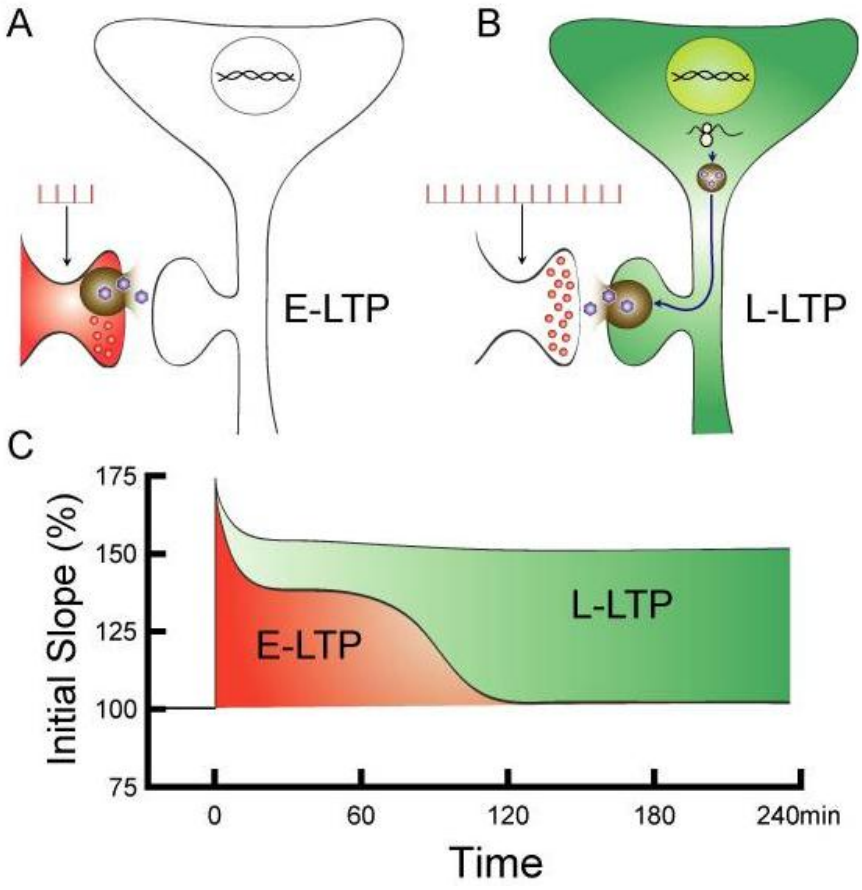


Figure 3. The timeline of the LTP phases. Panel A shows that a weak, high frequency stimulation can evoke an increase in synaptic efficacy (E-LTP), while triggering the L-LTP requires repeated, strong high frequency stimulation and dependent on gene transcription (B). The morphological changes in spines reduced over a long time (C). This figure also shows the activity-dependent secretion of BDNF at the presynaptic side (red in A and C) may require development of E-LTP and L-LTP (green in B and C). Abbreviations: LTP (long-term potentiation), E-LTP (early-phase of long-term potentiation), L-LTP (late-phase of long-term potentiation) and BDNF (brain derived neurotrophic factor). Figure from Lu, Christian *et al.* 2008.

E-LTP involves Glu transmission and several postsynaptic processes that lead to enhanced Glu release from the presynapse and increased sensitivity to Glu on the postsynaptic side. When Glu is released from the presynaptic side onto the dendritic spine of the postsynaptic neuron, it can bind GluRs localised to the postsynaptic density (PSD) (reviewed by Byth 2014). The PSD involves iGluRs and several signaling proteins, and is linked to the actin filaments that support the structural framework of the spine. PSD contains NMDARs, which can enable Ca^{2+} ion influx to the spine, and they require for a binding Glu and its coagonist. These coagonists, namely glycine or D-serine can bind to the NMDARs gating its activity, moreover they may play a role in astroglial regulation of neural plasticity (Fossat, Turpin et al. 2012). The activation of NMDARs requires Mg^{2+} ion removal from the channel. This occurs when there is a strong activation of nearby AMPARs, which can mediate rapid synaptic transmission. Moreover, Glu has to bind to NMDARs in the presence of depolarized postsynaptic membrane in order to activate the receptor (Bliss and Collingridge 1993). During LTP induction, nonsynaptic AMPARs are rapidly trafficked into the postsynaptic membrane, which is mediated by the activation of various protein kinases. Activated CaMKII phosphorylates the GluR1-containing AMPARs, increasing their sensitivity to Glu and increasing the synaptic expression of AMPAR (Lisman, Yasuda et al. 2012). CaMKII activation also induces remodeling of the cytoskeleton and functions as retrograde signal generator along with protein kinase C (PKC): a second messenger, namely nitrogen-oxide (NO) is released to the presynaptic terminal to increase the probability of neurotransmitter vesicle release (Johnstone and Raymond 2011). The activity-dependent brain derived neurotrophic factor (BDNF) released from presynaptic sites may also be necessary for E-LTP and have a crucial role for L-LTP induction (see above, **Figure 3**).

Elevated intracellular Ca^{2+} triggers CaMKII, which in turn activates adenylyl-cyclase and cAMP kinase, required for L-LTP. The increased Ca^{2+} influx via L-type voltage-dependent Ca^{2+} channels (VDCC) is also critical for translation and for the activation of cAMP-mediated transcription (Morgan and Teyler 1999), while mitogen-activated protein kinase (MAPK) cascade can mediate cAMP response element binding protein (CREB) phosphorylation (Kanterewicz, Urban et al. 2000). The regulation of CREB has been shown to play a role in several protein kinases' activation including protein kinase A (PKA), which may contribute to transcription via serum response element (SRE) by activating the ERK-MAPK stream (Adams, Anderson et al. 2000, Davis, Vanhoutte et al. 2000). Protein synthesis occurs in dendrites too, and this type of local translation is necessary for L-LTP induction (Huang and Kandel 2005).

In summary, L-LTP differs from E-LTP in its requirement for gene transcription and in its association with structural and morphological changes at synapses. Induction of LTP results the functional and morphological development of synapses: the pre- and postsynaptic structures such as dendritic spines and axonal buttons are stabilized, the postsynaptic density via postsynaptic scaffolding proteins (PSD-95) are increased and also results enhanced postsynaptic sensitivity to Glu (Ehrlich, Klein et al. 2007). However, during induction and during E-LTP, the morphological changes of spine dynamics reduced progressively, and they are less stable over longer time (Yuste and Bonhoeffer 2001). The latter phase of LTP is associated with postranslational modifications that increase the excitatory postsynaptic current (EPSC). Moreover, presynaptic synthesis of synaptotagmin-1 and increased synaptic vesicle numbers suggest that protein synthesis is not restricted to the postsynaptic side (Ahmad, Polepalli et al. 2012). LTP induction also results in the growth of new spines, increased an dendritic spine number and formation of new synapses on the CA1 pyramidal cells.

3.5. INVESTIGATION OF EXCITATORY EXTRACELLULARLY EVENTS IN THE CA1

In my thesis I used multi-electrode electrophysiological recordings from hippocampal slices for investigating cellular events. Extracellular recording from hippocampal slices has long been a method of choice for determining the changes in excitability and synaptic plasticity of the CA1 microcircuitry (Dingledine, Dodd et al. 1980).

Spontaneous spikes and far more often, field excitatory postsynaptic potentials (fEPSPs) are recorded extracellularly from a local network of neurons, however, the relationship between these two phenomena is not fully clear. Intuitively, both electrophysiological events correlate with the excitability of the network under investigation, but these events are generated by different mechanisms. Evoked fEPSPs are mainly composed of subthreshold events from a population of neurons, like dendritic depolarizations (Tominaga, Tominaga et al. 2002) and glial contributions to the net extracellular charge-flow arising mainly from the function of transporters (Diamond, Bergles et al. 1998, Luscher, Malenka et al. 1998). In contrast, spontaneous firing represents only neuronal suprathreshold events (Brock, Coombs et al. 1952). Another difference is that a fEPSP is evoked by a stimulus, and therefore it is the result of a coordinated and synchronized electrical activity of a cell population mediated by synaptic connections. Spontaneous spikes, in contrast,

are not evoked by an external stimulus, and they are more likely to be dependent on intrinsic network connections and properties (Cohen and Miles 2000).

Several protocols have been introduced to evoke LTP since its original description. By definition, NMDA-dependent LTP is a process of Hebbian synaptic plasticity, where input specificity, associativity, cooperativity, and persistence are the governing rules.

3.6. AMYLOID-BETA INDUCES NEURONAL HYPEREXCITABILITY

Seizures in patients with AD have been investigated by many researchers over the last decades. It was shown that AD patients can exhibit more than one spontaneous seizures, but the relationship of epilepsy and with AD pathophysiology has remained unknown (Rao, Dove et al. 2009). Recent studies show that the occurrence of unprovoked seizures is 5-10-fold greater in SAD, and 87-fold greater in the less elderly cohort of patients (50-59 years old) than in the reference population (Amatniek, Hauser et al. 2006). Minkeviciene *et al.* have also demonstrated that Abeta-induced neuronal hyperexcitability culminates in epileptiform activity (Minkeviciene, Rheims et al. 2009). This fact shows that the brain network shifts towards hyperexcitability in AD, leading to several effects: neuronal dysfunction, cell death and cognitive decline. Importantly, this process enhances the production of Abeta, because Abeta release is dependent on the network activity (Cirrito, Kang et al. 2008, Kang, Lim et al. 2009).

Several animal models were used to elucidate the relationship between AD and epilepsy. Numerous findings confirmed that excitotoxic effects of Glu contribute to progressive neuronal loss in AD (Koh, Yang et al. 1990, Pomara, Singh et al. 1992, Harkany, Abraham et al. 2000, Amatniek, Hauser et al. 2006). Alternations in Glu uptake regulatory system can lead to increased Glu level and subsequent increase in the excitability of the network. EAATs may be central players in this mechanism, and indeed, recent findings suggest that Abeta oligomers inhibit normal Glu recycling at the synapse (Awobuluyi, Yang et al. 2007, Li, Hong et al. 2009, Chen, Reese et al. 2011), resulting in elevated ambient extracellular Glu level in the brain (Matos, Augusto et al. 2008, Mura, Zappettini et al. 2012), which might be responsible for the overexcitation seen in AD. Abeta blocks Glu reuptake by inhibiting both neuronal and glial EAATs (Li, Hong et al. 2009, Matos, Augusto et al. 2012), which might lead to esyn NMDAR activation. The activation of esyn NMDARs impairs LTP (Li, Jin et al. 2011), enhances LTD (Liu, Yang et al. 2013) and promotes apoptosis (Hardingham, Fukunaga et al. 2002). Recent findings suggest that Abeta binds to prion protein, mGluR5 and integrin receptors, and this

complex initiates a molecular cascade mediated by Fyn kinase (Larson, Sherman et al. 2012, Um, Nygaard et al. 2012, Um, Kaufman et al. 2013), which subsequently phosphorylates NMDARs. Increased phosphorylation of NMDARs enhances their sensitivity to Glu and modifies their opening kinetics.

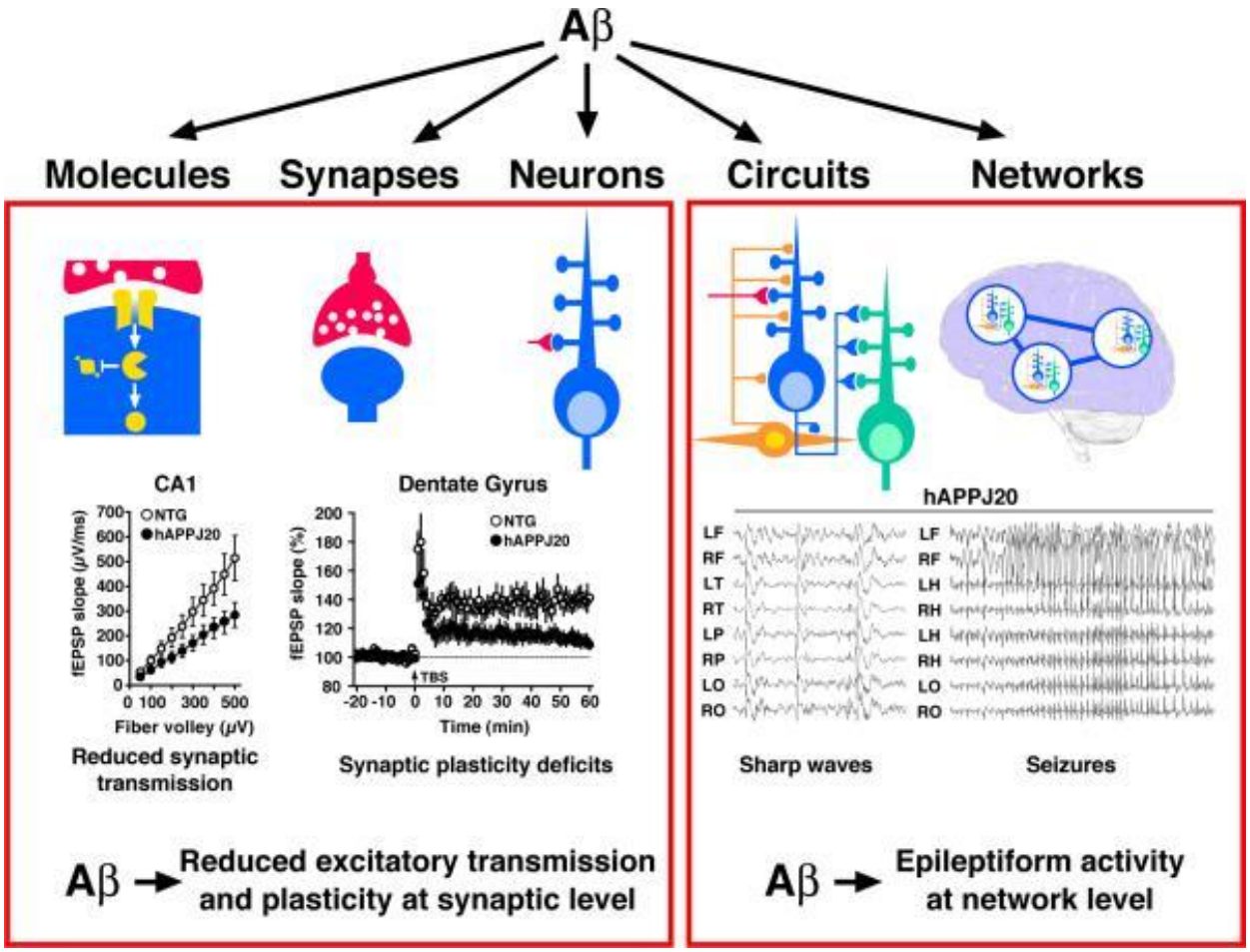


Figure 4. Effect of Abeta at multiple levels of complexity. Abeta causes failure of excitatory neurotransmission and impairs synaptic plasticity at the level of specific synapses (left). The hAPP-J20 transgenic mice also show epileptiform activity and seizures at the network level (right). These results suggest that aberrant network synchronization is a primary effect of high Abeta level. Abbreviations: Abeta (amyloid-beta), hAPP-J20 (human amyloid precursor protein transgenic mice), F (frontal), fEPSPs (field excitatory postsynaptic potentials), H (hippocampal), L (left), NTG (nontransgenic mice), O (posterior-parietal), P (parietal), R (right), T (temporal), TBS (theta-burst stimulus). Figure from Palop and Mucke 2009.

The brain can be described as a complex system of networks and is classified by multiple levels of complexity. Molecular and synaptic alternations can influence the network function in higher levels manifesting in rhythmic neural activity. It is well characterized that Abeta interferes with excitatory neurotransmission at synaptic level, however its effect on the network is still

undetermined. Figure 4. is a good summary of the effect of Abeta on synaptic transmission and on the network (see above, **Figure 4**) (reviewed by Palop and Mucke 2009). The authors have shown that paired-pulse ratio (PPF) and LTP was depressed in human APP (hAPP) expressing transgenic mice, as well as these mice also exhibited frequent generalized cortical epileptiform spike discharges that may lead to cognitive deficit (Palop, Chin et al. 2007). Although, their experimental data also show that the excitatory transmission was reduced in the hippocampus of transgenic mice, epileptiform activity was recorded from both hippocampal and cortical regions of the same mouse strain.

3.7. THERAPEUTIC APPROACHES IN ALZHEIMER'S DISEASE: FOCUSING ON GLUTAMATE TOXICITY AND THE SCAVENGER SYSTEM

Several studies have clearly indicated that Abeta toxicity is mediated by Glu excitotoxicity, involving Glu spillover and esyn NMDARs activation, excessive Ca^{2+} influx and consequent activation of cascade pathways resulting in cell death. Thus, Glu plays a critical role in Abeta pathology, therefore GluR antagonists could be promising therapeutic targets against AD.

Currently, the only drug that targets the Glu system and is used for treating AD in the clinic is memantine, a non-competitive NMDAR antagonist. This compound exhibits higher affinity to NMDARs than the endogenous NMDAR antagonist, Mg^{2+} ion (Chen, Pellegrini et al. 1992). Memantine can attenuate the excessive Ca^{2+} flow into the cell without interfering with normal physiological processes. It was also shown that memantine favorably binds to and blocks NMDARs during its prolonged activation, however this process takes place during a few milliseconds, thus memantine is not able to act or accumulate in the channel under normal synaptic activity (reviewed by Revett, Baker et al. 2013). Furthermore, it was also shown that memantine is able to reverse Abeta-induced LTP damage (Klyubin, Wang et al. 2011), reduces Abeta plaque number and increases synaptic density in a mouse model of AD (Dong, Yuede et al. 2008). Despite these findings, the clinical efficiency of the drug is still debated (Schneider, Dagerman et al. 2011).

Increased extracellular Glu level may activate esyn NMDARs leading to neuronal overactivation and cell death. More recently, Abeta-induced neuronal hyperexcitability via esyn NMDARs has been suggested to play a critical role in hyperexcitation (Costa, Lacor et al. 2012, Koeglsperger, Li et al. 2013). Indeed, p38-MAPK pathway is altered by Abeta resulting in LTD facilitation via both esyn NMDARs and mGluRs (Chen, Lin et al. 2013). Moreover, blocking

NR2B subunits by ifenprodil or Ro-6981 rescued Abeta-induced LTP disruption (Li, Jin et al. 2011, Zhang, Wang et al. 2013), suggesting that it might be a potential therapeutic target against AD. Indeed, recent papers have suggested that memantine itself has greater affinity for esyn NMDARs that to synaptic NMDARs (Xia, Chen et al. 2010).

It is also likely that Abeta increases extracellular Glu levels by damaging the function of Glu transporters. Jacob *et al.* have found that gene expression and protein levels of EAATs are altered in AD brains, especially in the hippocampus and frontal cortex (Jacob, Koutsilieri et al. 2007). This suggests that by restoring the normal level of EAAT in the brain, a possible protection can be reached. Interestingly, ceftriaxone, a β-lactam antibiotic was shown to have a side effect that increases the expression of EAATs in the brain (Rothstein, Patel et al. 2005), and this might be exploited as a treatment against AD.

Another approach might be to reduce brain Glu level through the BBB. The blood Glu level is regulated by two Glu transaminases, glutamic-pyruvic transaminase (GPT) and glutamic-oxaloacetate transaminase (GOT). These enzymes play a critical role in reducing Glu levels in the blood by converting Glu into other molecules along with their substrates, pyruvate (Pyr) and oxaloacetate (Oxa) (**Figure 5**) (Palmada and Centelles 1998). If the blood Glu level is decreasing, the EAATs localized on the brain capillaries can pump out the excess Glu from the CNS with higher efficiency.

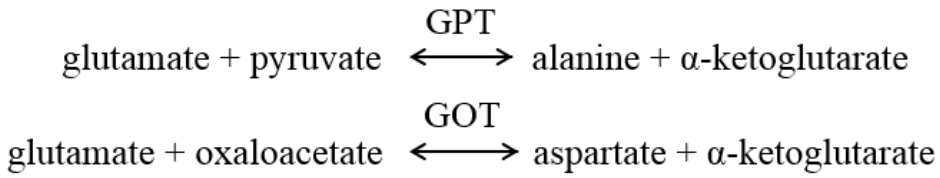


Figure 5. Glu metabolism. Glu can be converted to alanine and α-ketoglutarate (by GPT) or aspartate and α-ketoglutarate (by GOT) by reversible mechanism in the brain. These processes require the substrates of the enzymes. Abbreviations: Glu (glutamate), glutamic-pyruvic transminase (GPT) and glutamic-oxaloacetate transminase (GOT).

It was shown that the intravenous administration of either Pyr or Oxa decreases blood Glu level and subsequently reduces brain Glu concentration (Teichberg, Cohen-Kashi-Malina et al. 2009, Boyko, Stepensky et al. 2012). Based on this fact, blood Glu scavenger system was proposed by Vivian Teichberg as a new approach for the treatment of neurological conditions associated with excitotoxicity (Zlotnik, Gurevich et al. 2007). Indeed, these Glu scavengers have protective effect in several disorders, such as traumatic brain injury (Zlotnik, Gurevich et al.

2007), stroke (Castellanos, Sobrino et al. 2008), ischemia (Marosi, Fuzik et al. 2009) and neuropathy (Nie and Weng 2010). We suggest that this method may be a promising treatment against AD. Indeed, GPT could prevent Abeta-induced LTD enhancement in a slice experiment without affecting baseline synaptic activity (Li, Hong et al. 2009). Moreover, application of a Glu uptake inhibitor mimicked the action of Abeta on LTD enhancement, indicating that Abeta oligomers perturb synaptic plasticity by altering Glu recycling (Li, Hong et al. 2009).

Based on these findings, we aimed to investigate further the connection between Glu homeostasis and Abeta-induced synaptic plasticity impairment.

IV. AIMS

There is growing evidence that Abeta oligomers mediate synaptic impairment in AD, however the exact mechanism of synaptic depression is still unknown.

My Ph.D. work aims to identify a possible pathway of increased neuronal excitability and impaired synaptic plasticity caused by Abeta(1-42) using electrophysiological recordings by a multi-electrode array setup.

We aimed to clarify the following points:

1) Is there a difference between the mechanism of evoked and spontaneous neuronal events?
2) Which receptors are involved in the generation of fEPSP and spontaneous spiking activity?
3) How does Abeta influence evoked and spontaneous neuronal activity?
4) Can we mimick the effect of Abeta by blocking Glu reuptake?
5) Can we prevent the effect of Abeta by reducing the extracellular Glu level in the brain?

V. MATERIALS AND METHODS

5.1. COMPOUNDS

For the preparation of artificial cerebrospinal fluid (ACSF), all salts, glucose, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), (+)-MK801 hydrogen maleate (MK801), α-(4-hydroxyphenyl)-β-methyl-4-benzyl-1-piperidineethanol (+)-tartrate (ifenprodil), sodium pyruvate (Pyr), GPT and DL-threo-β-benzyloxyaspartate (TBOA) were purchased from Sigma-Aldrich (St Louis, MO).

5.2. ANIMALS

The study conformed to EU Directive 2010/63/EU and was approved by the regional Station for Animal Health and Food Control under Project License XVI/8/2013. BALB/c mice were housed in groups of 2-3 under standard conditions (24°C, 12 h light-dark cycle) with food and water available *ad libitum*.

5.3. *EX VIVO* ELECTROPHYSIOLOGY

5.3.1. EXTRACELLULAR RECORDING USING MULTI-ELECTRODE ARRAY

Transverse acute hippocampal slices of 400 µm in thickness were prepared from the brains of 3 month old mice using a McIlwain tissue chopper (Campden Instrument, Loughborough, UK). Slices were incubated in ACSF gassed with 95% O_2, 5% CO_2 at 35°C for 60 min. ACSF was composed of (in mM): 130 NaCl, 3.5 KCl, 3 $CaCl_2$, 1.5 $MgSO_4$, 0.96 NaH_2PO_4, 24 $NaHCO_3$ and 10 D-glucose, pH 7.4. Individual slices were transferred to a 3D multi-electrode array (MEA) chip with 60 tip-shaped and 60 µm high electrodes spaced by 200 µm (Qwane Biosciences, Lausanne, Switzerland, **Figure 6**). The surrounding solution was removed quickly, and the slice was immobilized by placing a grid onto it. The slices were continuously perfused with oxygenated ACSF (3 ml/min at 36°C) throughout the entire recording session. Data were recorded using a standard, commercially available MEA 60 setup (Multi Channel Systems MCS GmbH, Reutlingen, Germany).

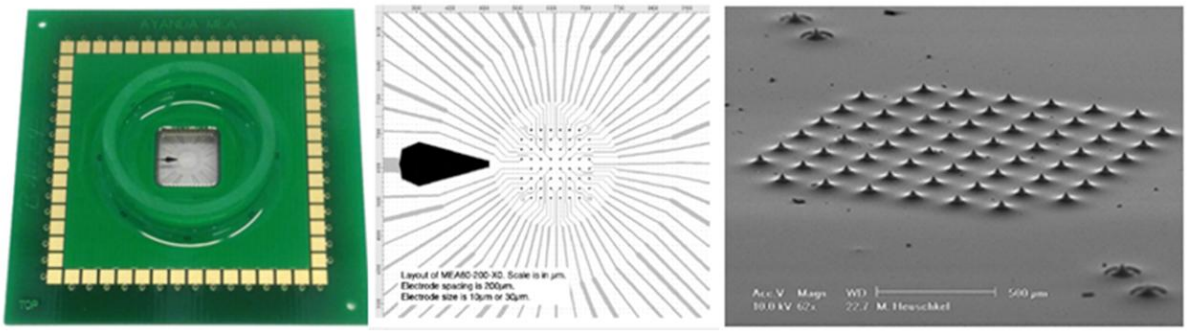


Figure 6. 3D tip-shaped MEA60 biochip. MEA biochips are realised on small glass substrate that consist of micro-fabricated platinum electrodes that are used to measure electrical activity from hippocampal slices (left). Middle figure shows the schematic array of the chip. The tip-shape electrodes penetrate through the upper outer dead cell layer of the slice thereby enabling to record signals that come from healthy neurons in the deeper layer of the slice (right) (Qwane Biosciences, Lausanne, Switzerland). Abbreviation: MEA (multi-electrode array).

5.3.2. STIMULATION PROTOCOL AND DATA ANALYSIS

Care was taken to place the stimulating electrode in the same region at every slice. The Schaffer-collateral was stimulated by injecting a biphasic voltage waveform (−100/+100-µs) through one selected electrode at 0.033 Hz. Evoked fEPSPs were recorded from the *proximal stratum radiatum* at 10 kHz (**Figure 7A**), while spontaneous spiking activity from the *stratum pyramidale* in the CA1 at a frequency of 25 kHz for 5 min epochs (**Figure 7B**). After a 30 min incubation period, the threshold and maximum stimulation intensities for evoked responses were determined. To evoke responses, 30% of the maximal stimulation intensity was used. For analyzing the peak-to-peak amplitudes of fEPSPs MC_Rack program, while for sorting and analyzing the spiking activity, Spike2 software package (Cambridge Electronic Design, Cambridge, UK) was used. Evoked fEPSPs recordings were filtered above 10 Hz, while spontaneous activity was filtered between 300-3000 Hz offline.

Before any treatment, fEPSPs were recorded for 1 hour followed by a 5 min spike recording (0 h). Electric stimulation was ceased during spontaneous activity recordings. After 30 min treatment, spiking activity was also recorded, and the number of spikes after treatment (1.5 h) was normalized to the initial firing activity (0 h). On the other hand, the level of evoked fEPSPs (the last 10 peak-to-peak amplitudes) was compared to the average of the 20 peak-to-peak amplitudes of the control level in all cases (**Figure 8A**).

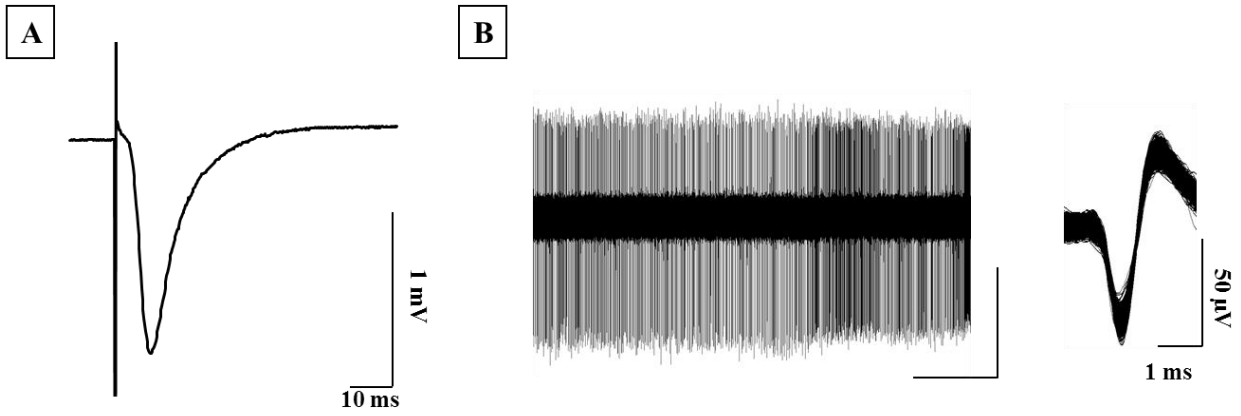


Figure 7. Exemplar recordings in the CA1. Both evoked fEPSPs and spiking activity recording electrodes were located in the CA1 region of a mouse hippocampal slice. The fEPSPs were recorded from the *proximal stratum radiatum* (**A**) and the spontaneous spiking was recorded from the *stratum pyramidale* (**B**). Both fEPSPs and spiking activity were recorded from the same slice. Data were considered as multi-unit activity. Abbreviation: fEPSPs (field excitatory postsynaptic potentials).

In the other groups, which were treated with Abeta, spontaneous activity was recorded before treatment (0 h) followed by 25 min fEPSPs recording, than spiking activity was also recorded for 5 min (0.5 h). Number of spikes was normalized to the initial firing activity (0 h), which was taken as 100% in all channels. The threshold for spike detection was set to 2.5-fold of noise level. The data of those electrodes were included in the analysis where the initial spiking activity was above 0.5 Hz. Firing activity ranged from 150 to 1000 spikes/5 min. Data were considered as multi-unit activity. Field EPSPs were recorded for 25 min, followed by a 5 min spiking recording, then again fEPSPs were recorded. The level of evoked fEPSPs (the last 10 peak-to-peak amplitudes) was compared to the control level in all cases (**Figure 8B**).

In a second set of experiments, where we focused on LTP, slices were treated for 1 h before inducing LTP. The level of LTP (the last 10 peak-to-peak amplitudes) was compared to the average of the last 20 peak-to-peak amplitudes of evoked fEPSPs before applying theta-burst stimulation (TBS). TBS comprised of 15 trains administered at 5 Hz, the individual trains contained 4 pulses separated by 10-ms (**Figure 9**).

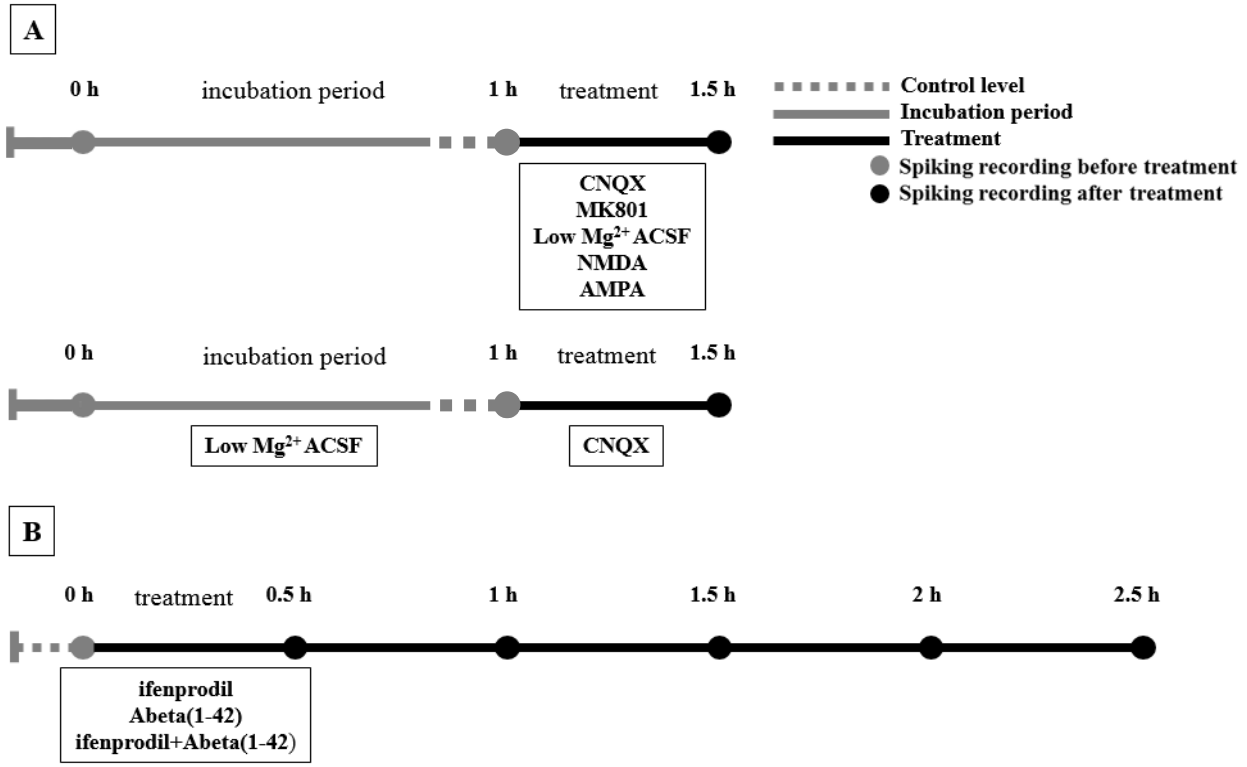


Figure 8. The timeline of the experiments. The fEPSPs were recorded from the *proximal stratum radiatum* and the spontaneous spiking was recorded from the *stratum pyramidale*. Before any treatments, slices were left to incubate for 60 min (grey line), then they were applied (black line). The effect of CNQX was also investigated in Low Mg^{2+} ACSF (middle panel). In this set of experiment, the last 10 min of the incubation period was the control level (dashed line) (**A**). In the other groups, following 10 min control level, slices were applied both Abeta(1-42) and ifenprodil for 2.5 h, and the spiking activity was recorded at every 30 min for 5 min (**B**). Abbreviations: fEPSPs (field excitatory postsynaptic potentials), GluRs (glutamate receptors), Abeta(1-42) (amyloid-beta(1-42)), AMPA (α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), NMDA (N-methyl-D-aspartate), CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), Low Mg^{2+} ACSF (Low Mg_2SO_4-containing ACSF), MK801 ((+)-MK801 hydrogen maleate) and ifenprodil (α-(4-hydroxyphenyl)-β-methyl-4-benzyl-1-piperidineethanol (+)-tartrate).

5.3.3. DRUG TREATMENTS

All slices were incubated for 30 min without stimulation in the recording chamber before any recording was done. Following the first spike-recording (0 h), slices were left to incubate a further 60 min with continuous electric stimulation, then slices were applied with 10 μM CNQX or 10 μM MK801 or 25 μM MK801 or ACSF containing low concentration of Mg_2SO_4 (Low Mg^{2+} ACSF; 0.25 mM) or 0.5 μM NMDA or 0.05 μM AMPA for 30 min (**Figure 8A**). Spontaneous spiking was recorded before and 30 min after the 5 min treatment epochs. The effect of CNQX was also tested in Low Mg_2SO_4-containing ACSF (0.25 mM), where modified ACSF was perfused to the slices from the beginning of the experiment.

Other cohorts of slices were treated with 1 μM oligomer Abeta(1-42), ifenprodil (3 μM) and ifenprodil+Abeta(1-42) for 2.5 h. The spiking activity was also recorded at every 30 min for 5 min (**Figure 8B**).

Next, slices were treated with 1 μM oligomer Abeta(1-42) for 60 min, then LTP was induced by the TBS protocol. LTP was followed for 1.5 hour after TBS. During treatment, evoked fEPSPs were recorded. In the other hand, after 10 min control level, slices were treated with 1 μM Abeta(1-42) or 5 μM TBOA for 60 min before LTP was induced (**Figure 9**). Another cohort of slices was treated with 3 μM ifenprodil or 0.82 mM Pyr for 10 min then 2.06 U/ml GPT for 60 min before LTP induction. Separate groups of slices were treated with these compounds together with Abeta(1-42) or TBOA.

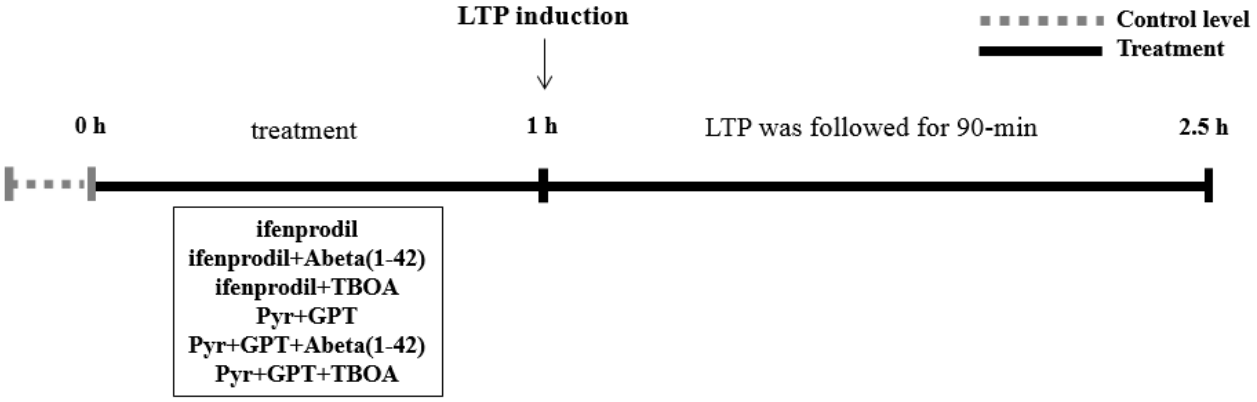


Figure 9. The timeline of the LTP experiments. Evoked fEPSPs were recorded from the *proximal stratum radiatum*. After 10 min control level (grey line), slices were applied different treatments for 60 min (black line), then LTP was evoked by TBS and was followed for 90 min. Abbreviations: LTP (long-term potentiation), fEPSPs (field excitatory postsynaptic potentials), TBS (theta burst stimulus), ifenprodil (α-(4-hydroxyphenyl)-β-methyl-4-benzyl-1-piperidineethanol-(+)-tartrate), Abeta(1-42 (amyloid-beta(1-42)), TBOA (DL-threo-β-benzyloxyaspartate), Pyr (sodium pyruvate) and GPT (glutamic-pyruvic transaminase).

5.4. STATISTICS

Testing for normality was done with the Kolmogorov-Smirnov normality test. Our fEPSPs data have shown normal distribution, hence independent samples t-test and one-way repeated measures analysis of variance (ANOVA) was used with the Bonferroni test for *post-hoc* analysis.

Despite of this, spiking data have shown nonparametric distribution, thus we used nonparametric tests (Kruskal-Wallis test was followed by Mann-Whitney U-test) for determining differences between two and several groups.

The p value ≤ 0.05 was considered significant in all cases. Data were analyzed using SPSS statistical software.

5.5. SYNTHESIS AND CHARACTERIZATION OF AMYLOID-BETA

Detailed description of the synthesis and characterization of Abeta(1-42) is reported in Bozso and his coworkers and in Fulop and her coworkers (Bozso, Penke et al. 2010, Fulop, Mandity et al. 2012). To summarize shortly, a depsipeptide derivative of Abeta(1-42) was synthesized and after purification, it was used in lyophilized form. A 200 μM stock solution of the peptide was prepared in 0.1 mM NaOH, and the pH was set to 11.0. After incubation for 2 hour at ambient temperature, the stock solution was diluted into ACSF to a concentration of 50 μM and the pH was set to 7.3. The peptide solution was incubated for 12 hour at 37°C and prior to use, it was diluted with ACSF to a final concentration of 1 μM.

Aggregation grade and the size distribution of the oligomers were checked by Western Blots (WB) by following the methods described in Fulop, Mandity *et al*. 2012. Oligomers were detected either with sequence-specific BAM10 antibodies (Sigma-Aldrich), or with conformation specific OC antibodies (Millipore), which detects the oligomers of fibrillar nature, i.e. of beta-sheet structure.

VI. RESULTS

6.1. EVOKED FIELD POTENTIALS, BUT NOT SPONTANEOUS SPIKES ARE MEDIATED BY AMPA RECEPTORS

Evoked fEPSP are regarded as the input, while spiking rate as the output of the neuronal network; however, the relationship between these two phenomena is not fully clear. We recorded fEPSPs from the *proximal stratum radiatum* (see above, **Figure 7A**) and in parallel spontaneous spikes from the *stratum pyramidale* (see above, **Figure 7B**) of the CA1 using MEA electrodes. Both fEPSPs and spontaneous activity could be abolished by application of 1 μM tetrodotoxin (data not shown).

First, we investigated the contribution of AMPARs to evoked fEPSPs and spontaneous activity using CNQX (10 μM), an AMPAR inhibitor. Blocking AMPARs resulted in a complete reduction of evoked fEPSPs (untreated: n=5; 98.84±2.41% vs. CNQX: n=5; 4.62±0.45%, $p<0.001$, independent samples t-test; **Figure 10A,B**). Conversely, spontaneous activity remained unaffected (untreated: n=5; 139.55±44.95% vs. CNQX: n=5; 182.18±56.76%, Mann-Whitney U-test; **Figure 10C**), suggesting that AMPARs have a key role in generating fEPSPs, but not spiking activity.

Under physiological conditions, NMDARs activation is dependent on depolarization, e.g. on previous AMPARs activation (Bliss and Collingridge 1993). Thus we changed to Low Mg^{2+} ACSF (0.25 mM) to remove the depolarization-dependent Mg^{2+} plug from the NMDARs (Derchansky, Shahar et al. 2004). Consistent with our previous findings, applying CNQX in Low Mg^{2+} ACSF for 30 min, fEPSPs were completely abolished (untreated: n=7; 101.54±1.69% vs. CNQX in Low Mg^{2+} ACSF: n=6; 6.44±0.36%, $p<0.001$, independent samples t-test; **Supplementary Figure 1**), suggesting that CNQX-inhibited fEPSPs are independent from NMDARs activation.

We have also tried to activate AMPARs by applying a low concentration of AMPA (0.05 μM), but we observed epileptiform field responses and a huge elevation of basal activity which hindered the unambiguous detection of action potentials (**Supplementary Figure 2**).

These findings suggest that evoked fEPSPs, but not spontaneous spikes require AMPARs activity.

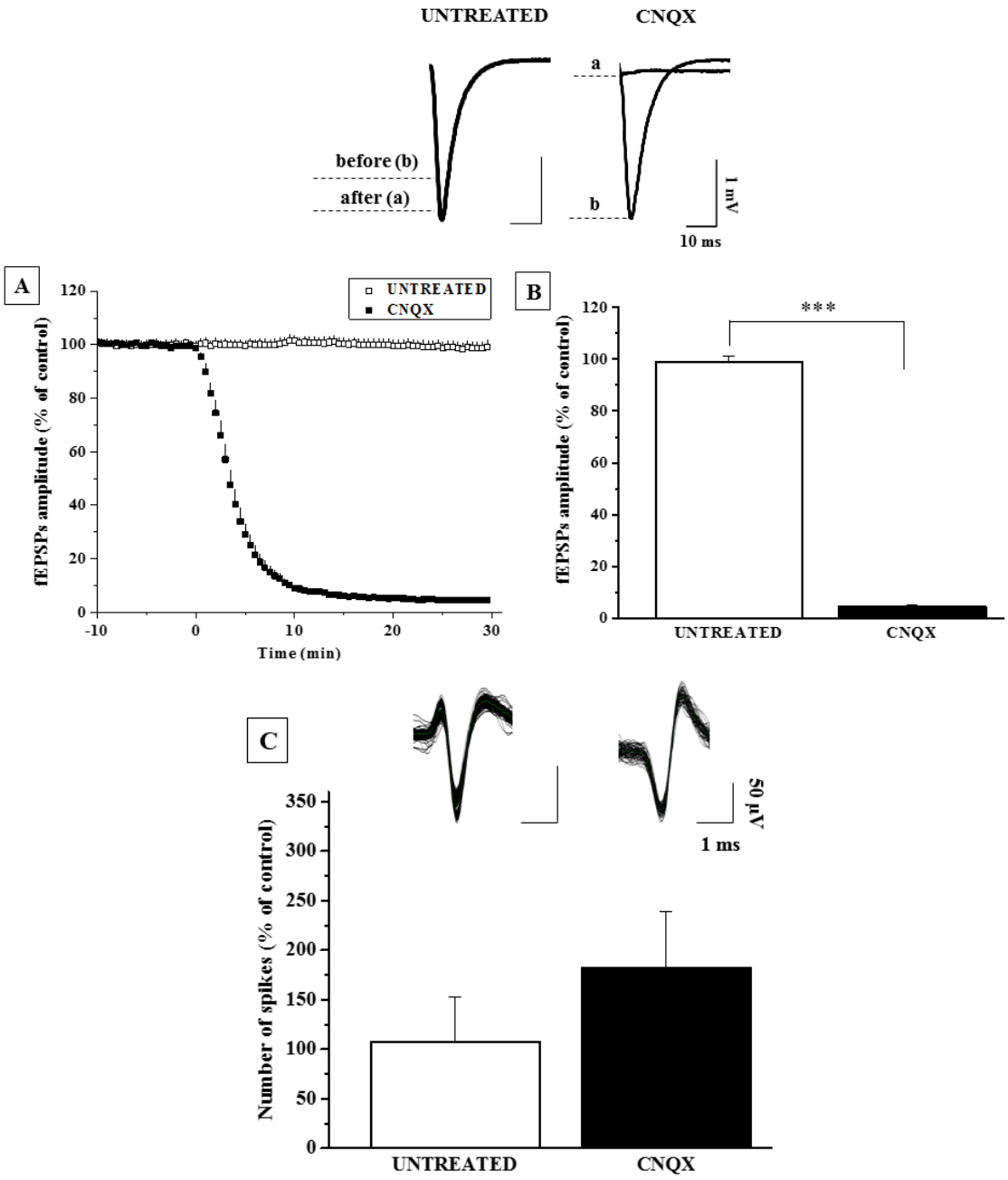
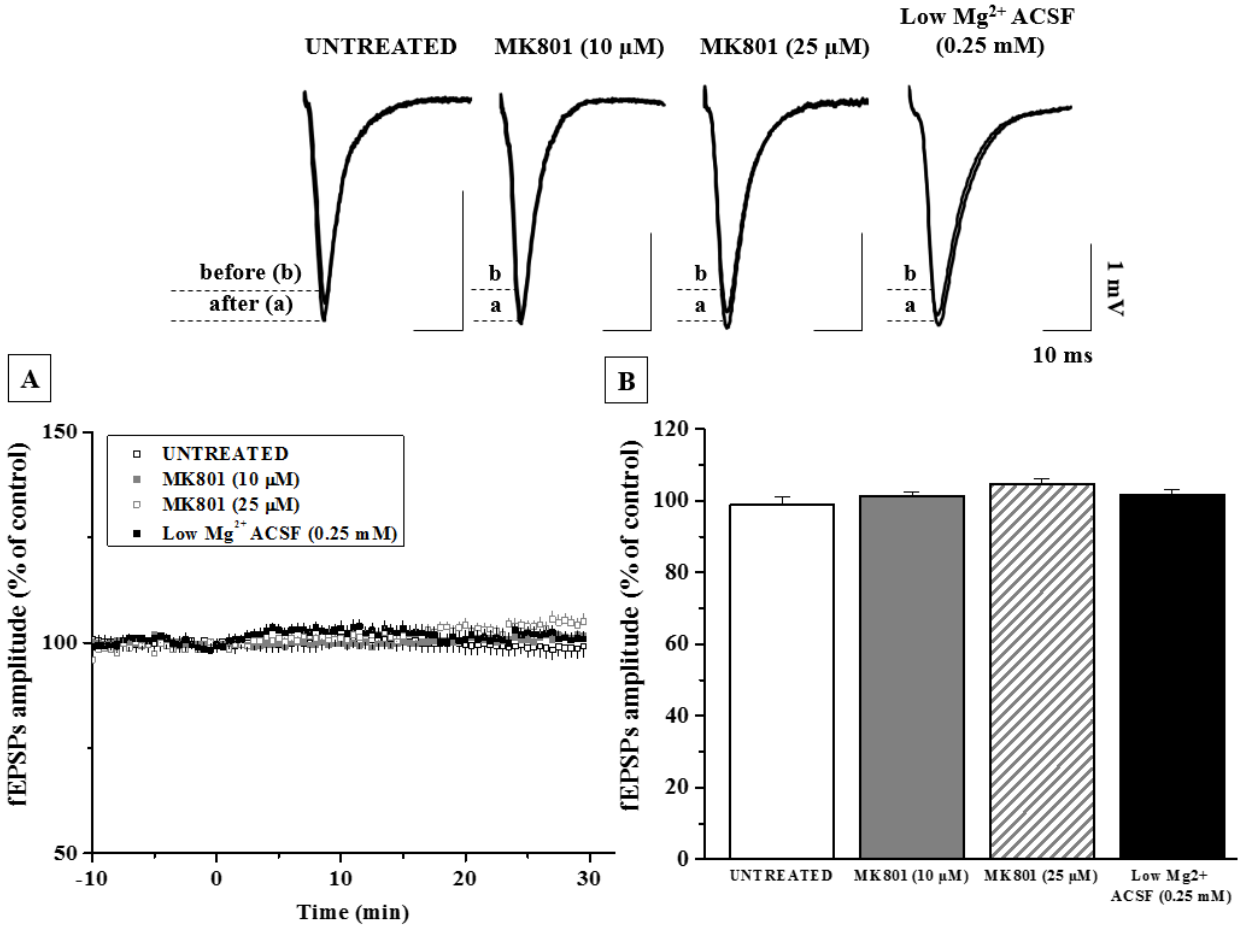


Figure 10. AMPARs regulate mainly fEPSPs but not spontaneous spikes. Panel **A** shows the changes of fEPSPs amplitudes over time: blocking AMPARs completely inhibited fEPSPs compare to untreated slices. The upper panel shows representative fEPSPs before (b) and after (a) treatment. Bar graphs represent the average of the fEPSPs amplitudes of the 25-30 min period after treatment (**B**). CNQX induced a complete reduction of evoked fEPSPs (n=5, $p<0.001$), however the spontaneous activity was not affected (**C**). Above panel C, superimposed representative spontaneous activities. Error bars represent SEM; ***$p\leq0.001$. Abbreviations: fEPSP (field excitatory postsynaptic potential), AMPARs (α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors) and CNQX (6-cyano-7-nitroquinoxaline-2,3-dione).

6.2. SPONTANEOUS FIRING, BUT NOT FIELD POTENTIAL IS GOVERNED BY NMDA RECEPTOR FUNCTION

Next, we focused on NMDARs to test how the modulation of these receptors affect both evoked fEPSPs and spiking activity. Applying an NMDAR antagonist, MK801 did change evoked fEPSP amplitudes in neither 10 µM nor in 25 µM (untreated: n=5; 98.8±2.4% vs. MK801 in 10 µM: n=5; 101.23±1.17% vs. MK801 in 25 µM: n=5; 104.49±1.72, one-way ANOVA, Bonferroni *post-hoc* test; **Figure 11A,B**). In contrast, MK801 caused a dose-dependent inhibition of spiking rate (untreated: n=5; 139.55±44.95% vs. MK801 in 10 µM: n=5; 96.73%±32.22, p=0.48 vs. MK801 in 25 µM: n=5; 29.23±22.93, p=0.035, Mann-Whitney U-test; **Figure 11C**).



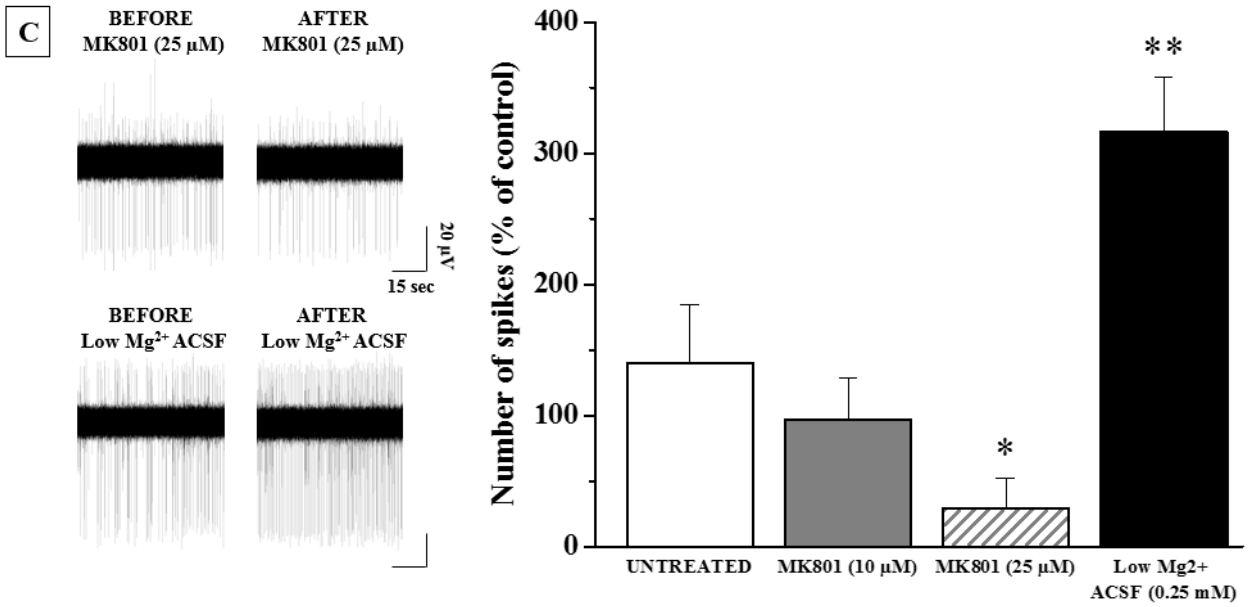


Figure 11. NMDARs influence spontaneous spiking but not fEPSPs. Panel **A** shows the changes of fEPSPs amplitudes over time and the upper panel depicts representative fEPSPs before (b) and after (a) treatments. Blocking (MK801) or enhancing (Low Mg^{2+} ACSF) NMDAR function did not change the fEPSPs amplitudes. Bar graphs represent the average of the fEPSPs amplitudes of the 25-30 min period after treatments (**B**). MK801 dose-dependently reduced spiking frequency, while Low Mg^{2+} ACSF increased firing rate (untreated vs. MK801 in 25 µM: p=0.035 and untreated vs. Low Mg^{2+} ACSF: p=0.004) (**C**). Representative spikes are shown at the left from the respective groups. Error bars represent SEM; *$p\leq0.05$ and **$p\leq0.01$. Abbreviations: NMDAR (N-methyl-D-aspartate receptor), fEPSPs (field excitatory postsynaptic potentials), MK801 (+)-MK801 hydrogen maleate) and Low Mg_2SO_4 ACSF (low Mg_2SO_4-containing artificial cerebrospinal fluid).

Next, we tried to enhance NMDAR function. We have supposed that removal the depolarization-dependent Mg^{2+} plug from the NMDARs may induce elevated spiking activity without any effect on evoked fEPSPs. Indeed, Low Mg^{2+} ACSF induced a massively elevated frequency of the spontaneous activity compared to the untreated slices (n=7, 316.34±41.89%, p=0.004, Mann-Whitney U-test; **Figure 11C**), but evoked fEPSP responses remained unchanged (n=7, 101.54±1.69%, one-way ANOVA, Bonferroni *post-hoc* test).

Based on these results we hypothesized that spontaneous firing is mediated by the ambient Glu concentration acting on the esyn NMDARs.

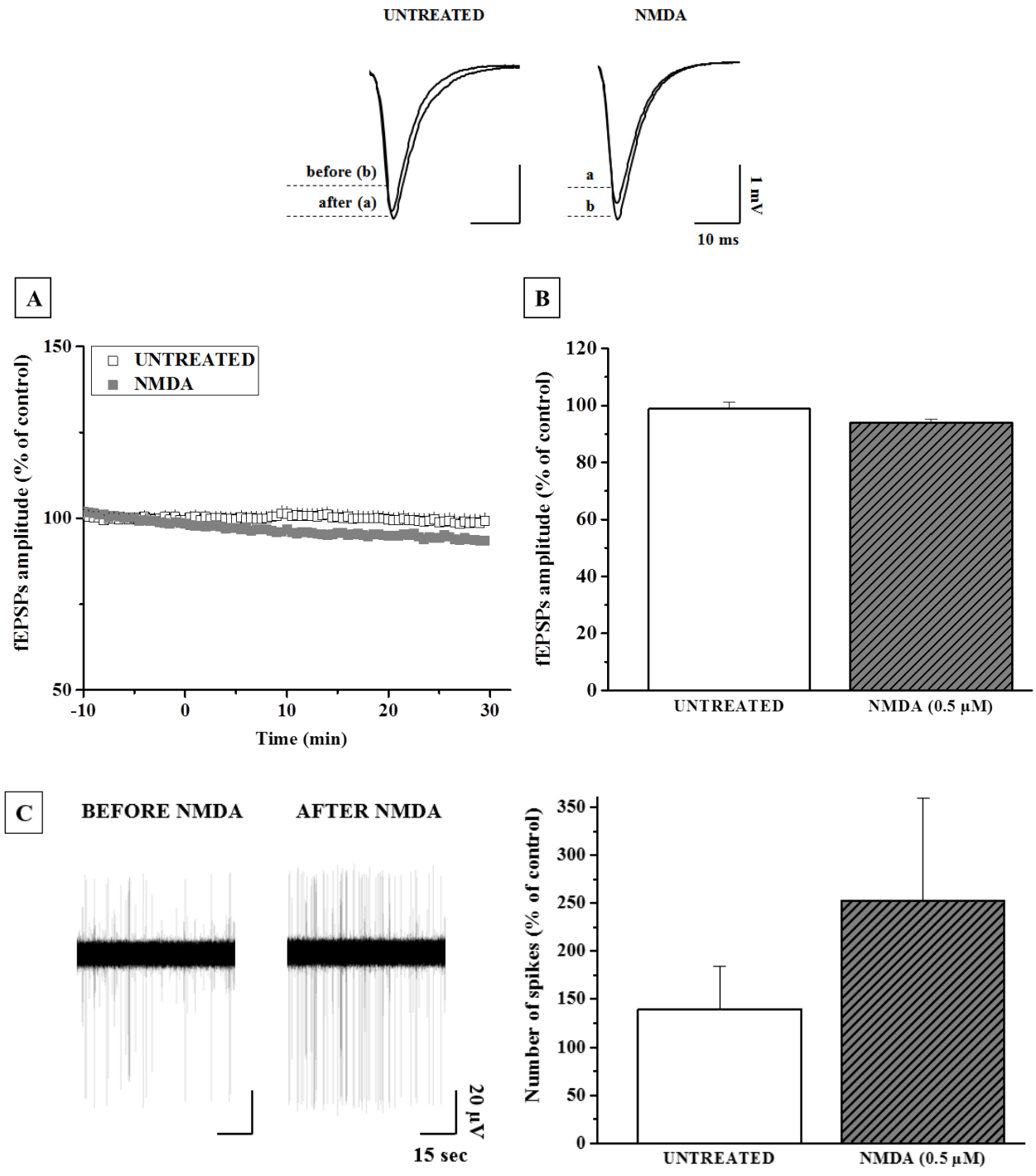


Figure 12. NMDA induces a trend of elevated firing rate without affecting fEPSPs. Figure **A** represents the change of fEPSP amplitudes and the upper panel shows raw fEPSPs before (b) and after (a) treatment. Small concentration of NMDA (0.5 µM), which was reported to activate esyn NMDARs, did not affect fEPSPs. Bar graphs show the average of the fEPSPs amplitudes of the 25-30 min period after treatment (**B**). Low concentration of NMDA elevated spiking frequency, although not significantly (**C**). Left panel shows exemplar spikes from the respective group. Error bars represent SEM. Abbreviations: esyn NMDAR (extrasynaptic N-methyl-D-aspartate receptor) and fEPSPs (field excitatory postsynaptic potential).

Therefore, we tried to activate selectively this set of receptors by applying low concentration of NMDA (0.5 μM) in normal ACSF (Harney, Jane et al. 2008). This treatment resulted in unaltered fEPSPs (untreated: n=5; 98.8±2.40% vs. NMDA: n=9; 94.03±1.04%; independent samples t-test; see above, **Figure 12A,B**), but influenced spiking activity (untreated: n=5; 139.55±44.95% vs. NMDA: n=9; 253.02±105.9%, *p*=0.51, Mann-Whitney U-test; **Figure 12C**). This change is almost significant, thus low concentration of NMDA induced a trend of elevated firing rate.

These various findings suggest that evoked fEPSPs are mediated by AMPARs, but spontaneous spiking mainly requires esyn NMDARs activity.

6.3. AMYLOID-BETA(1-42) INDUCES HYPEREXCITATION VIA NR2B

Excessive neuronal activity is a phenomenon associated with early AD, however underlying mechanisms are not well understood. After establishing that fEPSPs and spontaneous firing are mediated by distinct set of receptors, we tried to investigate the effect of Abeta(1-42) on evoked fEPSPs and spontaneous activity. Spiking rate was determined every 30 min in 5 min epochs within the time frame of the recordings.

In the untreated slices the amplitude of fEPSPs increased slightly until 1.5 h reaching 103.36±2.67% of the initial amplitude, then decreased to initial value (n=5; 0.5 h: 100.8±1.65%; 1 h: 102.9±1.84%; 2 h: 100.86±3.38% and 2.5 h: 98.18±4.04%, one-way repeated measures ANOVA, Bonferroni *post-hoc* test; **Figure 13**). Similarly, spiking frequency did not change over time (n=5; 0.5 h: 124.34±29.72%; 1 h: 129.57±53.28%; 1.5 h: 139.55±44.95%; 2 h: 102.48±28.42 and 2.5 h: 76.16±28.08%, Mann-Whitney U-test; **Figure 14**).

Abeta(1-42) applied in 1 μM did not change fEPSPs amplitudes (Abeta(1-42): n=7; 0.5 h: 107.08±2.43%; 1 h: 106.41±2.93%; 1.5 h: 103.54±3.07%; 2 h: 100.92±3.06% and 2.5 h: 97.1±2.97%, one-way repeated measures ANOVA, Bonferroni *post-hoc* test), compared to untreated slices (see above; **Figure 13**), suggesting that Abeta(1-42) did not affect the AMPAR-mediated synaptic transmission. On the other hand, Abeta(1-42) induced a massively elevated firing (n=7; 0.5 h: 182.6±30.91%; 1 h: 242.29±83.31%, *p*=0.043; 1.5 h: 233.29±83.31%; 2 h: 240.32±85.0%, *p*=0.036 and 2.5 h: 244.72±64.21%, *p*=0.001, Mann-Whitney U-test; **Figure 14**) compared to untreated slices, suggesting that NMDARs are involved in the effect of Abeta(1-42).

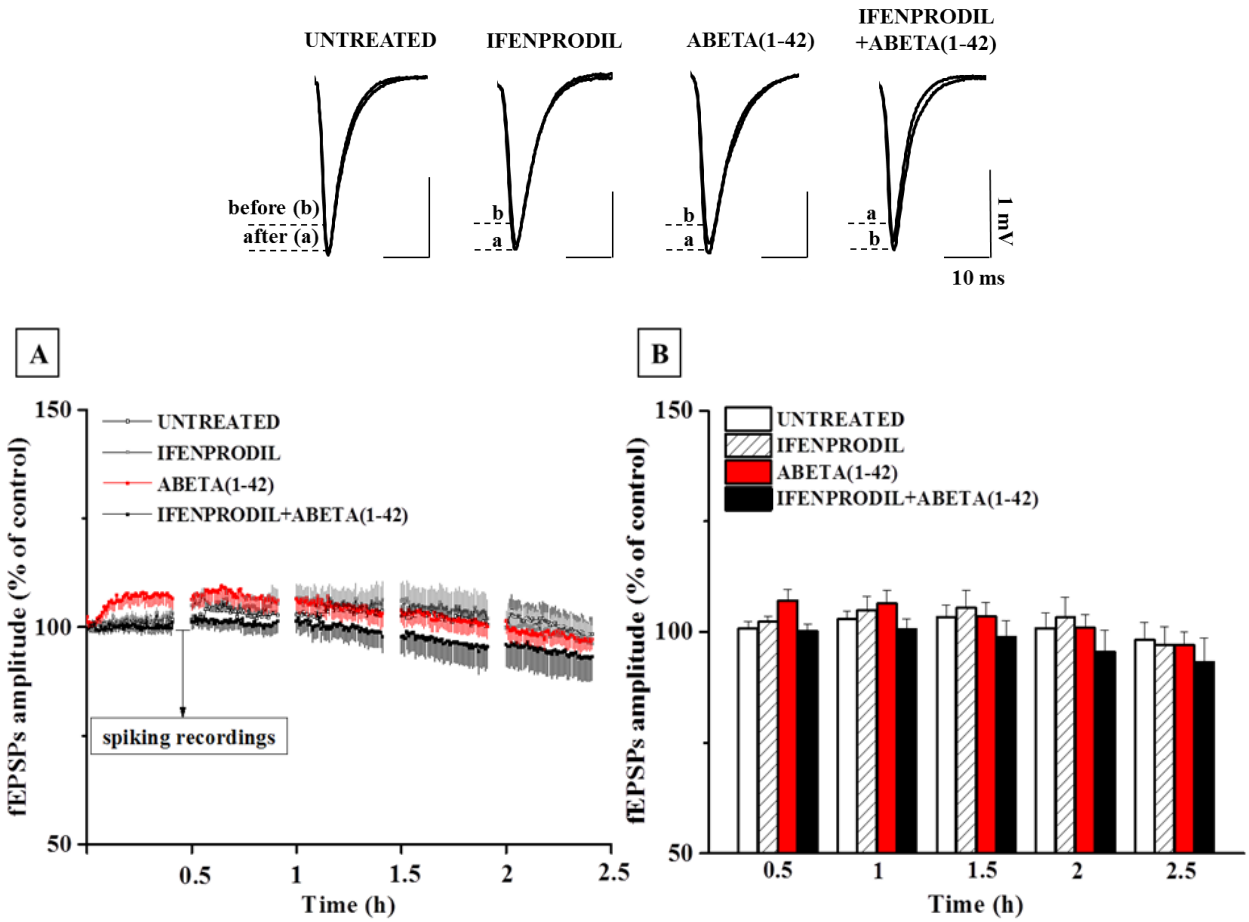


Figure 13. Neither Abeta(1-42) nor ifenprodil changed fEPSPs amplitudes. Representative fEPSPs are shown before (b) and after (a) treatment in the upper panel, and panel **A** shows the changes of fEPSPs amplitudes over time. Neither Abeta(1-42) nor ifenprodil affected fEPSPs amplitudes (one-way repeated measures ANOVA with Bonferroni correction) (**A**). Bar graphs depict the mean of the last 5 min of every recording epoch. The level of fEPSPs amplitudes (the last 10 peak-to-peak amplitudes) were compared to the average 20 peak-to-peak amplitudes of the control level (0 h) in all cases. Error bars represent SEM. Abbreviations: Abeta(1-42) (amyloid-beta(1-42)), ifenprodil (α-(4-Hydroxyphenyl)-β-methyl-4-benzyl-1-piperidineethanol (+)-tartrate) and fEPSPs (field excitatory postsynaptic potential).

Several recent findings suggest that the deleterious effect of Abeta is mediated via the NR2B subunit-containing NMDARs (Tackenberg, Grinschgl et al. 2013, Zhang, Wang et al. 2013). To test whether the observed hyperexcitation in our experimental setup is sensitive to NR2B antagonism, we have applied ifenprodil, an antagonist of the NR2B. Ifenprodil did not change either fEPSPs (ifenprodil: n=5; 0.5 h: 102.26±1.35%; 1 h: 104.95±3.00%; 1.5 h: 105.45±3.91%; 2 h: 103.28±4.53% and 2.5 h: 96.99±4.15%, one-way repeated measures ANOVA, Bonferroni *post-hoc* test; **Figure 13**) compared to the untreated slices, or spiking activity (ifenprodil: n=5; 0.5 h: 127.11±53.62%, 1 h: 163.58±101.77%; 1.5 h: 81.37±37.37%, 2 h: 110.35±65.81% and 2.5 h: 120.26±64.58%, Mann-Whitney U-test; **Figure 14**), suggesting that NR2B-activation is not

required for basic synaptic transmission in the CA1. However, Abeta(1-42) induced elevated spiking activity was prevented by ifenprodil (ifenprodil+Abeta(1-42): n=5; 0.5 h: 157.44±84.19%; 1 h: 196.87±79.61%; 1.5 h: 116.36±61.71%; 2 h: 101.72±76.26% and 2.5 h: 88.88±59.67%, p=0.038, Mann-Whitney U-test; **Figure 14**) without changing of fEPSPs amplitudes (ifenprodil+Abeta(1-42): n=5; 0.5 h: 100.17±1.53%; 1 h: 100.57±2.38%; 1.5 h: 98.85±3.66%; 2 h: 95.45±4.97% and 2.5 h: 93.15±5.47%, one-way repeated measures ANOVA, Bonferroni *post-hoc* test; **Figure 13**).

These results suggest that the hyperexcitability caused by Abeta(1-42) requires the activation of NR2B-subunit containing NMDARs, but not AMPARs.

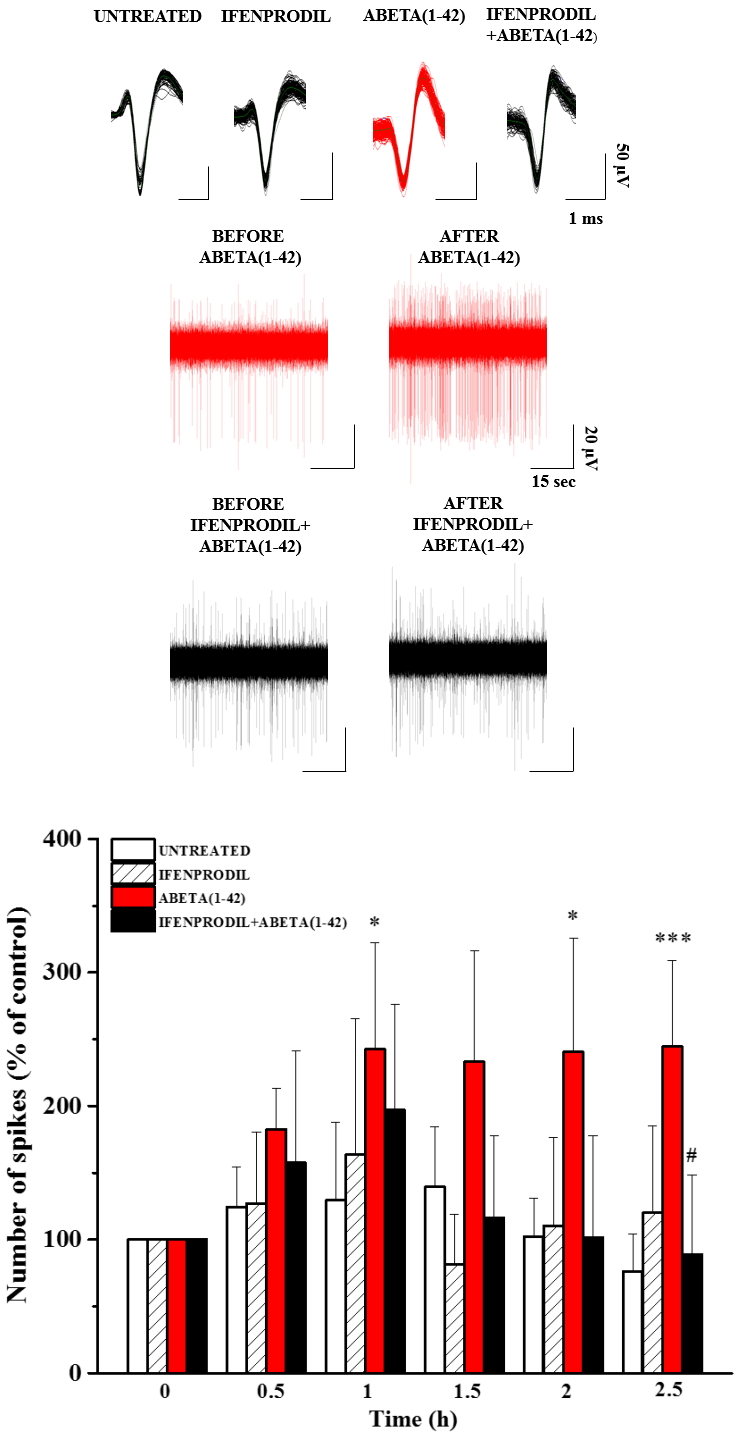


Figure 14. Abeta(1-42) causes hyperexcitation via NR2B. Upper panel shows exemplar units from the respective recordings. Abeta(1-42) induced elevated spiking activity (untreated vs. Abeta(1-42): 1 h: **p*=0.043; 2 h: **p*=0.036 and 2.5 h: ****p*=0.001; Mann-Whitney U-test). Ifenprodil did not affect spontaneous activity, but prevented the increased spiking rate caused by Abeta(1-42) (Abeta(1-42) vs. ifenprodil+Abeta(1-42) 2.5 h $^{\#}p$=0.038; Mann-Whitney U-test). Error bars represent SEM; **p*≤0.05; ****p*≤0.001 and $^{\#}p$≤0.05. Abbreviations: Abeta(1-42) (amyloid-beta(1-42)) and ifenprodil (α-(4-Hydroxyphenyl)-β-methyl-4-benzyl-1-piperidineethanol (+)-tartrate).

6.4. AMYLOID-BETA(1-42) INDUCED IMPAIRMENT OF LTP REQUIRES NR2B ACTIVATION

There is growing evidence that oligomer Abeta impairs synaptic plasticity in AD. Despite intense research, the exact mechanisms involved in Abeta-mediated synaptic dysfunction are not fully clear, thus we investigated the effect of Abeta(1-42) preparation on LTP in the hippocampal slices.

Untreated slices showed a persistent elevated level of fEPSPs after LTP induction (168.33±5.58%, n=12), while Abeta(1-42) reduced the magnitude of LTP (124.35±4.88%, n=9; $p<0.001$, one-way ANOVA, Bonferroni *post-hoc* test; **Figure 15**).

Several studies suggest that different NR2 subunits of NMDARs may have divergent roles in NMDAR-dependent LTP activation and Abeta pathology (reviewed by Koffie, Hyman et al. 2011). To test whether LTP activation requires NR2B-containing NMDARs function, slices were treated with an NR2B antagonist, ifenprodil. We have observed that ifenprodil did not alter the level of LTP compared to untreated slices (176.81±4.93%, n=5; one-way ANOVA, Bonferroni *post-hoc* test), suggesting that NR2B-activation is not required for LTP in the CA1. Furthermore, ifenprodil prevents the Abeta(1-42) induced reduction of LTP magnitude (166.03±12.38%, n=5; $p=0.003$, one-way ANOVA, Bonferroni *post-hoc* test; **Figure 15**), suggesting that Abeta(1-42) induces LTP damage via NR2B-containing NMDARs.

Based on these findings, we propose that NR2B-containing NMDARs activity is not required for LTP, but is essential for the effect of Abeta(1-42).

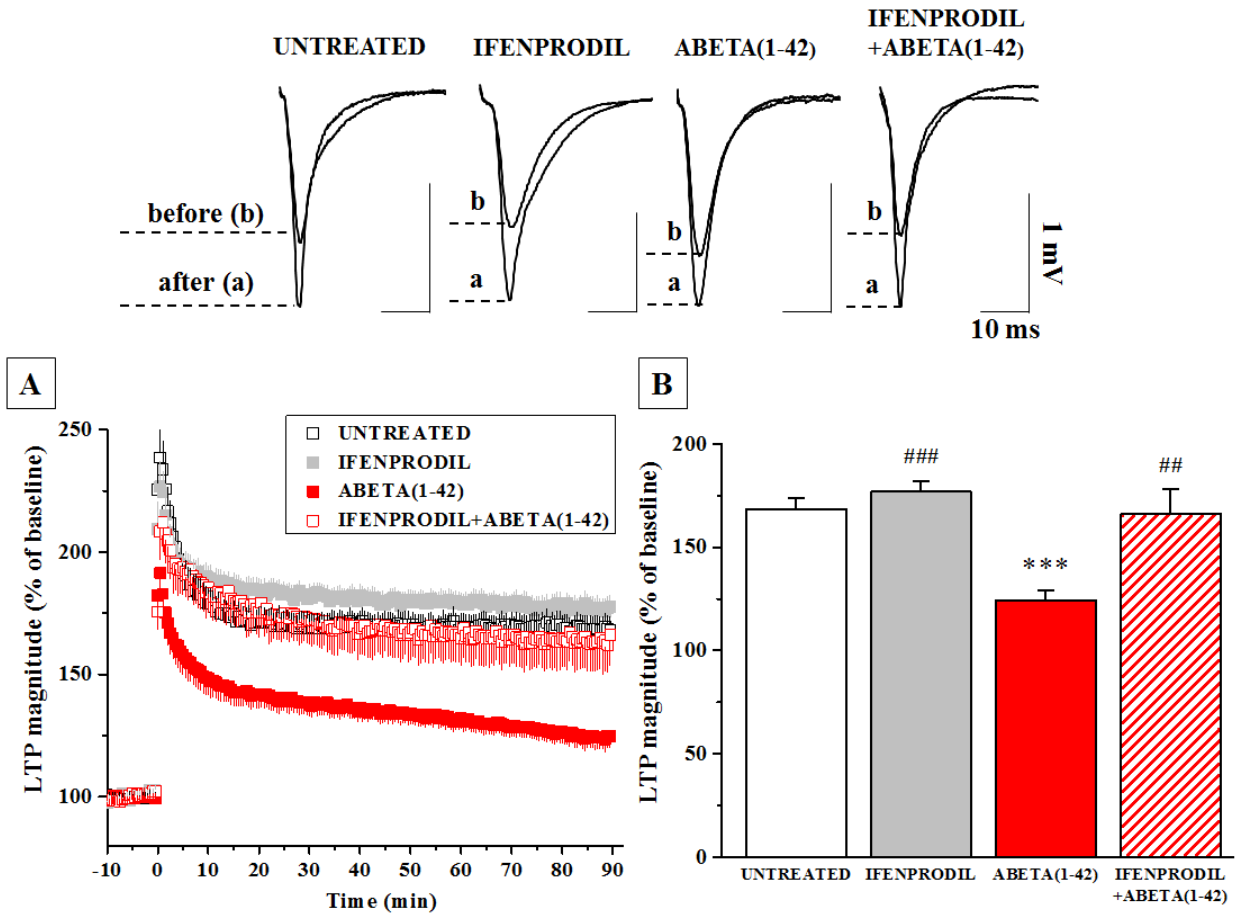


Figure 15. Ifenprodil prevents Abeta(1-42)-induced LTP impairment. Upper panel shows representative fEPSPs before (b) and after (a) treatment. Panel **A** shows the changes of fEPSPs amplitudes over time. LTP was altered in Abeta(1-42) treated slices compared to untreated group (untreated vs. Abeta(1-42): ***p<0.001; one-way ANOVA, Bonferroni *post-hoc* test) (**B**). Ifenprodil did not affect the level of LTP, however it prevented Abeta(1-42)-impaired LTP (Abeta(1-42) vs. ifenprodil+Abeta(1-42): $^{\#\#}p$=0.003; one-way ANOVA, Bonferroni *post-hoc* test). Error bars represent SEM; ***$p\leq$0.001 vs. untreated; $^{\#\#}p\leq$0.01; $^{\#\#\#}p\leq$0.001 vs. Abeta(1-42). Abbreviations: Abeta(1-42) (amyloid-beta(1-42)), LTP (long-term potentiation), fEPSPs (field excitatory postsynaptic potential) and ifenprodil (α-(4-Hydroxyphenyl)-β-methyl-4-benzyl-1-piperidineethanol (+)-tartrate).

6.5. GLUTAMATE SCAVENGER RESCUES AMYLOID-BETA(1-42)-IMPAIRED LTP

Li S *et al.*, suggest a novel mechanism by which Abeta(1-42) oligomers induce synaptic impairment. The authors have shown that the peptide blocks the Glu uptake regulatory system which can lead to synapse depression (Li, Hong et al. 2009). To investigate this possibility, slices were treated with GPT and its substrate, Pyr for 10 min followed by Abeta(1-42) for 60 min, then LTP was induced.

We have found that Pyr+GPT treatment did not change the magnitude of LTP compared to untreated slices (Pyr+GPT: 157.32±6.68%, n=5; one-way ANOVA, Bonferroni *post-hoc* test, **Figure 16**). However, Abeta(1-42)-induced LTP damage was prevented by Glu scavenger (Abeta(1-42) vs. Pyr+GPT+Abeta(1-42): 159.66±6.37%, n=5; p=0.006; one-way ANOVA, Bonferroni *post-hoc* test; **Figure 16**).

Our data suggest that applying Glu scavenger restores the Abeta(1-42)-induced reduction of LTP.

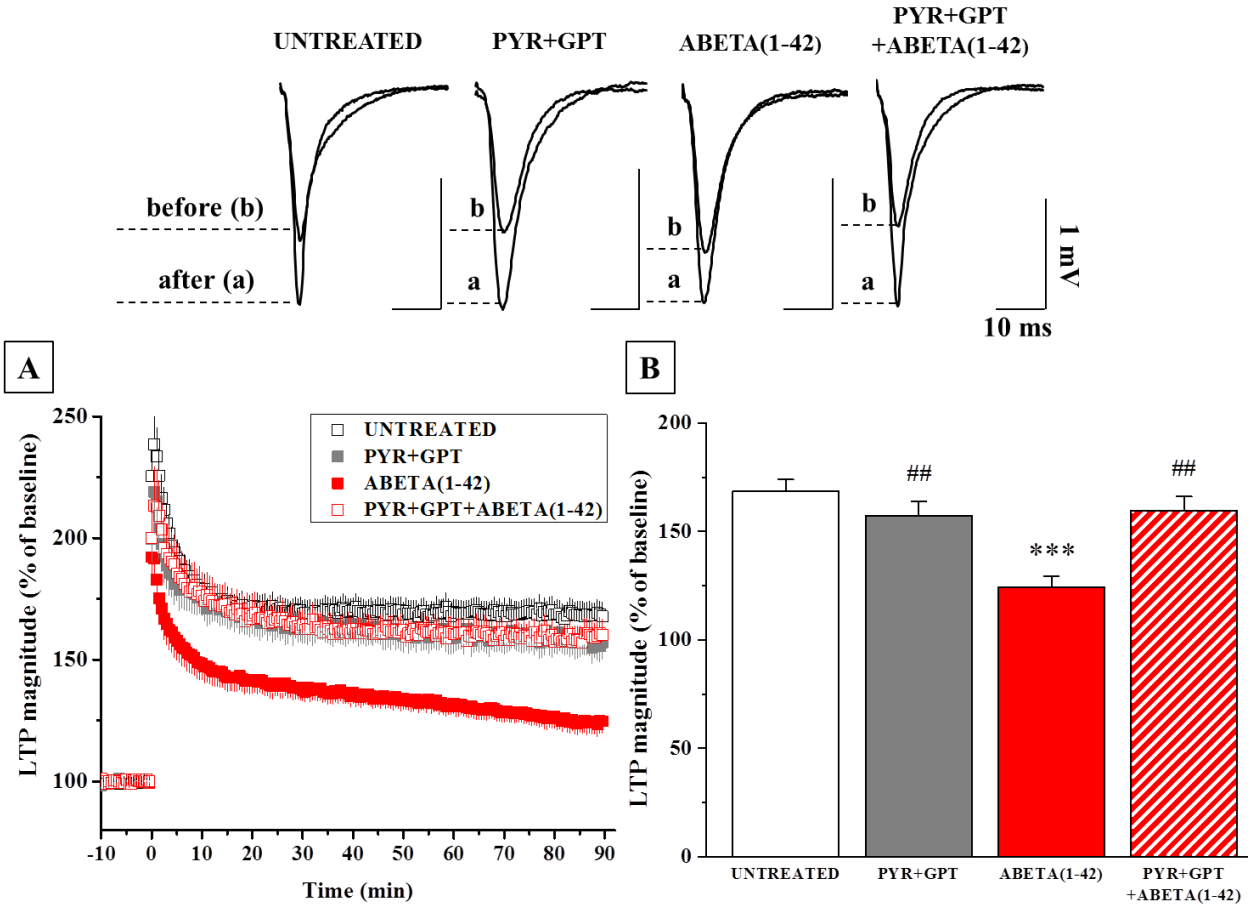


Figure 16. Glu scavenger rescues Abeta(1-42)-impaired LTP. Insets show representative fEPSPs before (b) and after (a) treatment. Panel **A** represents the changes of fEPSPs amplitudes over time. Pyr+GPT treatment did not change the LTP level compared to untreated slices, however Abeta(1-42)-induced LTP damage was prevented by Glu scavenger (Abeta(1-42) vs. Pyr+GPT+Abeta(1-42): $^{\#\#}p$=0.006; one-way ANOVA, Bonferroni *post-hoc* test) (**B**). Error bars represent SEM; ***$p\leq$0.001 vs. untreated; $^{\#\#}p\leq$0.01 vs. Abeta(1-42). Abbreviations: Abeta(1-42) (amyloid-beta(1-42)), LTP (long-term potentiation), fEPSPs (field excitatory postsynaptic potential), Pyr (sodium pyruvate) and GPT (glutamic-pyruvic transaminase).

6.6. THE EFFECT OF AMYLOID-BETA(1-42) IS MIMICKED BY TBOA, A GLUTAMATE REUPTAKE INHIBITOR

Based on previous findings, we supposed that Abeta may interrupt Glu uptake. In order to address, whether a Glu reuptake inhibitor, TBOA can mimick the effect of Abeta(1-42), we applied the slices with TBOA for 60 min before LTP induction. Indeed, LTP was impaired by TBOA compared to untreated slice (123.22±3.48%, n=6; $p<0.001$; one-way ANOVA, Bonferroni *post-hoc* test, **Figure 17**).

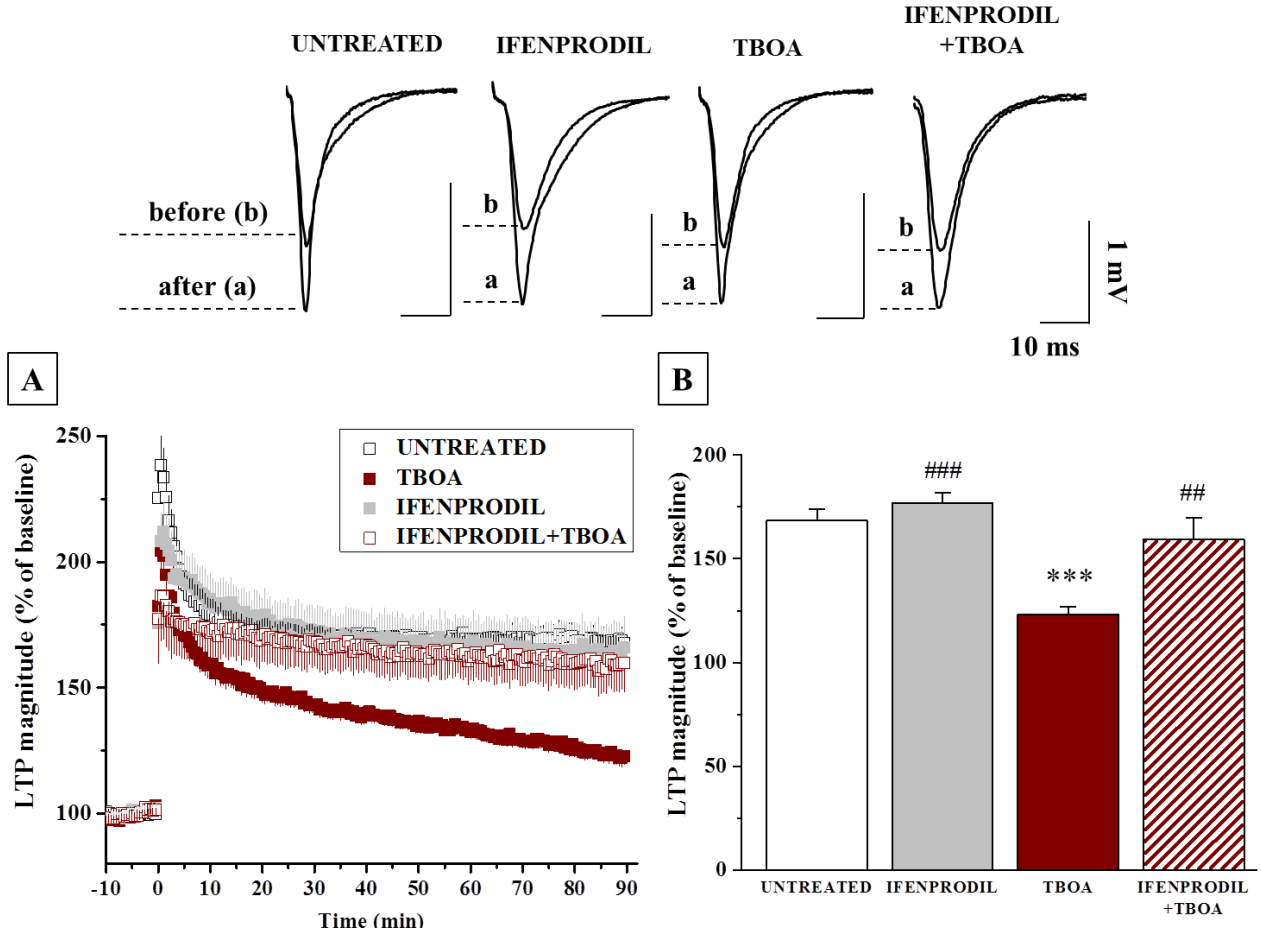


Figure 17. Blocking NR2B restores TBOA-induced LTP impairment. Upper panel shows exemplar fEPSPs before (b) and after (a) treatment. Panel **A** represents the changes of fEPSPs amplitudes over time. LTP was impaired by TBOA compared to untreated group (untreated vs. TBOA: ***$p<0.001$; one-way ANOVA, Bonferroni *post-hoc* test), which was prevented by ifenprodil (TBOA vs. ifenprodil+TBOA: $^{\#\#}p=0.013$; one-way ANOVA, Bonferroni *post-hoc* test) (**B**). Error bars show SEM; ***$p\leq0.001$ vs. untreated; $^{\#\#}p\leq0.01$ and $^{\#\#\#}p\leq0.001$ vs. TBOA. Abbreviations: ifenprodil (α-(4-Hydroxyphenyl)-β-methyl-4-benzyl-1-piperidineethanol(+)-tartrate), TBOA (DL-threo-β-benzyloxyaspartate), LTP (long-term potentiation) and fEPSPs (field excitatory postsynaptic potential).

Next, we tested whether NR2B subunit activation is required for the effect of TBOA. We have found that ifenprodil prevents TBOA-induced LTP damage, which suggests that NR2B subunit activation is essential for the effect of TBOA on LTP (TBOA vs. ifenprodil+TBOA: 159.29±10.67%, n=4; p=0.013; one-way ANOVA, Bonferroni *post-hoc* test, see above, **Figure 17**).

We proceeded to apply Glu scavenger to test whether the inhibitory effect of TBOA was due to the elevated extracellular Glu level. To investigate this possibility, slices were treated with GPT and its substrate, Pyr for 10 min followed by TBOA for 60 min, then LTP was induced. We have found that TBOA failed to impair LTP after Glu scavenger treatment (TBOA vs. Pyr+GPT+TBOA: 169.28±8.18, n=5; p=0.001; one-way ANOVA, Bonferroni *post-hoc* test, **Figure 18**).

Collectively these results suggest that TBOA and Abeta(1-42) share common pathway in synaptotoxicity. The effect of Abeta(1-42) is mimicked by Glu reuptake inhibition, however both could be prevented by a Glu scavenger and NR2B inhibition, suggesting that Abeta(1-42) disrupts synaptic plasticity by altering Glu recycling at the synapse in the CA1.

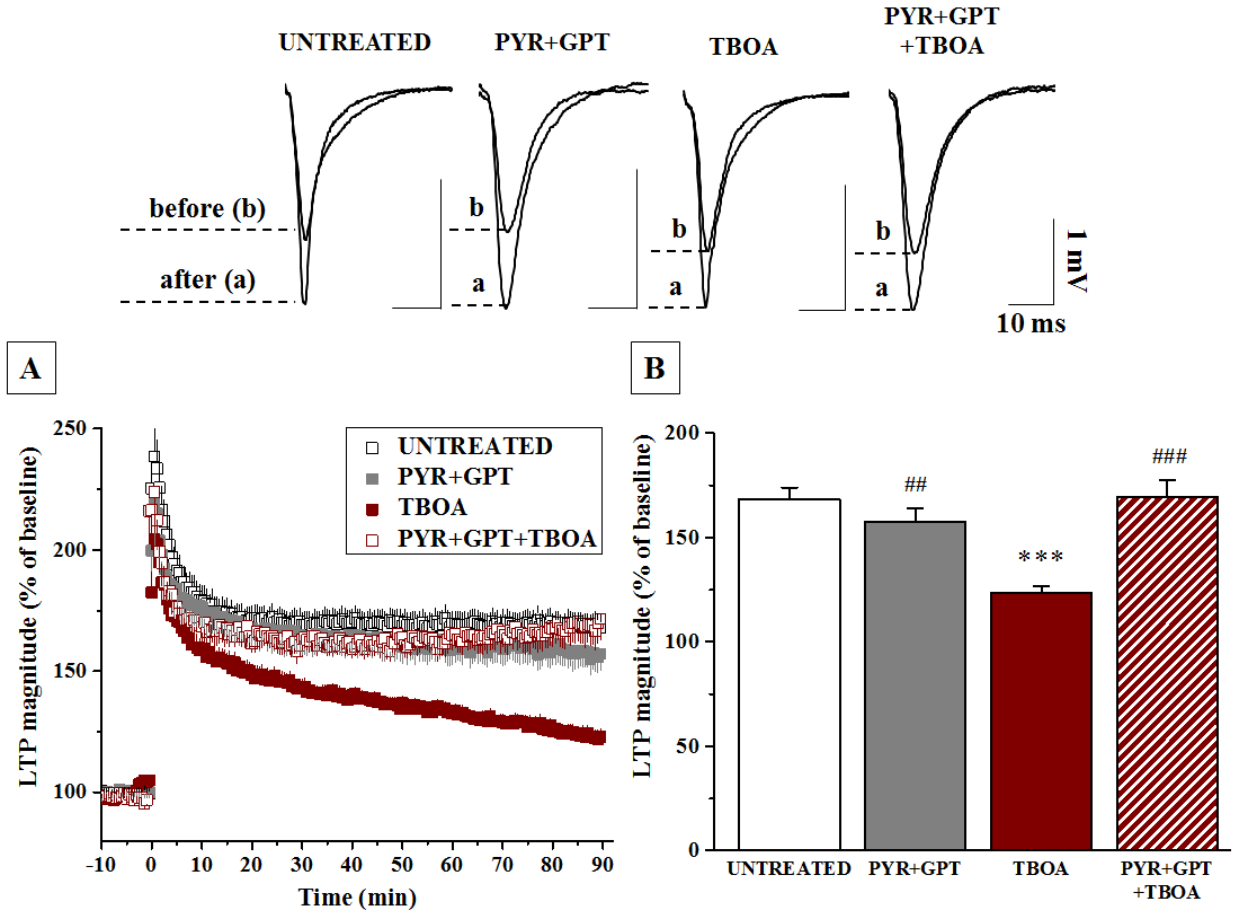


Figure 18. Glu scavenger restores the effect of TBOA. Insets show representative fEPSPs before (b) and after (a) treatment. Panel **A** represents the changes of fEPSPs amplitudes over time. TBOA impaired LTP, which was rescued by Glu scavenger (TBOA vs. Pyr+GPT+TBOA: $^{###}p$=0.001; Bonferroni *post-hoc* test). Error bars represent SEM; ***$p\leq0.001$ vs. untreated; $^{##}p\leq0.01$ and $^{###}p\leq0.001$ vs. TBOA. Abbreviations: TBOA (DL-threo-β-benzyloxyaspartate), LTP (long-term potentiation), fEPSPs (field excitatory postsynaptic potential), Pyr (sodium pyruvate) and GPT (glutamic-pyruvic transaminase).

6.7. CHARACTERIZATION OF AMYLOID-BETA

The size distribution of Abeta(1-42) oligomers formed in 50 μM after incubation at 37°C was studied on WB by using two different antibodies: the monoclonal BAM10 antibody is sequence specific and binds to the N-terminal end of the peptide, while OC stains the oligomers of fibrillar nature (protofibrils, oligomers with beta-sheet structure (Glabe 2008).

Supplementary Fig. 3 shows the results of the WB experiments (**Supplementary Figure 3**). BAM10 staining reveals the presence of both low- and high- molecular weight oligomers in the sample, while their positive staining with the OC antibody indicates that they have protofibrillar characteristics. The presence of SDS stable dimers and trimers in the sample closely resembles to Abeta(1-42) derived from biological sources, namely from transfected 7PA2 cells (Davis,

Marsden et al. 2011) and from human brain (Shankar, Bloodgood et al. 2007, Klyubin, Betts et al. 2008). These species were shown to have strong synaptotoxic properties (Townsend, Shankar et al. 2006). On the other hand, protofibrillar species, which are also abundant in our sample, were also reported to be synaptotoxic (Minkeviciene, Rheims et al. 2009).

VII. DISCUSSION

Neuronal hyperexcitability in early AD and in AD modeling mice is an emerging finding that points to a network dysfunction as a critical component of the pathomechanism. Soluble Abeta species have been increasingly implicated as the key pathologic components of the disease, thus elucidating the mechanisms by which these species alter neuronal function is important. There is growing evidence that soluble Abeta oligomers mediate synaptic impairment in AD, but the exact mechanism of synaptotoxicity remains to be determined.

In my thesis work, we examined a possible pathway of neuronal excitability and impaired synaptic plasticity caused by Abeta. First, we identified two possible mechanisms that regulate extracellular excitatory events in the CA1, and then we examined how Abeta affects these events. Second, we investigated the receptors and receptor subunits that are required for Abeta-induced excitotoxicity and LTP impairment. Third, we tested whether interfering with the pathway we identified previously can prevent the effect of Abeta peptide.

7.1. THE UNDERLYING MECHANISM OF EVOKED FIELD POTENTIAL AND SPONTANEOUS SPIKING GENERATION ARE NOT CORRELATED

Extracellular recordings of fEPSP is one of the most common ways for determining the excitation and synaptic plasticity in hippocampal slices. Evoked fEPSPs are commonly evoked by electric stimulation at Schaffer-collaterals and are recorded from the proximal apical dendrites of the CA1 *stratum radiatum*. Monitoring the changes in the peak-to-peak amplitudes of fEPSPs is widely used as a readout for investigation of synaptic plasticity. The changes of spontaneous spiking activity under conditions that modify network excitability are, however, less well studied in acute slices. In these sets of experiments we have studied the involvement of AMPARs and NMDARs in fEPSPs evoked by Schaffer-stimulation and in spontaneous firing in the CA1.

We shown that these two electrophysiological markers previously thought to underlie measures of excitation, are not correlated. Under our conditions, inhibiting AMPAR with CNQX blocked fEPSPs, however, the spiking rate remained unaffected. In contrast, modulating NMDAR function influenced spontaneous activity without any change in evoked fEPSPs. Blocking NMDARs ablated, while enhancing NMDAR function increased spontaneous activity.

Importantly, the spontaneous spikes we recorded here were almost exclusively localized to the *stratum pyramidale* layer of CA1; hence they were presumably generated by principal cells.

Multi-unit activity recordings do not allow to separate the firing from individual neurons and may include bursts of spikes fired by the same neuron. Thus in our study, spiking represents the global amount of activity within this network, and most probably the activity of principal cells. The firing rates observed in this study were comparable to previously reported values in slices of CA1 of the guinea pig (0.22 and 1.8 spikes/sec) (Cohen and Miles 2000).

7.2. POSSIBLE INVOLVEMENT OF THE CA1 LOCAL MICROCIRCUITRY IN THE REGULATION OF SPONTANEOUS SPIKING

The activity of CA1 pyramidal cells is regulated by the excitation arriving from CA3 through the Schaffer-collateral, and by the inhibition of the local interneuron microcircuitry. Although the activity of CA1 inhibitory cells has been reported to be scarce compared to CA3 in acute slices, powerful pyramidal somatic inhibition could be detected upon minimal electric stimulation (Beyeler, Retailleau et al. 2013), showing that the inhibition is very effective.

What is the driving force of the interneuronal activity? One possible explanation is that the spontaneous activity of the incoming axons of the CA3 principal cells recruits a CA1 inhibitory microcircuitry via the Schaffer-collateral. This is unlikely, because there is no correlation between CA3 multi-unit activity and the extracellularly recorded inhibitory post-synaptic potential in the CA1 (Beyeler, Retailleau et al. 2013). Another possibility is that the spontaneous discharge of CA1 interneurons, although scarce, is keeping a strong blockade of the principal cells. Favoring this hypothesis, the resting membrane potential is more hyperpolarized in CA1 compared to CA3 pyramidal neurons (Fricker, Verheugen et al. 1999, Tyzio, Ivanov et al. 2003). These spontaneous discharges might be regulated by the ambient Glu concentration via NMDARs. Indeed, tonic activation of NMDARs by ambient Glu has been described in virtually all pyramidal cells of the CA1 of the hippocampus in slice models (Sah, Hestrin et al. 1989, Dalby and Mody 2003, Angulo, Kozlov et al. 2004, Cavelier, Hamann et al. 2005). Importantly, as Le Meur et al. has shown (Le Meur, Galante et al. 2007), neither AMPA/kainate receptors nor mGluRs contributes to this tonic excitation of pyramidal neurons. This tonic current is not dependent on vesicular release of transmitters from neurons, but is affected by inhibition of the enzyme converting Glu in glutamine in glial cells, indicating that ambient Glu is mainly of glial origin. Consistent with these findings, we have also shown that spontaneous spiking is driven by NMDAR activity, but not by AMPARs, indicating that the tonic activation could reach the threshold of spike generation.

The question arises that why do not AMPARs mediate spontaneous activity? NMDARs have a much higher affinity for Glu than do AMPARs; in steady-state conditions, the EC50 for Glu at NMDARs is over two orders of magnitude lower than at AMPARs (Patneau and Mayer 1990). Esyn NMDARs containing the NR2B subunit have even higher affinity than synaptic NR2A-containing receptors (Priestley, Laughton et al. 1995). It should be noted, that evoked firing, which is usually detected in the form of population spikes (pop-spikes for short) in CA1 extracellular recordings, is mediated by AMPAR signaling (Wood and Tattersall 2001). Under our conditions, action potentials were not inhibited by AMPAR blockade (CNQX). The reason behind this discrepancy might be that evoked pop-spikes require a synchronous discharge of a population of cells. This coordinated function might be based on the fast AMPAR function.

7.3. AMYLOID-BETA(1-42)-INDUCED HYPEREXCITATION AND LTP IMPAIRMENT REQUIRES NR2B ACTIVATION

Numerous studies have reported that Abeta can affect the function of NMDARs (Shankar, Bloodgood et al. 2007, Juhasz, Marki et al. 2009, Dinamarca, Rios et al. 2012, Tackenberg, Grinschgl et al. 2013, Xu, Cao et al. 2014), which may lead to excitotoxicity and neuronal hyperactivation seen in the early stage of AD. Abeta can bind to integrin receptors and this complex initiates a molecular cascade mediated by Fyn kinase, which subsequently phosphorylates NMDARs (Larson, Sherman et al. 2012). Furthermore, there is evidence that Abeta affects esyn NMDAR function. Memantine, a clinically effective drug in the treatment of AD, was shown to block preferentially esyn NMDARs, further arguing for the Abeta and esyn NMDARs connection (Szegedi, Juhasz et al. 2010, Xia, Chen et al. 2010).

Next, we investigated the effect of Abeta(1-42) on spontaneous firing, and found that the peptide increased the rate of spontaneous discharges, but did not alter evoked fEPSPs. These results suggest that NMDARs are involved in the hyperexcitability induced by Abeta(1-42). Similar results have been recently reported by Varghese *et al*. using cultured embryonic rat hippocampal cells. Abeta increased spontaneous firing activity dose-dependently, reaching the maximum after 1 hour of treatment, which was followed by a complete cessation of spikes following a few hours (Varghese, Molnar et al. 2010). The discrepancy between this and our result may be due to the fact that the authors have used a cell culture largely devoid of glial elements, while we have used a complex microcircuitry built up from all of the neuronal cell types (including glia) forming elaborate connections.

Abeta induced enhancement of spontaneous spiking activity could be prevented by blocking the NR2B subunits, suggesting that Abeta activates esyn NMDARs. The role of esyn or perisynaptic NMDARs in Abeta(1-42)-induced changes in functional synaptic plasticity and subsequent cell death has been received much attention recently (Palop and Mucke 2009, Li, Jin et al. 2011, Talantova, Sanz-Blasco et al. 2013). Esyn NMDARs contain predominantly NR2B subunits, which in contrast to NR2A-containing synaptic NMDARs trigger apoptotic signaling cascade (Hardingham, Fukunaga et al. 2002). Recently, it was shown using organotypic hippocampal cell cultures, that Abeta induces neuronal death and subsequent tau hyperphosporylation via esyn NR2B subunit (Tackenberg, Grinschgl et al. 2013).

Blocking NR2B subunits does not only prevent hyperexcitation caused by Abeta, but also rescues Abeta induced LTP impairment, suggesting that blocking NR2B subunits might be a promising target in AD. Tackenberg *et al*. also suggest that esyn NR2B-enriched NMDARs activation is essential for tau-dependent neurodegeneration (Tackenberg, Grinschgl et al. 2013). It was also shown that the opposite form of synaptic plasticity, LTD requires both syn and esyn receptor activation (Liu, Yang et al. 2013), while LTP is not mediated by esyn NMDARs (Hu, Klyubin et al. 2009, Zhang, Wang et al. 2013). It should be noted, however, that contradictory data is available on the role of NR2B-subunit containing NMDARs on LTP: esyn NDMARs are also recruited to the synapse during LTP (Harney, Jane et al. 2008) and may play an essential role in LTP maintenance (Foster, McLaughlin et al. 2010, Hasegawa, Mukai et al. 2014). However, others have reported that Abeta oligomers directly activate NMDARs especially via NR2A subunit (Domingues, Almeida et al. 2007, Texido, Martin-Satue et al. 2011). In our hands, ifenprodil is not required the LTP magnitude suggesting that NR2B activation do not require for LTP.

7.4. GLUTAMATE SCAVENGER AS A NOVEL THERAPEUTIC OPPORTUNITY AGAINST ALZHEIMER'S DISEASE

Since the late 1980's, disturbances in the Glu uptake system has been suggested as a cause for AD which leads to excitatoxicity in the hippocampal and the cortical regions (Hardy, Cowburn et al. 1987, Perry, Perry et al. 1987). The spillover of Glu from the synapse contributes to normal physiological processes in the hippocampus (Asztely, Erdemli et al. 1997, Min, Rusakov et al. 1998, Scimemi, Fine et al. 2004). However, under certain pathological conditions,

increased spillover results in excessive esyn receptor activation, which enhances network excitability and promotes pro-apoptotic pathways.

Abeta has been shown to elevate extracellular Glu concentration in the brain without altering gamma-aminobutyric acid (GABA) level (Mura, Zappettini et al. 2012). The mechanism behind this is probably the inhibition of the transporters mediating Glu clearance (**Figure 19A**). The level of brain extracellular Glu is regulated by EAATs expressed mainly on the astrocytes, which efficiently remove the excess of this neurotransmitter from the synaptic cleft (reviewed by (Anderson and Swanson 2000), thus Glu transporters play a critical role in limiting esyn diffusion of Glu and provide normal function of glutamatergic neurons (Hertz and Zielke 2004). Indeed, Abeta was shown to inhibit both glial and neuronal EAATs (Bashir, Alford et al. 1991, Masliah, Alford et al. 2000, Li, Hong et al. 2009, Marosi, Fuzik et al. 2009). Rossi and coworkers have reported that under certain pathological conditions, for example during epileptic activity, ischaemia or AD, EAAT may reverse its function leading to Glu release into the extracellular side (Rossi, Oshima et al. 2000). Furthermore, down-regulation or abnormal expression and protein levels of EAAT1 and EAAT2 are altered in the hippocampus and frontal cortex of AD patients (Scott, Pow et al. 2002, Jacob, Koutsilieri et al. 2007) and in APP transgenic mice (Masliah, Alford et al. 2000), further supporting that Glu level regulation fails during AD pathomechanism. These effects may lead to Glu spillover from the synapses and subsequent activation of esyn receptors, however the exact mechanism by which Abeta impairs EAATs remains unknown. Some available data suggest an indirect mechanism, namely Abeta interaction with the cell membrane or after being endocytosed, the peptide can trigger intracellular signaling pathways in astrocytes (Wyss-Coray, Loike et al. 2003), which may modulate Glu transporters levels/activity by alternating the phosphorylation states of EAATs (Daniels and Vickroy 1999, Gosselin, Meylan et al. 2013). Moreover, Abeta induced hyperexcitability appears to involve tau, because deletion of tau reduces seizures in some of the transgenic mouse models of AD (Roberson, Scearce-Levie et al. 2007). Li S *et al.* suggest that Abeta depresses peak NMDAR currents as a result of desensitization of the receptor (Li, Shankar et al. 2010). Interesting result is that several Glu uptake inhibitors are able to reduce NMDAR desensitization (Arnth-Jensen, Jabaudon et al. 2002).

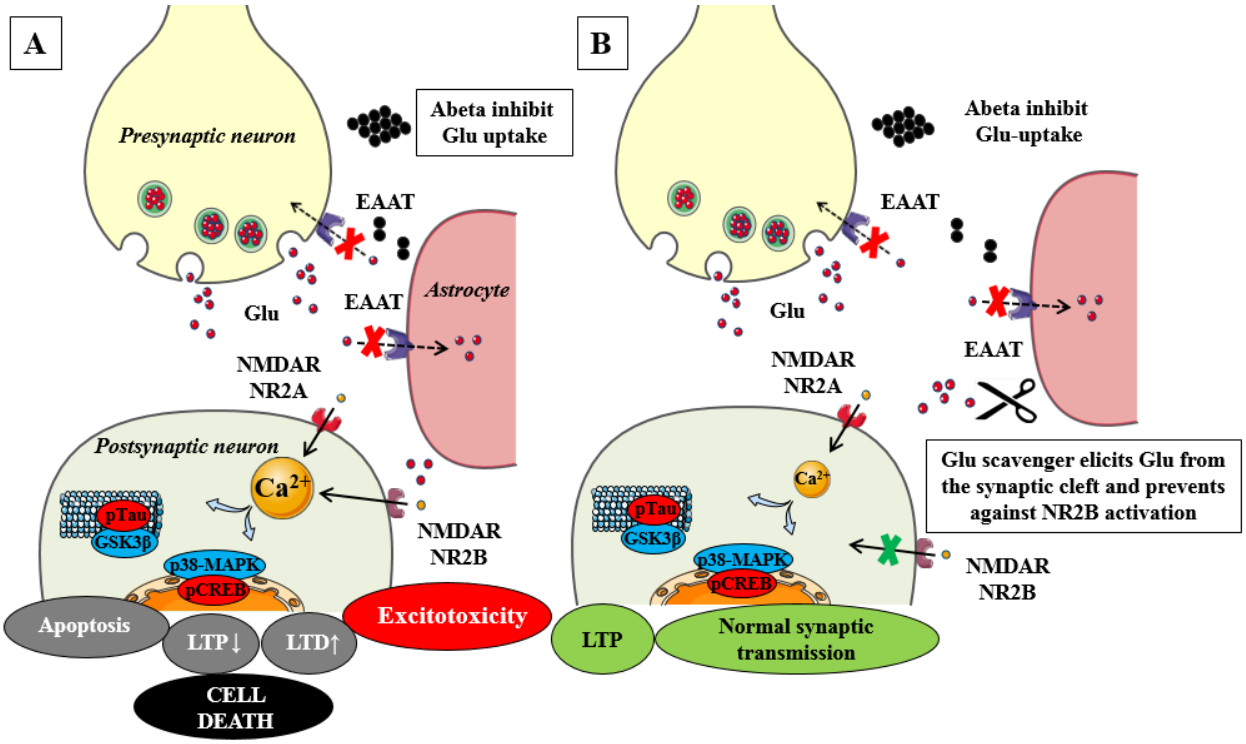


Figure 19. Abeta(1-42) perturbs Glu recycling at synapses. Abeta enhances the ambient Glu level by the blockade of Glu transporters (EAATs). Elevated ambient Glu level would be a spillover and subsequent esyn NR2B-enriched esyn NMDAR activation. Excessive activation of NMDARs increases the influx of Ca^{2+} and activates signaling pathways responsible for tau hyperphosphorylation (GSK3β), inhibited LTP (p38-MAPK), enhanced LTD, apoptosis and finally cell death (**A**). Decreasing extracellular Glu level using Glu scavenger may be able to prevent the synaptic plasticity impairment caused by Abeta (**B**). Abbreviations: Abeta(1-42) (amyloid-beta(1-42)), Glu (glutamate), EAATs (excitatory amino-acid glutamate transporters), esyn (extrasynaptic), NMDAR (N-methyl-D-aspartate receptor), GSK3β (glycogen synthase kinase 3β), LTP (long-term potentiation), LTD (long-term depression), pTau (hyperphosporylated tau protein), pCREB (phosporylated cyclic adenosine monophosphate response element binding protein) and p38-MAPK (p38-mitogen-activated protein kinase).

Our results extend this line of research by showing that reducing Glu that has been spilled-over from the synapse, with a Glu scavenger enzyme also prevents Abeta-induced LTP impairment (**Figure 19B**). We have also shown that the effect of Abeta(1-42) is mimicked by TBOA, a Glu reuptake inhibitor. Blocking NR2B-subunit or using a Glu scavenger restores impaired LTP caused by either of the compounds. These results agree with Li and his coworkers' findings: reducing ambient Glu in the brain is protective against Abeta induced synaptic depression (LTD) (Li, Hong et al. 2009). A recent paper by Chen and Herrup makes the suggestion that although the level of glutamine synthase is elevated in AD brains, its activity is severely compromised by oxidative damage, leading to impaired Glu metabolism (Chen and Herrup 2012). It is also reported that the interaction between Abeta and glutamine synthase can

induce the inactivation of the enzyme leading to excessive depolarization of the neurons via mGluRs and NMDARs (Blanchard, Stockwell et al. 2002, Blanchard, Thomas et al. 2002).

Neuronal hyperactivity and the appearance of seizures could further aggravate memory loss through a synergic mechanism. The secretion of Abeta is governed by neuronal activity (Kamenetz, Tomita et al. 2003, Cirrito, Kang et al. 2008) during pathological events, such as epileptiform activity induced by electrical stimulation, as well as during normal physiological processes, such as the sleep-wake cycle (Kang, Lim et al. 2009). Increased excitability induced by Abeta may result in a "vicious cycle" leading to massive neuronal loss and the formation of Abeta deposits. Excessive Glu release activates NMDARs which in turn stimulate a shift from α- to β-secretase cleavage of the APP facilitating further Abeta(1-42) production (Lesne, Ali et al. 2005).

VIII. SUMMARY

Taken together, we have shown that two electrophysiological events recorded from hippocampal slices, evoked fEPSPs and spontaneous spikes are not generated by the same mechanisms (Varga, Juhasz et al. 2014). Evoked fEPSPs, but not firing activity was mainly regulated by AMPARs. In contrast, spontaneous spikes are mediated by NMDAR function. Bath application of synaptotoxic Abeta(1-42)-enhanced firing activity in an NR2B-dependent manner without altering evoked fEPSPs. These effects may contribute to synaptic dysfunctions seen in early AD.

Our results also provide evidence that Abeta(1-42) impairs Glu recycling at the synapse, which leads to Glu spillover and NR2B-subunit containing NMDAR activation (Varga, Juhasz et al. 2014). Excessive activation of NMDARs increases Ca^{2+} influx and activates signaling pathway responsible for synaptic loss, apoptosis and neuronal death. Blocking NR2B or decreasing extracellular Glu level offers protection against the synaptic plasticity impairment caused by Abeta.

Based on our results, we suggest a possible therapeutic targets against Abeta. Glu scavengers may be a novel therapeutic opportunities for AD, because reducing ambient Glu in the brain may be protective against Abeta-induced synaptic dysfunction. Abeta and Glu toxicity mediated dysfunction have been closely associated, however decreasing the extracellular Glu level can be also protective in other conditions such as brain ischemia (Marosi, Fuzik et al. 2009), stroke (Campos, Rodriguez-Yanez et al. 2011), traumatic brain injury (Boyko, Stepensky et al. 2012) or certain psychiatric disorders (Mitani, Shirayama et al. 2006, Shinohe, Hashimoto et al. 2006).

IX. CONCLUSION

The main conclusions of my thesis are:

1) Evoked fEPSPs, but not spontaneous firing is mainly regulated by AMPARs. In contrast, spontaneous spikes are mediated by NMDAR function.
2) Abeta(1-42)-induced hyperexcitation and LTP impairment requires NR2B-subunit containing NMDARs activation.
3) Abeta(1-42) perturbs Glu recycling that is prevented by a Glu scavenger.
4) Blocking Glu reuptake mimics the effects of Abeta.
5) Glu scavenger may be novel therapeutic opportunity for AD.

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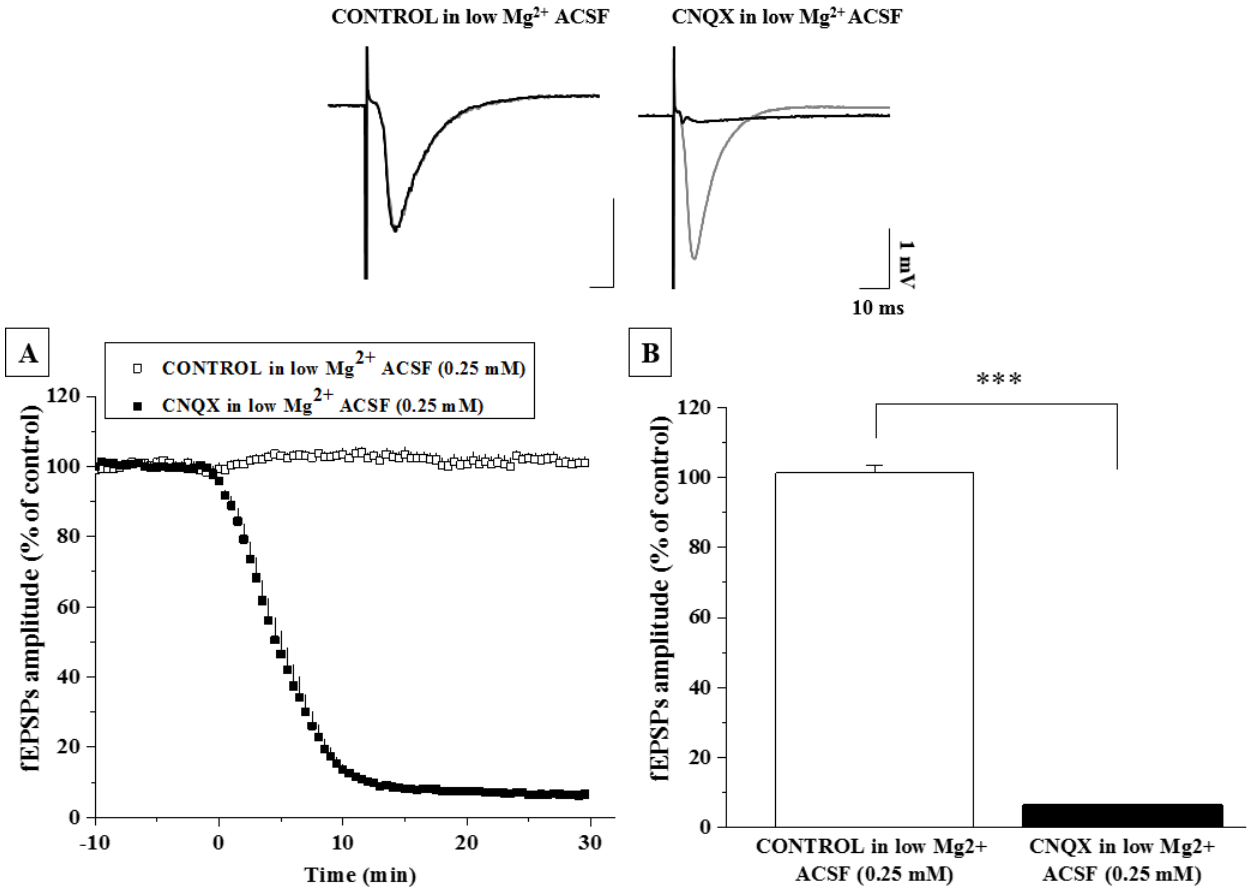
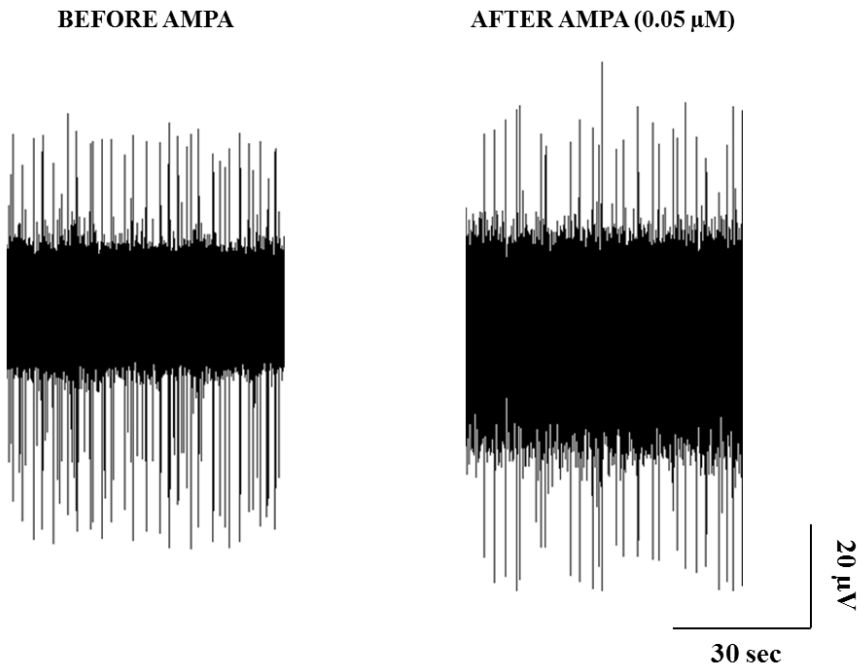
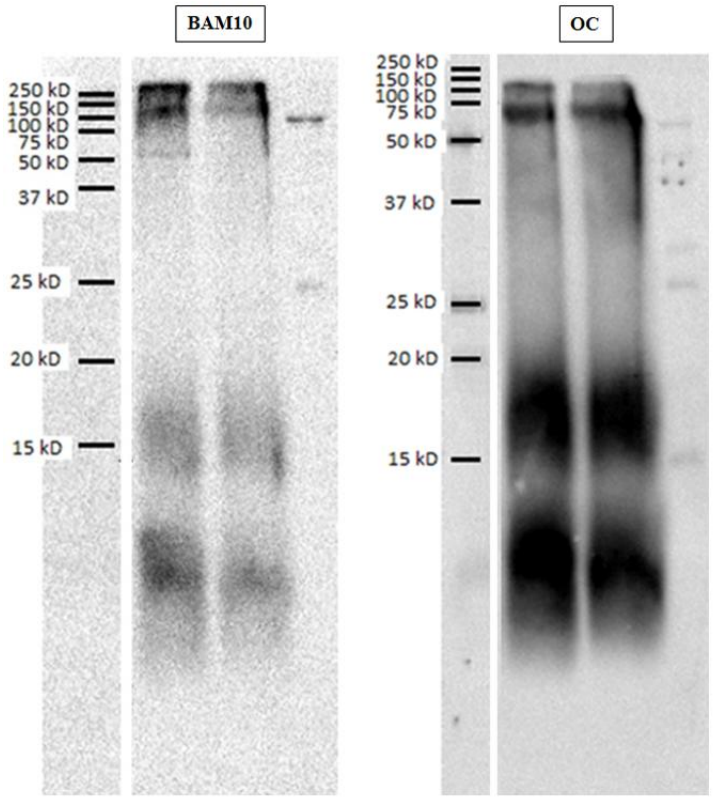
XII. SUPPLEMENTARY MATERIAL



Supplementary Figure 1. Panel **A** represents the changes of fEPSPs amplitudes over time: CNQX abolished evoked fEPSPs even under condition that promotes NMDAR activation (in Low Mg^{2+} ACSF) (**A**). Upper panel shows representative fEPSPs before (gray) and after (black) treatment. Bar graphs show the average of the fEPSPs amplitudes of the 25-30 min period after treatment (**B**). Error bars show SEM; ****p*<0.001. Abbreviations: fEPSPs (field excitatory postsynaptic potential), CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), NMDAR (N-methyl-D-aspartate receptor) and Low Mg^{2+} ACSF (low Mg_2SO_4-containing artificial cerebrospinal fluid).



Supplementary Figure 2. Low concentration of AMPA (0.05 µM) increased basal activity which hindered the effective detection of spikes. Figure shows representative traces before (left) and after (right) AMPA application. Abbreviation: AMPA (α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid).



Supplementary Figure 3. Abeta(1-42) samples used for the recordings contained clearly detectable SDS-stable trimers (≈15 kDa) and higher molecular weight protofibrils. Staining with OC antibody, which is specific for species having beta-sheet conformation, shows that the small low-n oligomers are of prefibrillar nature. Abbreviation: Abeta(1-42) (amyloid-beta(1-42)).

XIII. APPENDIX

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Amyloid-$\beta_{1\text{-}42}$ Disrupts Synaptic Plasticity by Altering Glutamate Recycling at the Synapse

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Abstract. Alzheimer's disease (AD) is the most prevalent form of neurodegenerative disorders characterized by neuritic plaques containing amyloid-β peptide (Aβ) and neurofibrillary tangles. Evidence has been reported that $A\beta_{1\text{-}42}$ plays an essential pathogenic role in decreased spine density, impairment of synaptic plasticity, and neuronal loss with disruption of memory-related synapse function, all associated with AD. Experimentally, $A\beta_{1\text{-}42}$ oligomers perturb hippocampal long-term potentiation (LTP), an electrophysiological correlate of learning and memory. Aβ was also reported to perturb synaptic glutamate (Glu)-recycling by inhibiting excitatory-amino-acid-transporters. Elevated level of extracellular Glu leads to activation of perisynaptic receptors, including NR2B subunit containing NMDARs. These receptors were shown to induce impaired LTP and enhanced long-term depression and proapoptotic pathways, all central features of AD. In the present study, we investigated the role of Glu-recycling on $A\beta_{1\text{-}42}$-induced LTP deficit in the CA1. We found that Aβ-induced LTP damage, which was mimicked by the Glu-reuptake inhibitor TBOA, could be rescued by blocking the NR2B subunit of NMDA receptors. Furthermore, decreasing the level of extracellular Glu using a Glu scavenger also restores TBOA or Aβ induces LTP damage. Overall, these results suggest that reducing ambient Glu in the brain can be protective against Aβ-induced synaptic disruption.

Keywords: Alzheimer's disease, glutamate scavenger, glutamate-reuptake, long-term potentiation, NR2B, TBOA

INTRODUCTION

Amyloid-β (Aβ), a misfolded peptide, is widely regarded as a central player in the pathogenesis of Alzheimer's disease (AD). The accumulation of soluble Aβ [1] in the brain of patients and animal models of AD is associated with impairments of cognition and memory [2–4]. In addition, both the synthetic and brain-derived soluble Aβ have been shown to damage certain forms of synaptic plasticity, correlates of learning and memory [5, 6]. Despite intense research, the mechanisms involved in Aβ-mediated neuronal degeneration and dysfunction are not well understood.

The hippocampus is especially affected in AD including hippocampal-dependent cognitive abilities such as learning and memory. Long-term potentiation (LTP), a form of synaptic plasticity in the CA1 field of the hippocampus, is impaired in animal models of AD. Numerous studies reported that $A\beta_{1\text{-}42}$ oligomers block hippocampal LTP *ex vivo* [7–9] and *in vivo* [10, 11].

Although the increased neuronal excitability caused by Aβ seems to contribute to and to be a key part of the pathomechanism of AD, the exact mechanisms by which neuronal over-activity develops is unknown. Glutamate (Glu) excitotoxicity has been established to have a major role in AD pathogenesis; however, how Aβ induces its effects is poorly understood. Numerous findings confirmed that excitotoxic effects of Glu contribute to progressive neuronal loss in AD [12–14]. Inhibited excitatory-amino-acid-transporters

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(EAATs) may be a central player in this mechanism, and indeed, recent findings suggest that Aβ oligomers perturb synaptic plasticity by altering Glu-recycling at the synapse [15, 16], resulting in elevated ambient extracellular Glu-level in the brain [17, 18], which might be responsible for the overexcitation seen in AD. Aβ blocks Glu-reuptake by inhibiting both neuronal and glial Glu transporters [16, 19], which might lead to extrasynaptic NMDAR (esyn NMDAR) activation. Esyn NMDAR activation causes inhibited LTP [5], enhanced long-term depression (LTD) [20], and apoptosis [21].

The aim of this study was to confirm that Aβ causes synaptic Glu-spillover and esyn NMDAR activation, which leads to impaired synaptic plasticity in the CA1. We show that blocking Glu-reuptake with TBOA also impairs LTP, and both TBOA- and Aβ-induced synaptic damage could be rescued by blocking NR2B subunit. Moreover, reducing the level of extracellular Glu by applying a glutamate-scavenger enzyme GPT also provides protection against impaired synaptic plasticity by TBOA and Aβ.

MATERIALS AND METHODS

Compounds

For the preparation of artificial cerebrospinal fluid (ACSF), all salts, glucose, sodium pyruvate (Pyr), glutamic-pyruvic transaminase (GPT), DL-threo-β-benzyloxyaspartate (TBOA), and α-(4-Hydroxyphenyl)-β-methyl-4- benzyl-1-piperidineethanol (+)-tartrate salt (ifenprodil) were purchased from Sigma-Aldrich (St. Louis, MO). $A\beta_{1\text{-}42}$ was synthetized at the Department of Medical Chemistry University of Szeged, Hungary. Detailed description of the synthesis and characterization of $A\beta_{1\text{-}42}$ is reported in [7].

Animals

The study conformed to EU Directive 2010/63/EU and was approved by the regional Station for Animal Health and Food Control under Project License XVI/8/2013. BALB/c mice were housed in groups of 2-3 under standard conditions (24°C, 12-h light-dark cycle) with food and water available *ad libitum*.

Ex vivo electrophysiology

Hippocampal slices of 400 μm in thickness were prepared from the brains of 3-month old mice using a standard protocol [22]. Briefly, slices were incubated in ACSF gassed with 95% O_2, 5% CO_2 at 35°C for 60 min. ACSF was composed of (in mM) 130 NaCl, 3.5 KCl, 3 $CaCl_2$, 1.5 $MgSO_4$, 0.96 NaH_2PO_4, 24 $NaHCO_3$, and 10 D-glucose, pH 7.4. Individual slices were transferred to a 3D-MEA chip with 60 tip-shaped and 60 μm high electrodes spaced by 200 μm (Qwane Biosciences, Lausanne, Switzerland). The surrounding solution was quickly removed, and the slice was immobilized by placing a grid onto it. The slice was continuously perfused with oxygenated ACSF (3 ml/min at 36°C) throughout the entire recording session. Unfiltered data were recorded using a standard, commercially available MEA 60 setup (Multi Channel Systems MCS GmbH, Reutlingen, Germany). Field excitatory postsynaptic potentials (fEPSPs) were recorded from the proximal stratum radiatum at 5 kHz.

Stimulation protocol

The Schaffer-collateral was stimulated by injecting a biphasic voltage waveform (–100/+100 μs) through one selected electrode at 0.033 Hz. Care was taken to place the stimulating electrode in the same region at every slice. The peak-to-peak amplitudes of fEPSPs at the proximal stratum radiatum of CA1 were analyzed. After a 30-min incubation period, the threshold and maximum stimulation intensities for evoked responses were determined. To evoke responses, 30% of the maximal stimulation intensity was used. LTP was evoked by theta-burst stimulation (TBS). TBS comprised of 15 bursts given at 5 Hz and individual burst contained 4 pulses given at 100 Hz per burst. The level of LTP was compared to the average of the last 10 peak-to-peak amplitudes of evoked fEPSPs before TBS.

Drug treatments

After 10-min control level, slices were treated with 1 μM $A\beta_{1\text{-}42}$ or 5 μM TBOA for 60-min before LTP was induced. Other cohort of slices was treated with 3 μM ifenprodil or 0.82 mM Pyr for 10-min then 2.06 U/ml GPT for 60-min before LTP induction. Separate groups of slices were treated with these compounds together with $A\beta_{1\text{-}42}$ or TBOA.

Statistics

Statistical significance was determined by using ANOVA on ranks test with the *post hoc* Dunn's method (SigmaPlot 11 software package). The p value ≤ 0.05 was considered significant in all cases.

RESULTS

A$\beta_{1\text{-}42}$-impaired LTP requires NR2B activation

We recorded fEPSPs from the stratum radiatum of the CA1 using MEA electrodes. The peak-to-peak amplitudes of fEPSPs were analyzed from the proximal part of stratum radiatum.

First, we verified the effect of A$\beta_{1\text{-}42}$ preparation on LTP in the hippocampal slices. Untreated slices showed a persistent elevated level of fEPSPs after LTP induction ($168.33 \pm 5.58\%$, $n = 12$), while A$\beta_{1\text{-}42}$ reduced the magnitude of LTP ($124.35 \pm 4.88\%$, $n = 9$, $p < 0.05$, nonparametric ANOVA, Dunn *post-hoc* test; Fig. 1). Several recent studies suggested that different NR2 subunits of NMDARs may have divergent roles in NMDAR-dependent LTP activation and Aβ pathology (see discussion). To test whether LTP activation requires NR2B-containing NMDARs function, slices were treated with an NR2B antagonist, ifenprodil. We observed that ifenprodil did not alter the level of LTP compared to control ($176.81 \pm 4.93\%$, $n = 5$), suggesting that NR2B-activation is not required for LTP in the CA1. Furthermore, ifenprodil prevents the A$\beta_{1\text{-}42}$ effect on LTP ($166.03 \pm 12.38\%$, $n = 5$, $p < 0.05$, ANOVA on ranks, Dunn *post-hoc* test; Fig. 1), suggesting A$\beta_{1\text{-}42}$ induce LTP damage is via NR2B-containing NMDARs. None of the applied compounds altered the amplitude of fEPSPs during the wash-in period.

Glu-scavenger rescues the A$\beta_{1\text{-}42}$-impaired LTP

To determine whether A$\beta_{1\text{-}42}$ affects Glu-reuptake, we used an enzymatic Glu-scavenger system to reduce extracellular Glu-levels. Slices were treated

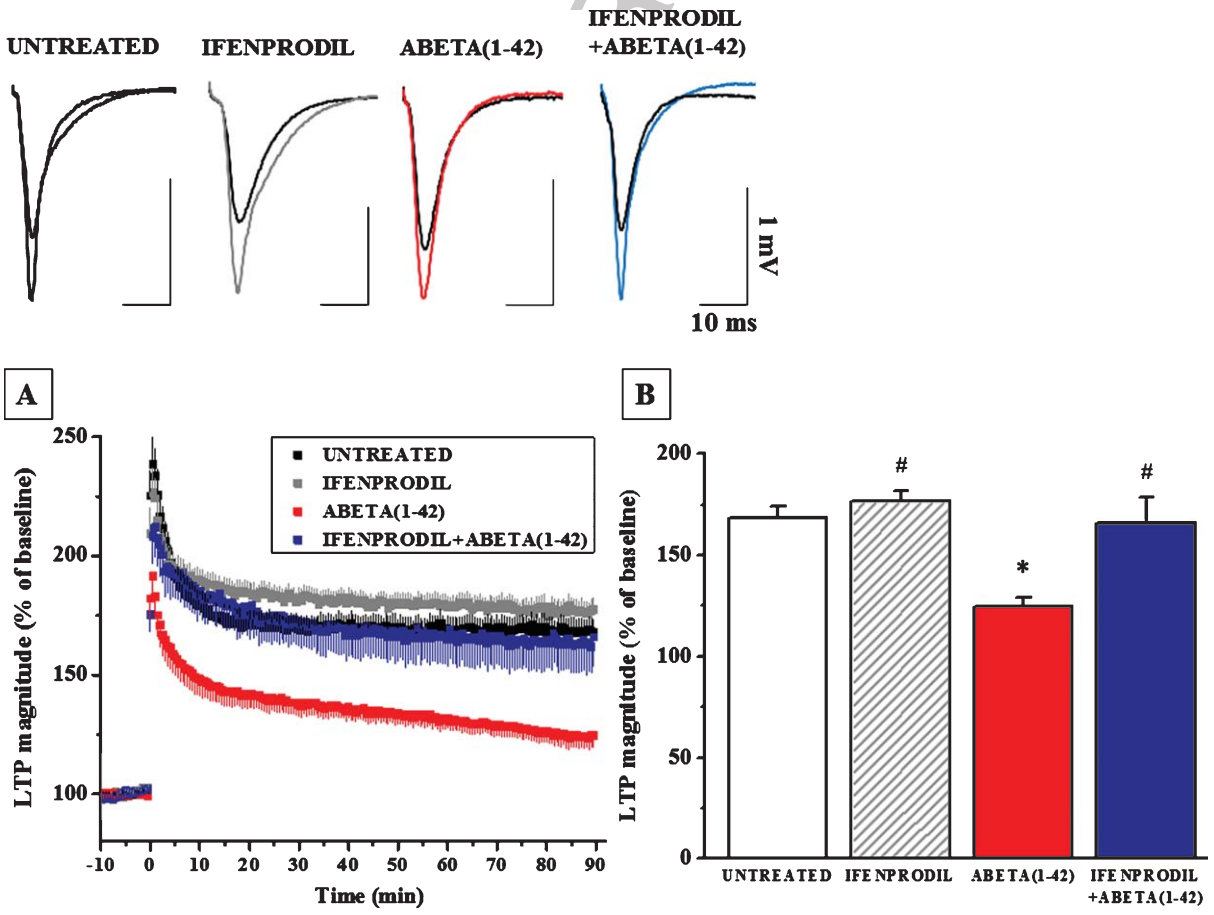

Fig. 1. Blocking NR2B subunit prevents A$\beta_{1\text{-}42}$-induced LTP damage. Insets show representative fEPSPs before (black) and after treatment. LTP was altered in A$\beta_{1\text{-}42}$ treated slices compared to untreated group (untreated versus A$\beta_{1\text{-}42}$: $^{*}p < 0.05$; ANOVA on ranks, Dunn *post-hoc* test). Ifenprodil did not change the level of LTP, however, it rescued the A$\beta_{1\text{-}42}$-impaired LTP. Error bars represent SEM; $^{\#}p < 0.05$ versus A$\beta_{1\text{-}42}$.

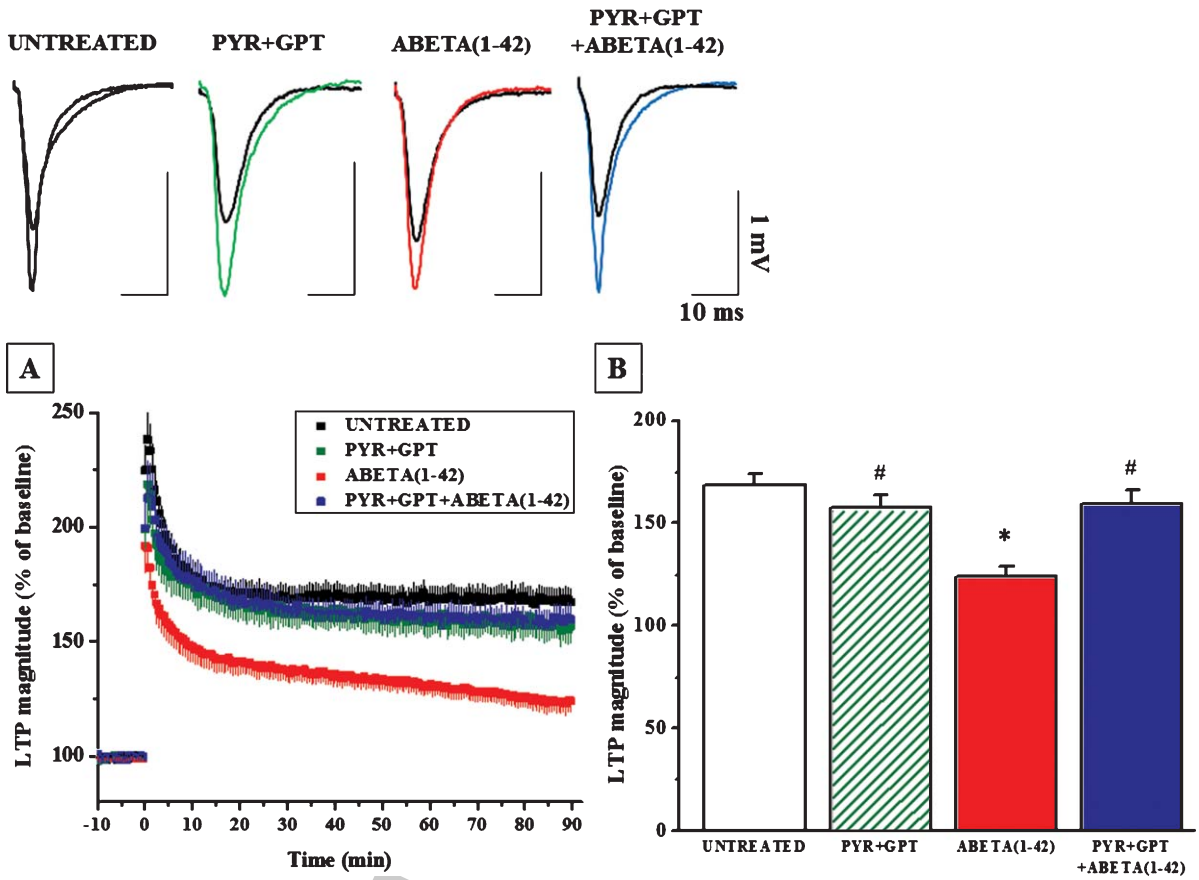

Fig. 2. Glu-scavenger restores $A\beta_{1\text{-}42}$-induced LTP damage. Insets show representative fEPSPs before (black) and after treatment. Pyr+GPT treatment did not affect the level of LTP compared to untreated slices, however $A\beta_{1\text{-}42}$ induced LTP impairment was prevented by Glu-scavenger ($A\beta_{1\text{-}42}$ versus Pyr+GPT+$A\beta_{1\text{-}42}$: $^{\#}p<0.05$; ANOVA on ranks, Dunn *post-hoc* test). Error bars represent SEM; $^{*}p<0.05$ versus untreated; $^{\#}p<0.05$ versus $A\beta_{1\text{-}42}$.

with GPT and its substrate, Pyr for 10 min followed by $A\beta_{1\text{-}42}$ for 60 min, then LTP was induced. We have found that Pyr+GPT treatment does not affect the level of LTP compared to control slices ($157.32 \pm 6.68\%$, $n=5$; Fig. 2). However, $A\beta_{1\text{-}42}$-induced LTP damage was prevented by Glu-scavenger (Pyr+GPT+$A\beta_{1\text{-}42}$: $159.66 \pm 6.37\%$, $n=5$ versus $A\beta_{1\text{-}42}$, $p<0.05$, ANOVA on ranks, Dunn *post-hoc* test; Fig. 2).

The effect of $A\beta_{1\text{-}42}$ is mimicked by TBOA, a Glu-reuptake inhibitor

TBOA was applied for 60 min before LTP induction. LTP was impaired by TBOA compared to untreated slice ($123.22 \pm 3.48\%$, $n=6$, $p<0.05$; ANOVA on ranks, Dunn *post-hoc* test, Fig. 3). Next, we tested whether NR2B subunit activation is required for the effect of TBOA. We have found that ifenprodil prevents TBOA-induced LTP damage suggesting NR2B subunit activation is essential for the effect of TBOA on LTP (ifenprodil+TBOA: $159.29 \pm 10.67\%$, $n=4$ versus TBOA: $p<0.05$; ANOVA on ranks, Dunn *post-hoc* test, Fig. 3). We proceeded to apply Glu-scavenger to test whether the inhibitory effect of TBOA was due to the elevated extracellular Glu-level. Indeed, TBOA-failed to impair LTP after Glu-scavenger treatment (Pyr+GPT+TBOA: 169.28 ± 8.18, $n=5$ versus TBOA, $p<0.05$; ANOVA on ranks, Dunn *post-hoc* test, Fig. 4). Collectively these results suggest that TBOA and Aβ share common pathway in synaptotoxicity. The effect of $A\beta_{1\text{-}42}$ is mimicked by Glu-reuptake inhibition; however both could be prevented by a Glu-scavenger and NR2B inhibition suggesting that $A\beta_{1\text{-}42}$ disrupts synaptic plasticity by altering Glu-recycling at the synapse in the CA1. Again, none of the applied compounds altered fEPSP amplitude during the wash-in period.

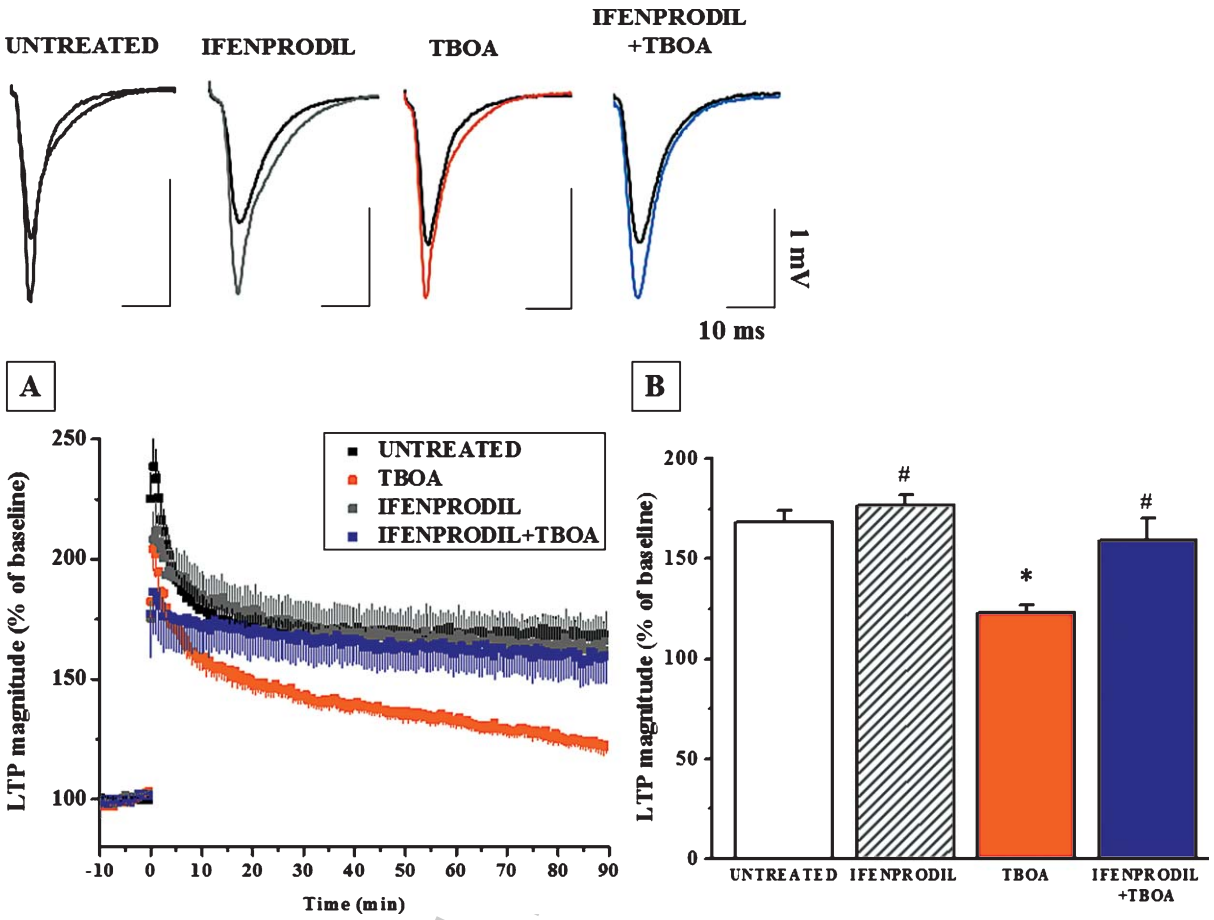

Fig. 3. Ifenprodil prevents TBOA-impaired LTP. Insets show representative fEPSPs before (black) and after treatment. LTP was impaired by TBOA compared to untreated group (untreated versus TBOA: $^{*}p < 0.05$; ANOVA on ranks, Dunn *post-hoc* test), however TBOA-induced LTP impairment was restored by ifenprodil (TBOA versus ifenprodil+TBOA: $^{\#}p < 0.05$; ANOVA on ranks, Dunn *post-hoc* test). Error bars represent SEM; $^{*}p < 0.05$ versus untreated; $^{\#}p < 0.05$ versus TBOA.

DISCUSSION

There is growing evidence that soluble Aβ oligomers mediate synaptic impairment in AD, but the exact mechanism of synaptotoxicity remains to be determined. Numerous studies have reported that Aβ can affect the function of NMDARs [23–27], which may lead to excitotoxicity and neuronal hyperactivation seen in the early stage of AD. Recent findings suggest that Aβ binds to prion protein, metabotropic Glu receptor 5, and integrin receptors, and this complex initiates a molecular cascade mediated by fyn kinase [28–30], which subsequently phosphorylates NMDARs.

An additional pathway of Aβ-mediated hyperexcitation could be, however, that the concentration of extracellular Glu is increased by Aβ. We have shown previously, that the excitatory effect of Aβ, as was determined by the rate of spontaneous spiking in hippocampal slices, is mediated by extrasynaptic NMDARs [22]. In the present study, we show that Aβ causes Glu spillover and subsequent esyn NMDAR activation, which could be prevented by either NR2B blockade or by "mopping-up" Glu with a Glu-scavenger enzyme.

TBOA mimics the effects of Aβ

Aβ has been shown to elevate extracellular Glu concentration in the brain without altering gamma-aminobutyric acid (GABA) level [18]. The mechanism behind this is probably the inhibition of the transporters mediating Glu-clearance. The level of brain extracellular Glu is regulated by EAATs expressed mainly on the astrocytes, which efficiently remove the excess of this neurotransmitter from the synaptic

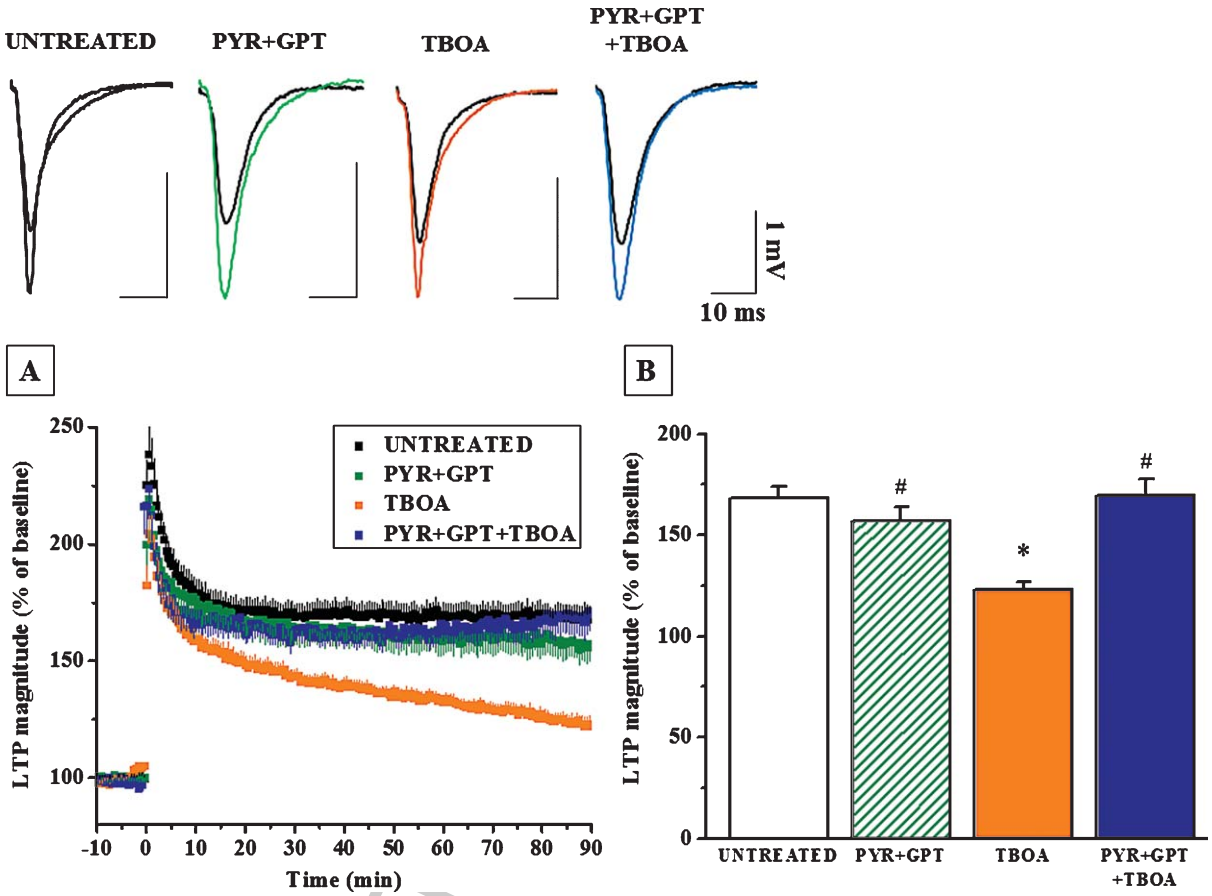

Fig. 4. Glu-scavenger prevents TBOA-induced LTP damage. Insets show representative fEPSPs before (black) and after treatment. TBOA-induced LTP impairment was restored by Glu-scavenger (TBOA versus Pyr+GPT+TBOA: $^{\#}p < 0.05$; ANOVA on ranks, Dunn *post-hoc* test). Error bars represent SEM; $^{*}p < 0.05$ versus untreated; $^{\#}p < 0.05$ versus TBOA.

cleft (reviewed by [31]). Indeed, Aβ was shown to inhibit both glial and neuronal EAATs [16, 17, 32]. Moreover, Noda and coworkers have reported that Aβ may even cause reverse functioning of EAAT, leading to Glu-release from glial cells [33]. Furthermore, down-regulation or abnormal expression and protein levels of EAAT1 and EAAT2 are altered in the hippocampus and frontal cortex of AD patients [34, 35] and in amyloid-β protein precursor transgenic mice [32], further supporting that Glu-level regulation fails during AD pathomechanism. These effects may lead to Glu-spillover from the synapses and subsequent activation of esyn receptors. Of particular interest here, esyn NMDARs containing mainly NR2B subunit were shown to activate apoptotic pathways and promote synaptic depression [21]. In contrast, NR2A subunit-containing NMDARs localized mainly at the synaptic domain are antiapoptotic and participate in the induction of LTP in the CA1. We previously reported that $A\beta_{1-42}$ induces hyperexcitability via NR2B-containing NMDARs [22]. Indeed, blocking selectively NR2B subunits protected against Aβ effects (including LTP impairment), suggesting that NR2B could be a promising target against AD [5, 8, 9, 36, 37]. Tackenberg et al. also suggest that esyn NR2B-containing NMDARs activation is essential for tau-dependent neurodegeneration [26]. It was also shown that the opposite form of synaptic plasticity, LTD requires both syn and esyn receptor activation [20], while LTP is not mediated by esyn NMDARs [8, 9]. It should be noted, however, that contradictory data is available on the role of NR2B NMDARs on LTP: esyn NDMARs are also recruited to the synapse during LTP [38] and may play an essential role in LTP maintenance [39, 40].

Our results extend this line of research by showing that reducing Glu that has been spilled-over from the synapse with a Glu-scavenger enzyme also prevents

Aβ induced LTP impairment. Moreover, we show that blocking EAATs by a selective inhibitor, TBOA, mimics the effects of Aβ: both compounds impair LTP, and this could be prevented by ifenprodil or GPT (Glu-scavenger).

Reducing ambient Glu in the brain is protective against Aβ induced LTD enhancement [16]. A recent paper by Chen and Herrup makes the suggestion that although the level of glutamine-synthase is elevated in AD brains, its activity is severely compromised by oxidative damage, leading to impaired Glu-metabolism [41].

Aβ and Glu-toxicity mediated dysfunction has been closely associated; however, decreasing the extracellular Glu-level can be protective in other conditions such as brain ischemia [42], stroke [43], traumatic brain injury [44] or certain psychiatric disorders [45, 46].

CONCLUSIONS

Collectively, our results provide evidence that Aβ impair Glu-recycling at the synapse, which leads to Glu-spillover and NR2B activation. Blocking NR2B or decreasing extracellular Glu offers protection against the synaptic plasticity impairment caused by Aβ.

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Research Article

Abeta(1-42) Enhances Neuronal Excitability in the CA1 via NR2B Subunit-Containing NMDA Receptors

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Neuronal hyperexcitability is a phenomenon associated with early Alzheimer's disease. The underlying mechanism is considered to involve excessive activation of glutamate receptors; however, the exact molecular pathway remains to be determined. Extracellular recording from the CA1 of hippocampal slices is a long-standing standard for a range of studies both in basic research and in neuropharmacology. Evoked field potentials (fEPSPs) are regarded as the input, while spiking rate is regarded as the output of the neuronal network; however, the relationship between these two phenomena is not fully clear. We investigated the relationship between spontaneous spiking and evoked fEPSPs using mouse hippocampal slices. Blocking AMPA receptors (AMPARs) with CNQX abolished fEPSPs, but left firing rate unchanged. NMDA receptor (NMDAR) blockade with MK801 decreased neuronal spiking dose dependently without altering fEPSPs. Activating NMDARs by small concentration of NMDA induced a trend of increased firing. These results suggest that fEPSPs are mediated by synaptic activation of AMPARs, while spontaneous firing is regulated by the activation of extrasynaptic NMDARs. Synaptotoxic Abeta(1-42) increased firing activity without modifying evoked fEPSPs. This hyperexcitation was prevented by ifenprodil, an antagonist of the NR2B NMDARs. Overall, these results suggest that Abeta(1-42) induced neuronal overactivity is not dependent on AMPARs but requires NR2B.

1. Introduction

Extracellular recording from hippocampal slices has long been a method of choice for determining the changes in excitability and synaptic plasticity of the CA1 microcircuitry [1]. Spontaneous spikes and, far more often, field excitatory postsynaptic potentials (fEPSPs) are recorded extracellularly from a local network of neurons; however, the relationship between these two phenomena is not fully clear. Intuitively, both electrophysiological events correlate with the excitability of the network under investigation, but these events are generated by different mechanisms. Field EPSPs are mainly composed of subthreshold events from a population of neurons, like dendritic depolarizations [2] and glial contributions to the net extracellular charge-flow, arising mainly from the function of transporters [3, 4]. In contrast, spontaneous firing represents only neuronal suprathreshold events [5]. Another difference is that a fEPSP is evoked by a stimulus, and therefore it is the result of a coordinated and synchronized electrical activity of a cell population, mediated by synaptic connections. Spontaneous spikes, in contrast, are not evoked by an external stimulus, and they are more likely to be dependent on intrinsic network connections and properties [6].

N-Methyl-D-aspartate receptor (NMDAR) and α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) play a key role in generating rapid excitatory events in the CA1, but these receptors serve different purposes. Their relative contribution to fEPSPs is well described [7]; however, their involvement in spontaneous spike-generation remains unknown and data on the correlation of extracellularly recorded field responses and action potentials are scarce. Activation of glutamate (Glu) receptors is known to play an important role in the mechanisms of neuronal plasticity

that might be the cellular basis of learning and memory [8]. Several studies have shown that the synaptic plasticity is impaired in Alzheimer's disease (AD) [9], the most common form of neurodegenerative disease, characterized by the presence of insoluble amyloid deposits in the brain. Overwhelming evidence suggests that the main component of plaques, amyloid-beta (Abeta) peptide, is thought to be responsible for the synaptic and cellular pathology of AD (for review see [10]). Numerous studies have reported that the severity of AD strongly correlates with decreased synapse density in hippocampus and cortex and with disruption of memory-related synapse function. Literature data strongly support the synaptotoxicity of Abeta(1-42), and the accumulation of soluble Abeta(1-42) [11] in the brain of patients and animal models of AD is associated with impairments of cognition and memory [12–14]. Recently, Abeta(1-42) was shown to induce epileptiform activity, both *in vitro* [15] and *in vivo*, and transgenic mice overexpressing Abeta develop seizures over time [16]. Moreover, AD patients have an estimated 87 times higher probability of developing epileptic seizures compared to age-matched population [17]. Despite the recent advancement in the research of the pathomechanisms of AD, the exact mechanism by which neuronal overactivity develops is unknown.

Recent findings suggest that Abeta blocks neuronal glutamate (Glu) uptake at synapses, leading to increased Glu level at the synaptic cleft [18]. Increased brain extracellular Glu, but not γ-aminobutyric acid (GABA), concentration was found upon Abeta administration by use of microdialysis technique [19, 20]. A resultant rise in Glu levels would lead to a spillover and activation of extra- or perisynaptic NMDARs enriched in the B isoform of the NMDAR 2 subunit (NR2B), which play a major role in the induction of long-term depression (LTD) [21] and have also been shown to help mediate the inhibition of long-term potentiation (LTP) by soluble Abeta oligomers [22].

In this study we investigated the mechanisms by which fEPSPs and spontaneous firing are regulated in the CA1 of hippocampal slices. We confirmed that fEPSP, but not spiking activity, is mainly mediated by AMPARs. In contrast, spontaneous activity is regulated by NMDARs. Bath application of synaptotoxic Abeta(1-42) greatly enhanced spontaneous firing rate, which could be prevented by blocking NR2B subunits.

2. Experimental Procedures

2.1. Compounds. For the preparation of artificial cere-brospinal fluid (ACSF), all salts, glucose, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), (+)-MK-801 hydrogen maleate, and α-(4-Hydroxyphenyl)-β-methyl-4-benzyl-1-piperidineethanol (+)-tartrate salt (ifenprodil) were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Animals. The study conformed to EU Directive 2010/63/EU and was approved by the regional Station for Animal Health and Food Control under Project License XVI/8/2013. BALB/c mice were housed in groups of 2-3 under standard conditions (24°C, 12 h light-dark cycle) with food and water available *ad libitum*.

2.3. Ex Vivo Electrophysiology. Hippocampal slices of 400 μm in thickness were prepared from the brains of 3-month-old mice using a standard protocol [23]. Briefly, slices were incubated in artificial cerebrospinal fluid ACSF gassed with 95% O_2 and 5% CO_2 at 35°C for 60 min. ACSF was composed of (in mM) 130 NaCl, 3.5 KCl, 3 $CaCl_2$, 1.5 $MgSO_4$, 0.96 NaH_2PO_4, 24 $NaHCO_3$, and 10 D-glucose, pH 7.4. Individual slices were transferred to a 3D-MEA chip with 60 tip-shaped and 60 μm high electrodes spaced by 200 μm (Qwane Biosciences, Lausanne, Switzerland). The surrounding solution was quickly removed, and the slice was immobilized by placing a grid onto it. The slice was continuously perfused with oxygenated ACSF (3 mL/min at 36°C) throughout the entire recording session. Unfiltered data were recorded using a standard, commercially available MEA 60 setup (Multi Channel Systems MCS GmbH, Reutlingen, Germany). Field EPSPs were recorded from the proximal stratum radiatum at 5 kHz, while spontaneous spiking activity was recorded from the CA1 stratum pyramidale at a frequency of 25 kHz for 5 min epochs. For analyzing and sorting the spiking activity, Spike2 software package (Cambridge Electronic Design, Cambridge, UK) was used. Recordings were filtered between 300 and 3000 Hz offline, and the threshold for spike detection was 2.5 fold higher than the noise level. The data of those electrodes were included in the analysis where the initial spiking activity was above 0.5 Hz. Firing activity ranged from 150 to 1000 spikes/5 min. Data are considered as multiunit activity.

2.3.1. Stimulation Protocol. The Schaffer-collateral was stimulated by injecting a biphasic voltage waveform (−100/+100 μs) through one selected electrode at 0.033 Hz. Care was taken to place the stimulating electrode in the same region at every slice. The peak-to-peak amplitudes of fEPSPs at the proximal stratum radiatum of CA1 were analyzed. After a 30 min incubation period, the threshold and maximum stimulation intensities for evoked responses were determined. To evoke responses, 30% of the maximal stimulation intensity was used. The level of LTP (the last 10 peak-to-peak amplitudes) was compared to the average of the last 20 peak-to-peak amplitudes of evoked fEPSPs before applying theta-burst stimulation (TBS). TBS comprised of 15 bursts given at 5 Hz and individual burst contained 4 pulses given at 100 Hz per burst.

2.3.2. Drug Treatments. All slices were incubated for 30 min without stimulation in the recording chamber before any recording was done. Following the first spike-recording, slices were left to incubate a further 60 min with continuous fEPSP recording and then slices were treated with 10 μM CNQX or 10 μM MK801 or 25 μM MK801 or ACSF containing low concentration of Mg_2SO_4 (low Mg^{2+} ACSF; 0.25 mM) or 0.5 μM NMDA or 0.05 μM AMPA for 30 min (see Figure 1(c)). Spontaneous activity was recorded before and 30 min after treatment for 5 min and the spiking data

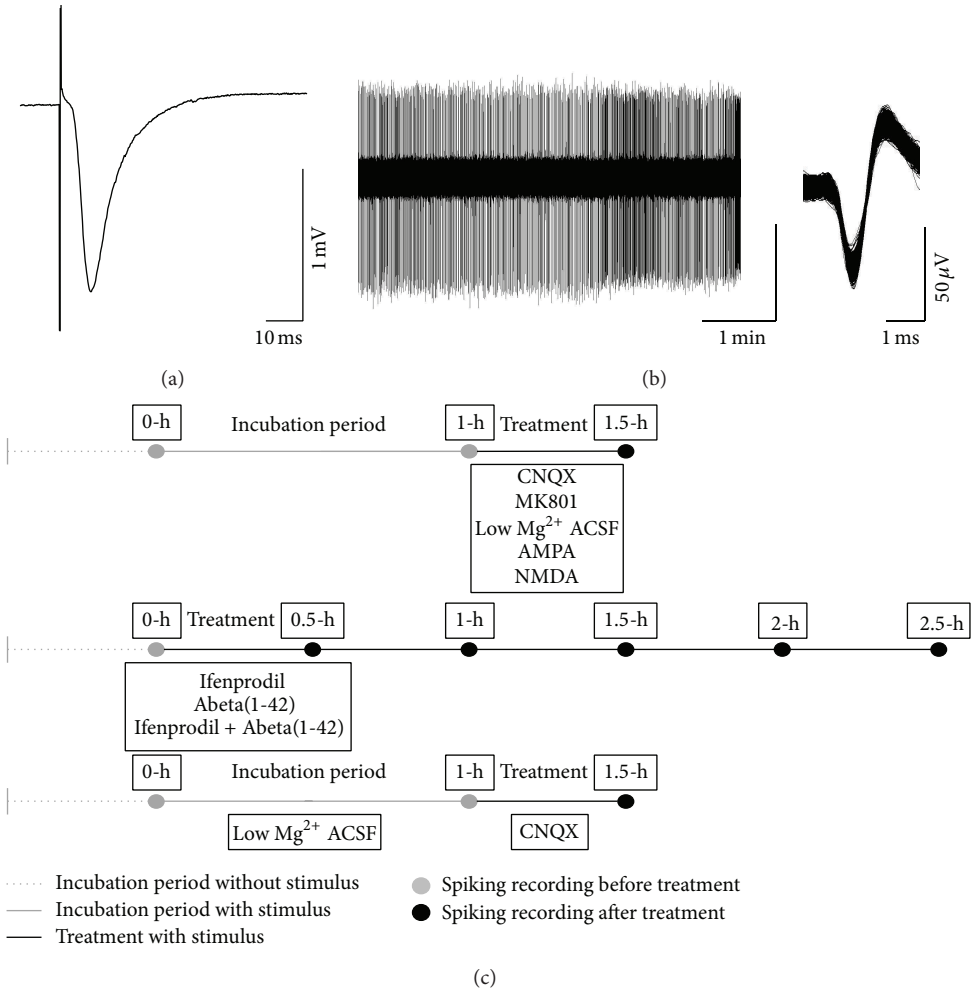

Figure 1: Exemplar recordings and the timeline of the experiments. Both evoked fEPSPs and spiking activity recording electrodes were located in the CA1 region of mouse hippocampal slice. The fEPSPs were recorded from the stratum radiatum (a) and the spontaneous spiking was recorded from the stratum pyramidale (b). Both fEPSPs and spiking activity were recorded from the same slice. Data were considered as multiunit activity. (c) shows the timeline of the recordings. The initial spiking activity (recorded at 0 h) was considered as 100% in each slice.

was compared to the untreated group recorded at the same time point. Other cohorts of slices were treated with 1 μM oligomer Abeta(1-42), ifenprodil (3 μM), and ifenprodil + Abeta(1-42) for 2.5 h. The spiking activity was recorded at every 30 min for 5 min. The effect of CNQX was also tested in low Mg_2SO_4-containing ACSF (0.25 mM), where modified ACSF was perfused to the slices from the beginning of the experiment. Electric stimulation was stopped during spontaneous activity recordings. Spiking frequency of each slice (number of spikes) was normalized to the initial firing activity (following the first 30 min incubation; 0 h), which was taken as 100% in all channels. Following this, slices were treated with 1 μM oligomer Abeta(1-42) for 60 min and then LTP was induced by the TBS protocol. LTP was followed for 90 min after TBS. During treatment, evoked fEPSPs were recorded.

2.4. Synthesis and Characterization of Abeta(1-42). Detailed description of the synthesis and characterization of Abeta(1-42) is reported in [23, 24]. Briefly, a depsipeptide derivative of Abeta(1-42) was synthesized and, after purification, it was used in lyophilized form. A 200 μM stock solution of the peptide was prepared in 0.1 mM NaOH, and the pH was set to 11.0. After incubation for 2 h at ambient temperature, the stock solution was diluted into ACSF to a concentration of 50 μM and the pH was set to 7.3. The peptide solution was incubated for 12 h at 37°C and, prior to use, it was diluted with ACSF to a final concentration of 1 μM. Aggregation grade and the size distribution of the oligomers were checked by Western blots by following the methods described in [23]. Oligomers were detected either with sequence-specific BAM10 antibody (Sigma-Aldrich) or with conformation specific OC antibody (Millipore), which detects the oligomers of fibrillar nature, that is, with beta sheet structure.

2.5. Statistics

2.5.1. Statistical Analysis for fEPSPs. Testing for normality was done with the Kolmogorov-Smirnov normality

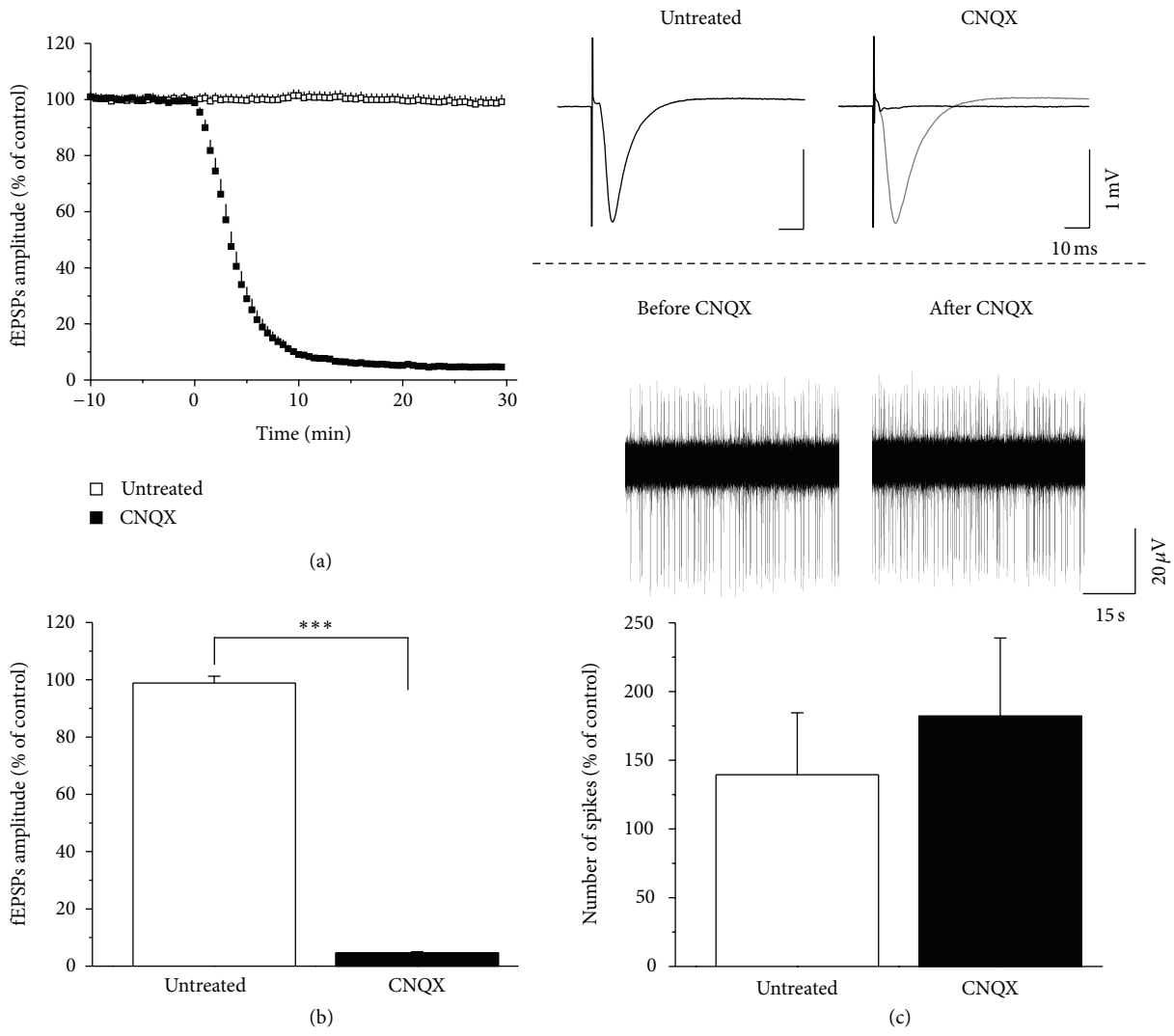

Figure 2: AMPA receptors mediate fEPSPs but not spontaneous activity. Blocking AMPARs abolished fEPSPs (a). Bar graphs show the average of the fEPSPs amplitudes of the 25–30 min period after treatment. CNQX induced a complete reduction of evoked fEPSPs ($n = 5$, $P < 0.001$) (b); however the spiking activity was not affected (c). Inset at the right panel shows representative fEPSPs before (grey) and after (black) treatment and representative spike trains. Error bars show SEM; $^{***}P \leq 0.001$.

test. Our data have shown normal distribution; hence, independent-samples t-test and one-way repeated measures analysis of variance (ANOVA) were used with the Bonferroni test for *post hoc* analysis. The P value ≤ 0.05 was considered significant in all cases. Data were analyzed using SPSS statistical software.

2.5.2. Statistical Analysis for Spiking Rate. Kolmogorov-Smirnov test was used also for testing normality of these data. Since our data have shown nonparametric distribution, we used nonparametric tests (Kruskal-Wallis test was followed by Mann-Whitney U test) for determining differences between two and several groups. Data were analyzed using SPSS. P value of ≤ 0.05 was considered significant in all cases.

3. Results

3.1. Field EPSPs, but Not Spontaneous Spikes, Are Mediated by AMPARs. We recorded fEPSPs from the stratum radiatum (Figure 1(a)) and in parallel spontaneous spikes from the stratum pyramidale (Figure 1(b)) of the CA1. Both fEPSPs and spontaneous activity could be abolished by application of 1 μM tetrodotoxin (data not shown).

First, we investigated the contribution of AMPARs to evoked fEPSPs and spontaneous activity, using CNQX, an AMPAR inhibitor. Blocking AMPARs resulted in a complete reduction of evoked fEPSPs (untreated: $n = 5$; 98.84 ± 2.41% versus CNQX: $n = 5$; 4.62 ± 0.45%, $P = 0.001$, independent samples t-test; Figures 2(a) and 2(b)). Conversely, spiking activity remained unchanged (untreated: $n = 5$; 139.55 ± 44.95% versus CNQX: $n = 5$; 182.18 ± 56.76%,

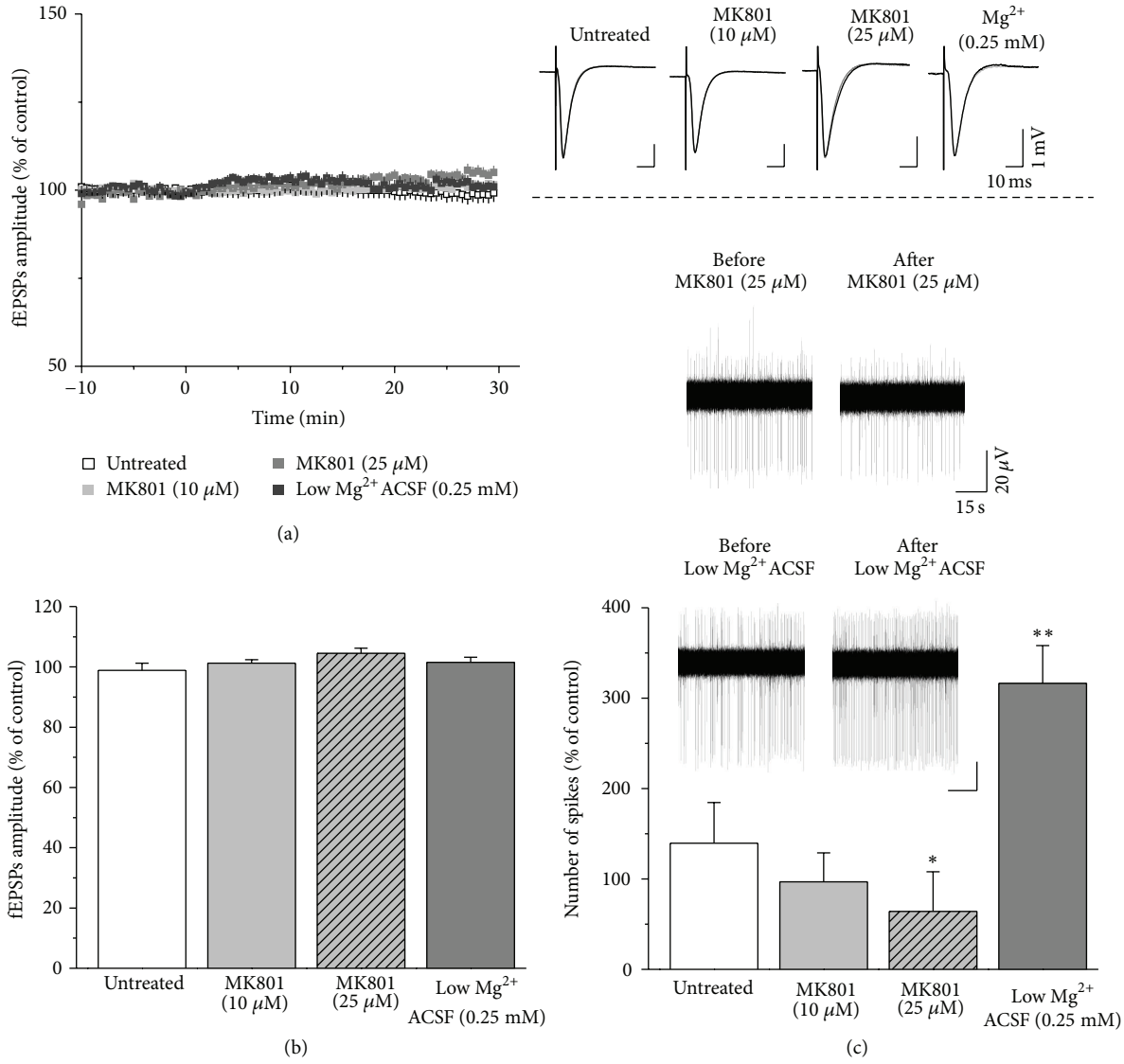

Figure 3: NMDA receptors mediate spontaneous activity but not fEPSPs. Blocking (MK801) or enhancing (low-Mg^{2+} ACSF) NMDAR function did not influence fEPSPs (a). Right panel shows representative fEPSPs before (grey) and after (black) treatment. Bar graphs show the average of the fEPSPs amplitudes of the 25–30 min period after treatment (b). In contrast, MK801 dose dependently reduced spiking frequency and low Mg^{2+} ACSF enhanced firing rate (untreated versus MK801 in 25 μM: $P = 0.035$ and untreated versus low Mg^{2+} ACSF: $P = 0.004$) (c). Inset shows representative spike trains. Error bars show SEM; $^{*}P \leq 0.05$, $^{**}P \leq 0.01$.

Mann-Whitney U test; Figure 2(c)), suggesting that AMPARs play a key role in generating fEPSPs but not spontaneous spiking. Under physiological conditions, NMDARs activation is dependent on depolarization, for example, on previous AMPARs activation. Thus we changed to low Mg^{2+} ACSF (0.25 mM) to remove the depolarization-dependent Mg^{2+} plug from the NMDAR. Applying 10 μM CNQX to low Mg^{2+} ACSF slices for 30 min, fEPSPs were completely blocked (control: $n = 6$; 95.54 ± 81% versus CNQX in low Mg^{2+}-containing ACSF: $n = 6$; 6.44±0.36%, $P < 0.001$, independent samples t-test; see Supplementary Figure 1 available online at http://dx.doi.org/10.1155/2014/584314). We have also tried to activate AMPARs by applying a low concentration of AMPA (0.05 μM), but we observed epileptiform field responses and a huge increase of basal activity which hindered the unambiguous detection of action potentials (Supplementary Figure 2).

3.2. Spontaneous Firing, but Not fEPSP, Is Governed by NMDAR Function. Next, we focused on NMDARs. Applying an NMDAR antagonist, MK801 resulted in a dose-dependent decrease of spiking rate (untreated: $n = 5$; 139.55 ± 44.95% versus MK801 in 10 μM: $n = 5$; 96.73%±32.22, $P = 0.48$; MK801 in 25 μM: $n = 5$; 29.23 ± 22.93, $P = 0.031$, Mann-Whitney U test; Figure 3(c)). MK801 did not have any effect on fEPSP in either 10 μM or 25 μM (untreated: $n = 5$; 98.8 ± 2.4% versus MK801 in 10 μM: $n = 5$; 101.23 ± 1.17% versus MK801 in 25 μM: $n = 5$, 104.49 ± 1.72%; one-way

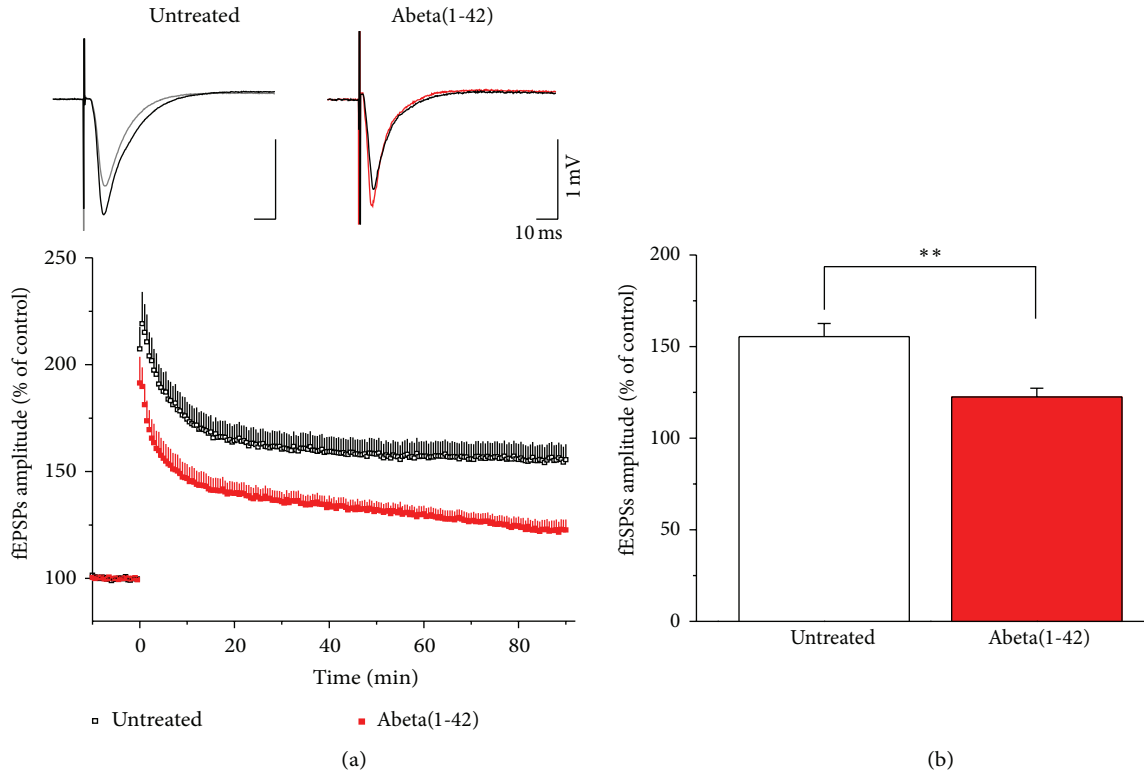

Figure 4: Abeta(1-42) impairs LTP. Representative fEPSPs from both control and Abeta(1-42) groups. Field EPSP was recorded before (grey) and 90 min after LTP induction (control is in black and Abeta(1-42) is in red) from the proximal stratum radiatum of CA1 (see above). Following 1 h of Abeta(1-42) treatment, LTP was induced by TBS protocol. LTP was reduced in Abeta(1-42) treated slices compared to controls 90 min after TBS (untreated versus Abeta: $P = 0.002$) (a). The amplitudes of fEPSPs after TBS were normalized to pre-TBS control. Bar graphs show the average of the last 5 min of LTP. Error bars represent SEM; $^{**}P \leq 0.01$ (b).

ANOVA and Bonferroni *post hoc* test; Figures 3(a) and 3(b)). Another cohort of slices was treated with ACSF having reduced Mg^{2+} concentration for 30 min. We have observed a massively elevated frequency of the spontaneous spiking activity compared to the untreated slices ($n = 7$, 316.34 ± 41.89%, $P = 0.004$, Mann-Whitney U test; Figure 3(c)), but evoked fEPSP responses remained unaltered ($n = 7$, 101.54 ± 1.69%, one-way ANOVA and Bonferroni *post hoc* test). Based on these results we hypothesized that spontaneous firing is mediated by the ambient Glu concentration acting on the extrasynaptic NMDARs. Therefore we tried to activate selectively this set of receptors by applying low concentration of NMDA (0.5 μM) in normal ACSF [25]. This treatment resulted in unchanged fEPSPs (untreated: $n = 5$; 98.8 ± 2.4% versus NMDA: $n = 9$; 94.03 ± 1.04%; independent-samples t-test) but induced a trend of elevated firing rate (untreated: $n = 5$; 139.55 ± 44.95% versus NMDA: $n = 9$; 253.02 ± 105.9%, $P = 0.51$, Mann-Whitney U test; Supplementary Figure 3).

3.3. Abeta(1-42) Impairs LTP. A major drawback of Abeta studies is that the activity of various Abeta preparations may vary between protocols and even between batches. We verified the activity of Abeta batches we have used for this study by determining their effect on CA1 LTP. Abeta(1-42) was applied for 60 min before inducing LTP by using TBS. Untreated slices showed a robust and permanent elevation of evoked fEPSPs after TBS ($n = 7$; 155.48 ± 7.16% 90 min after TBS), while slices having received Abeta(1-42) failed to exhibit permanent LTP ($n = 8$; 122.54 ± 4.75% 90 min after TBS, $P = 0.002$, independent-samples t-test; Figures 4(a) and 4(b)).

3.4. Abeta(1-42) Induces Hyperexcitation via NR2B. Firing rate was determined every 30 min in 5 min epochs within the time frame of the recordings. In the untreated slices the amplitude of fEPSPs increased slightly until 1.5 h reaching 103.36 ± 2.67% of the initial amplitude and then decreased to initial value ($n = 5$; 0.5 h: 100.8 ± 1.65%; 1 h: 102.9 ± 1.84%; 2 h: 100.86±3.38%; and 2.5 h: 98.18±4.04%, one-way repeated measures ANOVA and Bonferroni *post hoc* test; Figures 5(a) and 5(b)). Similarly, spiking frequency did not change over time ($n = 5$; 0.5 h: 124.34 ± 29.72%; 1 h: 129.57 ± 53.28%; 1.5 h: 139.55 ± 44.95%; 2 h: 102.48 ± 28.42; and 2.5 h: 76.16 ± 28.08%, Mann-Whitney U test; Figure 6).

Abeta(1-42) applied in 1 μM did not change fEPSPs amplitudes (Abeta(1-42): $n = 7$; 0.5 h: 107.08 ± 2.43%; 1 h: 106.41 ± 2.93%; 1.5 h: 103.54 ± 3.07%; 2 h: 100.92 ± 3.06%; and 2.5 h: 97.1 ± 2.97% compared to untreated slices, see above; Figure 5), suggesting that Abeta(1-42) did not affect the AMPAR-mediated synaptic transmission. On the other

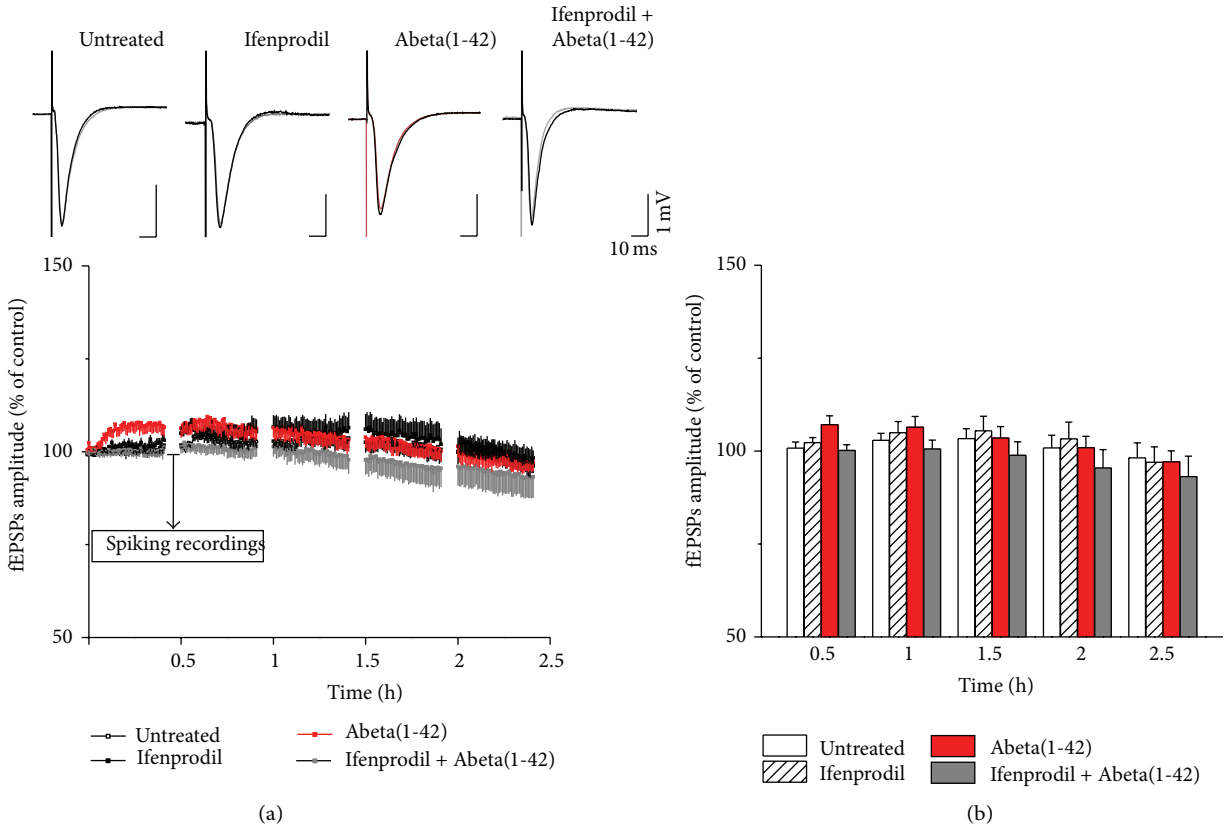

FIGURE 5: Neither Abeta(1-42) nor ifenprodil affects fEPSPs. Representative fEPSPs are shown before (grey) and after treatment in the upper panel. Neither Abeta(1-42) nor ifenprodil changed fEPSPs amplitudes (one-way repeated measures ANOVA with Bonferroni correction) (a). Bar graphs show the mean of the last 5 min of every recording epoch. Error bars represent SEM (b).

hand, Abeta(1-42) induced a massively elevated firing (n = 7; 0.5 h: 182.6 ± 30.91%; 1 h: 242.29 ± 83.31%, P = 0.043; 1.5 h: 233.29 ± 83.31%; 2 h: 240.32 ± 85.0%, P = 0.036; and 2.5 h: 244.72 ± 64.21%, P = 0.001, Mann-Whitney U test; Figure 6) compared to untreated slices, suggesting that NMDARs are involved in the effect of Abeta(1-42). Several recent reports suggested that the deleterious effect of Abeta is mediated via the NR2B subunit-containing NMDARs (see Section 4). To test whether the observed hyperexcitation in our experimental setup is sensitive to NR2B antagonism, we have applied ifenprodil (3 μM), an antagonist of the NR2B. Ifenprodil did not alter either fEPSPs (ifenprodil: n = 5; 0.5 h: 102.26 ± 1.35%; 1 h: 104.95 ± 3.00%; 1.5 h: 105.45 ± 3.91%; 2 h: 103.28 ± 4.53%; and 2.5 h: 96.99 ± 4.15% compared to control, see above; Figure 5) or spiking activity (n = 5; ifenprodil: 0.5 h: 127.11 ± 53.62%, 1 h: 163.58 ± 101.77%; 1.5 h: 81.37 ± 37.37%, 2 h: 110.35 ± 65.81%; and 2.5 h: 120.26 ± 64.58%; Figure 6), suggesting that NR2B-activation is not required for basic synaptic transmission in the CA1. However, Abeta(1-42) induced elevated spiking activity was prevented by ifenprodil (ifenprodil + Abeta(1-42): n = 5; 0.5 h: 157.44 ± 84.19%; 1 h: 196.87 ± 79.61%; 1.5 h: 116.36 ± 61.71%; 2 h: 101.72 ± 76.26%; and 2.5 h: 88.88 ± 59.67%, P = 0.048, Mann-Whitney U test compared to Abeta(1-42), see above; Figure 6) without changing of fEPSPs amplitudes (ifenprodil + Abeta(1-42): n = 5; 0.5 h: 100.17 ± 1.53%; 1 h: 100.57 ± 2.38%; 1.5 h: 98.85 ± 3.66%; 2 h: 95.45 ± 4.97%; and 2.5 h: 93.15 ± 5.47%; Figure 5), suggesting that the hyperexcitability caused by Abeta(1-42) requires the activation of extrasynaptic NR2B receptors, but not AMPARs.

3.5. Characterization of Abeta(1-42). The size distribution of Abeta(1-42) oligomers formed in 50 μM after incubation at 37°C was studied on Western blots by using two different antibodies; the monoclonal BAM10 antibody is sequence specific and binds to the N-terminal end of the peptide, while OC stains the oligomers of fibrillar nature (protofibrils, oligomers with beta sheet structure) [26]. Supplementary Figure 4 shows the results of the WB experiments. BAM10 staining reveals the presence of both low- and high-molecular-weight oligomers in the sample, while their positive staining with the OC antibody indicates that they have protofibrillar characteristics. The presence of SDS stable dimers and trimers in the sample closely resembles Abeta(1-42) derived from biological sources, namely, from transfected 7PA2 cells [27] and from human brain [28, 29]. These species were shown to have strong synaptotoxic properties [30]. On the other hand, protofibrillar species, which are also abundant in our sample, were also reported to be synaptotoxic [16].

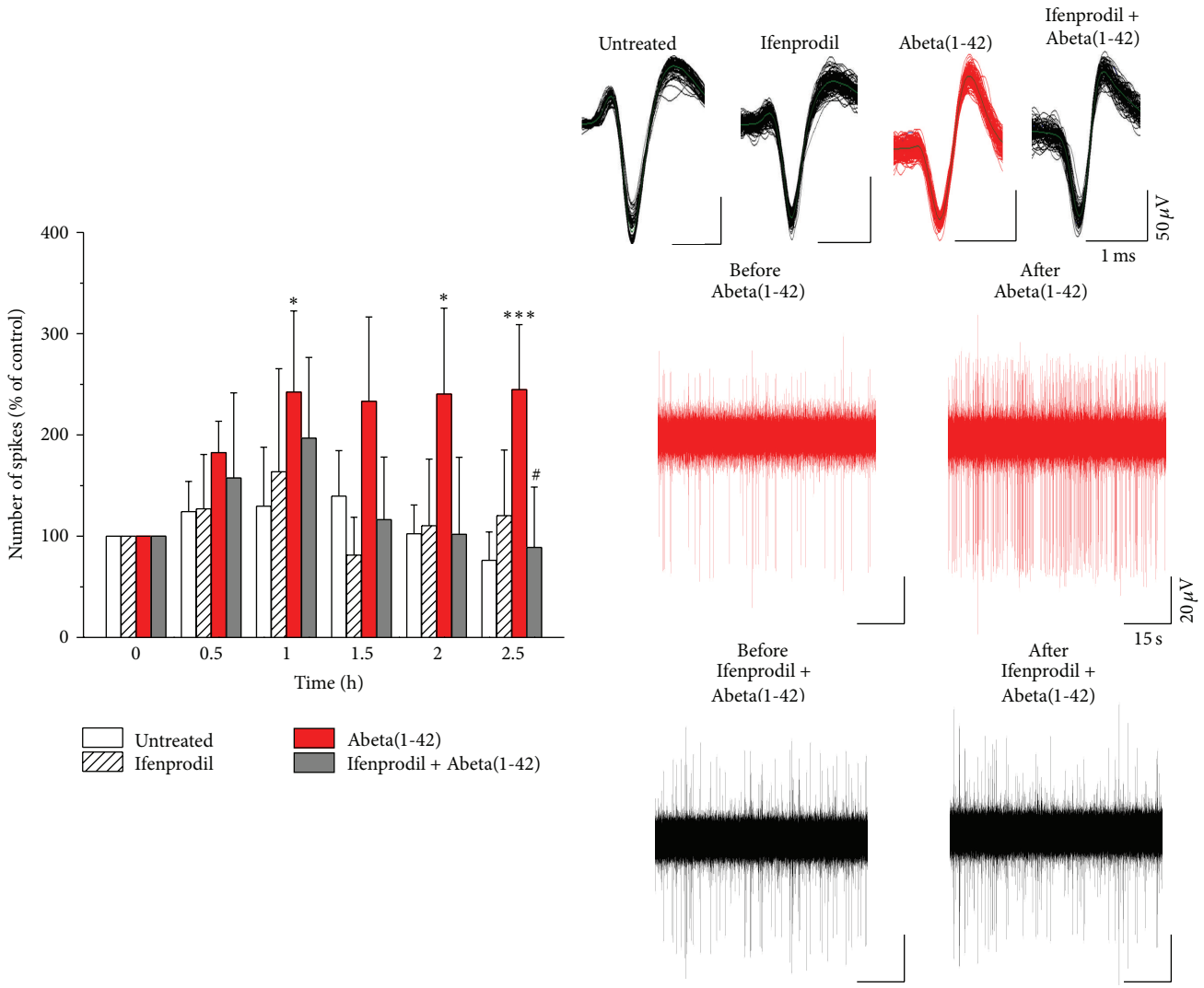

FIGURE 6: Abeta(1-42) induces hyperexcitation via NR2B. Abeta(1-42) induced increased spiking activity (untreated versus Abeta(1-42): 1 h: $^{*}P = 0.043$; 2 h: $^{*}P = 0.036$; 2.5 h: $^{***}P = 0.001$; Mann-Whitney U test). Ifenprodil did not affect spontaneous activity but prevented the elevated spiking rate caused by Abeta(1-42) (Abeta(1-42) versus ifenprodil + Abeta(1-42) 2.5 h, $^{\#}P = 0.038$; Mann-Whitney U test). Upper panel shows exemplar units from the respective recordings, while bottom panel illustrates representative spike trains. Error bars represent SEM; $^{*}P \leq 0.05$; $^{***}P \leq 0.001$; $^{\#}P \leq 0.05$.

4. Discussion

4.1. AMPA Receptors Regulate fEPSPs and NMDA Receptors Mediate Spontaneous Spiking. Extracellular fEPSP recordings are the gold standard for determining the excitation and synaptic plasticity in hippocampal slices. The changes of spontaneous spiking activity under conditions that modify network excitability are, however, less well studied in acute slices. In these sets of experiments we have studied the involvement of AMPARs and NMDARs in fEPSPs evoked by Schaffer-stimulation and in spontaneous firing in the CA1. We show that these two electrophysiological markers, previously thought to underlie measures of excitation, are not correlated. Importantly, the spikes we recorded here were almost exclusively localized to the pyramidal layer of CA1; hence, they were presumably from principal cells. Multiunit activity recordings do not allow separating the firing from individual neurons and may include bursts of spikes fired by the same neuron. Thus, in our study, spiking represents the global amount of activity within this network and most probably the activity of principal cells. The firing rates observed in this study were comparable to previously reported values in slices of CA1 of the guinea pig (0.22 and 1.8 spikes/sec) [6]. Under our conditions, blocking AMPAR with CNQX inhibited fEPSPs, however, the spiking activity did not change. In contrast, modulating NMDAR function affected spontaneous firing without any change in evoked fEPSPs. Blocking NMDARs ablated, while enhancing NMDAR function increased spontaneous activity.

4.2. Possible Involvement of the CA1 Local Microcircuitry in the Regulation of Spontaneous Spiking. Direct comparison of data from CA3 spiking regulation and our CA1 data is

difficult, because glutamatergic pyramidal cells of the CA3 form massive recurrent loops, a feature that is missing in the CA1. Therefore, the activity of CA1 pyramidal cells is regulated by the excitation arriving from CA3 through the Schaffer-collateral and by the inhibition of the local interneuron microcircuitry. Although the activity of CA1 inhibitory cells has been reported to be scarce, compared to CA3 in acute slices, powerful pyramidal somatic inhibition could be detected upon minimal electric stimulation [31], showing that the inhibition is very effective. What is the driving force of the interneuronal activity? One possible explanation is that the spontaneous activity of the incoming axons of the CA3 principal cells recruits a CA1 inhibitory microcircuitry via the Schaffer-collateral. This is unlikely, because there is no correlation between CA3 multiunit activity and the extracellularly recorded inhibitory postsynaptic potential in the CA1 [31]. Another possibility is that the spontaneous discharge of CA1 interneurons, although scarce, is keeping a strong blockade of the principal cells. Favoring this hypothesis, the resting membrane potential is more hyperpolarized in CA1 compared to CA3 pyramidal neurons [32, 33]. These spontaneous discharges might be regulated by the ambient Glu concentration via NMDARs. Indeed, tonic activation of NMDARs by ambient glutamate has been described in virtually all pyramidal cells of the CA1 of the hippocampus in slice models [34–37]. Importantly, as Le Meur et al. have shown [38], neither AMPA/kainate receptors nor metabotropic glutamate receptors contribute to this tonic excitation of pyramidal neurons. This tonic current is not dependent on vesicular release of transmitters from neurons but is affected by inhibition of the enzyme converting Glu, in glutamine in glial cells, indicating that ambient Glu is mainly of glial origin. Consistent with these findings, we have also shown that spontaneous spiking is driven by NMDAR activity, but not by AMPARs, indicating that the tonic activation could reach the threshold of spike generation. The question arises as follows: why do not AMPARs mediate spontaneous activity? NMDARs have a much higher affinity for Glu than AMPARs do; in steady-state conditions, the EC50 for Glu at NMDARs is over two orders of magnitude lower than that at AMPARs [39]. Extrasynaptic NMDARs containing the NR2B subunit have even higher affinity than synaptic NR2A-containing receptors [40]. It should be noted that evoked firing, which is usually detected in the form of population spikes (pop-spikes for short) in CA1 extracellular recordings, is mediated by AMPAR signaling [41]. Under our conditions, action potentials were not inhibited by AMPAR blockade. The reason behind this discrepancy might be that evoked pop-spikes require a synchronous discharge of a population of cells. This coordinated function might be based on the fast AMPAR function.

4.3. Abeta(1-42) Impairs LTP and Hyperexcites without Altering fEPSPs in the CA1 . Neuronal hyperexcitability in early AD and in AD modeling mice is an emerging finding that points to a network dysfunction as a critical component of the pathomechanism. Soluble Abeta species have been increasingly implicated as the key pathologic components of the disease, thus elucidating the mechanisms by which these species alter neuronal function is important. We confirmed that the Abeta(1-42) preparation we use is really synaptotoxic, impairing LTP. Next we tested its effect on spontaneous firing and found that it enhanced the rate of spontaneous discharges but did not alter evoked fEPSPs. These results suggest that NMDARs are involved in the hyperexcitability induced by Abeta(1-42). Similar results have been recently reported by Varghese et al. using cultured embryonic rat hippocampal cells. Abeta increased spontaneous firing activity dose dependently, reaching the maximum after 1 hour of treatment, which was followed by a complete cessation of spikes following a few hours [42]. The discrepancy between this and our result may be due to the fact that the authors have used a cell culture largely devoid of glial elements, while we have used a complex microcircuitry built up from all of the neuronal cell types (including glia) forming elaborate connections.

The forms of NMDAR are heterotetramer including two NR1 and two NR2 subunits [43]. One particular subunit, NR2B, is mainly localized in extrasynaptic side. Abeta induced elevation of spiking activity could be prevented by blocking the NR2B subunits, suggesting that Abeta activates extrasynaptic NMDARs. The role of extrasynaptic or perisynaptic NMDARs in Abeta(1-42)-induced changes in functional synaptic plasticity and subsequent cell death has received much attention recently [22, 44, 45]. Extrasynaptic NMDARs contain predominantly NR2B subunits, which in contrast to NR2A-containing synaptic NMDARs trigger apoptotic signaling cascade [46]. Recently it was shown, using organotypic hippocampal cell cultures, that Abeta induces neuronal death and subsequent tau hyperphosporilation via extrasynaptic NR2B subunit [47]. Blocking NR2B subunits not only prevents hyperexcitation caused by Abeta but also rescues Abeta induced LTP impairment [22, 48], suggesting that blocking NR2B subunits might be a promising target in AD. However, others have reported that Abeta oligomers directly activate NMDARs especially via NR2A subunit [49, 50].

5. Conclusions

Taken together, we show that two electrophysiological events, recorded from hippocampal slices, which evoked fEPSPs and spontaneous firing, are not mediated by the same mechanisms. Evoked fEPSPs, but not firing activity, were mainly regulated by AMPARs. In contrast, spontaneous spikes are governed by NMDAR function. Bath applications of synaptotoxic Abeta(1-42) enhanced firing activity in an NR2B-dependent manner without altering evoked fEPSPs. These effects may contribute to synaptic dysfunctions seen in early AD.

6. Highlights

(i) Spontaneous discharges and evoked fEPSPs were recorded from the CA1 of murine slices.

(ii) AMPA receptor blockade ablates fEPSPs without any effect on spiking rate.

(iii) NMDA receptor modulation affects spontaneous spiking, but not evoked fEPSPs.

(iv) Synaptotoxic amyloid-beta leaves evoked fEPSPs unaltered but increases firing activity via NR2B.

(v) These results confirm that amyloid-beta induces hyperexcitation through NR2B activation.

Abbreviations

Abeta(1-42):	Amyloid-beta(1-42)
ACSF:	Artificial cerebrospinal fluid
AD:	Alzheimer's disease
AMPA:	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR:	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA:	Analysis of variance
CNQX:	6-Cyano-7-nitroquinoxaline-2,3-dione
fEPSP:	Field excitatory postsynaptic potential
GABA:	Gamma-aminobutyric acid
Glu:	Glutamate
Low Mg^{2+} ACSF:	Low magnesium concentration-containing artificial cerebrospinal fluid
LTP:	Long-term potentiation
MEA:	Multielectrode array
NMDA:	N-Methyl-D-aspartate
NMDAR:	N-Methyl-D-aspartate receptor
TTX:	Tetrodotoxin
WB:	Western blot.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Zsolt Bozsó, Lívia Fülöp, and Botond Penke synthetized and characterized Abeta. Edina Varga performed the experiments, Edina Varga and Viktor Szegedi analyzed the data, and Edina Varga, Gábor Juhász, and Viktor Szegedi designed the experiments and wrote the paper.

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ORIGINAL PAPER

Overexpression of Hsp27 ameliorates symptoms of Alzheimer's disease in APP/PS1 mice

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Abstract Hsp27 belongs to the small heat shock protein family, which are ATP-independent chaperones. The most important function of Hsp27 is based on its ability to bind non-native proteins and inhibit the aggregation of incorrectly folded proteins maintaining them in a refolding-competent state. Additionally, it has anti-apoptotic and antioxidant activities. To study the effect of Hsp27 on memory and synaptic functions, amyloid-β (Aβ) accumulation, and neurodegeneration, we generated transgenic mice overexpressing human Hsp27 protein and crossed with APPswe/PS1dE9 mouse strain, a mouse model of Alzheimer's disease (AD). Using different behavioral tests, we found that spatial learning was impaired in AD model mice and was rescued by Hsp27 overexpression. Electrophysiological recordings have revealed that excitability of neurons was significantly increased, and long-term potentiation (LTP) was impaired in AD model mice, whereas they were normalized in Hsp27 overexpressing AD model mice. Using anti-amyloid antibody, we counted significantly less amyloid plaques in the brain of APPswe/PS1dE9/Hsp27 animals compared to AD model mice. These results suggest that overexpression of Hsp27 protein might ameliorate certain symptoms of AD.

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Introduction

AD is one of the most common neurodegenerative diseases, and its prevalence is strongly correlated with aging (Evans et al. 1989). AD is characterized by progressive loss of memory and cognitive functions (McKhann et al. 1984). The pathological hallmarks of AD are extracellular amyloid plaque deposition, formation of intracellular neurofibrillary tangles, and other alterations in the brain such as activation of microglial cells, oxidation of lipids, induction of reactive oxygen species, disruption of neuronal metabolic homeostasis, and neuronal cell death (Serrano-Pozo et al. 2011; Monji et al. 2001). Amyloid plaques and neurofibrillary tangles are present mainly in brain regions involved in learning, memory, and emotional behaviors, primarily in the hippocampus (Mattson 2004).

Heat shock proteins (Hsps) are ubiquitously expressed evolutionary conserved proteins which are upregulated by different stressors and also in various pathological conditions. Most of the stress-induced proteins are molecular chaperons, and they have an essential role in biosynthesis, transport, translocation, folding, and assembly of other proteins (Morimoto et al. 1992). Under stress, Hsps prevent protein misfolding and oligomerization, and they transfer misfolded proteins to the proteasome for degradation (Becker and Craig 1994). They also assist in the maintenance of normal cellular homeostasis and metabolism.

Hsp27 belongs to the small heat shock protein (sHsp) family, which are ATP-independent chaperones (Jakob et al. 1993). The most important function of sHsps is that they can

inhibit the aggregation of incorrectly folded proteins by binding to non-native proteins, and maintain them in a refolding-competent state (Haslbeck and Buchner 2002). Moreover, they also have anti-apoptotic and antioxidant activities and can bind to the actin cytoskeleton and stabilize it (Arrigo et al. 2002, Preville et al. 1999, Wang and Spector 1996).

Hsps are upregulated in several neurodegenerative diseases and can protect brain cells against free radical injury, oxidative stress, and misfolded proteins (Kalmar and Greensmith 2009). Several studies indicate that chaperones are potent suppressors of neurodegeneration and are, therefore, promising therapeutic targets for protein conformational disorders (reviewed in Muchowski and Wacker 2005). For example, treating a polyglutamine disease model with an antiulcer drug, geranylgeranylacetone (GGA), which can induce the expression of different Hsps, could suppress the accumulation of pathogenic proteins and ameliorate the related phenotype (Katsuno et al. 2005). In a rat model of Parkinson's disease, Hsp104 was found to be similarly efficient (Lo Bianco et al. 2008). An increased level of expression of small Hsps and Hsp70 in the brain of AD patients has been reported in a number of studies (Perez et al. 1991; Muchowski and Wacker 2005). Several studies suggest that expression of Hsps could suppress the progression of AD (Magrané et al. 2004; Muchowski and Wacker 2005; Evans et al. 2006). Hoshino et al. (2011) investigated the effect of overexpression of Hsp70 on AD-related phenotypes, using double transgenic mice overexpressing Hsp70 and a mutant (Swedish) type of APP (APPswe). They demonstrated that overexpression of Hsp70 inhibited the development of the pathological phenotype of AD and the resultant cognitive deficit, possibly through its effects on antiaggregation, neuroprotection, and stimulation of Aβ clearance.

To study the effect of a small heat shock protein, Hsp27, on memory and synaptic functions, Aβ accumulation, and neurodegeneration, we generated transgenic mice overexpressing the human Hsp27 protein and crossed them with the APPswe/PS1dE9 mouse strain, a widely used mouse model of AD.

Materials and methods

Animals

The study conformed to EU Directive 2010/63/EU and was approved by the regional Station for Animal Health and Food Control (Csongrád, Hungary) under project license XVI/02752/2009. Mice were housed in groups of two to three under standard conditions (24 °C, 12 h light–dark cycle) with food and water available ad libitum.

Generation of APPswe/PS1dE9/Hsp27 transgenic mice

We have previously generated transgenic mice overexpressing the human Hsp27 protein on FVB genetic background (Tóth et al. 2010). Transgenic DNA construct contained the human Hsp27 cDNA driven from a cytomegalovirus (CMV) promoter. This transgenic line was crossed with C57BL/6 mice four times in order to establish a Hsp27 transgenic line on a homogenous C57BL/6 genetic background. B6C3-Tg(APPswe/PS1dE9)85Dbo/Mmjax mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained on C57BL/6 genetic background. Hsp27 transgenic mice then were crossed with APPswe/PS1dE9 mice to generate APPswe/PS1dE9/Hsp27 multiple transgenic strain. To identify the genotype of the offsprings, polymerase chain reaction (PCR) was performed from tail biopsies using the following primers: 5′-GTC CCT GGA TGT CAA CCA CT-3′ and 5′-GAC TGG GAT GGT GAT CTC GT-3′ for Hsp27; 5′-GAC TGA CCA CTC GAC CAG GTT CTG-3′ and 5′-CTT GTA AGT TGG ATT CTC ATA TCC G-3′ for APP(Swe); and 5′-AAT AGA GAA CGG CAG GAG CA-3′ and 5′-GCC ATG AGG GCA CTA ATC AT-3′ for PS1dE9. The PCR amplification was done in a MJ Research PTC-200 Thermal Cycler using the following parameters: denaturation at 95 °C for 4 min followed by 29 cycles of denaturation at 95 °C for 1 min, annealing at 56 °C for 1 min and elongation at 72 °C 1 min, and a final extension at 72 °C for 4 min.

Western blot analysis

Expression of Hsp27 was determined from cortical and hippocampal tissues of 7 months old transgenic mice and wild type littermates. For ApoA1 protein level estimation, whole brain tissue was used. Total protein was isolated from frozen tissues homogenized in lysis buffer (containing 50 mM Tris–HCl, 150 mM NaCl, 1 % Triton X-100, 0.5 % Na deoxycholate, 0.1 % SDS, and protease inhibitor), centrifuged at 10,000 rpm for 10 min at 4 °C, and the supernatant was used for western blot analysis. Protein concentration was measured at 280 nm using a NanoDrop spectrophotometer (NanoDrop Technologies). Protein samples (50 μg) were separated by 10 % SDS-PAGE (Cambrex) and transferred electrophoretically onto Hybond-P PVDF membrane (Amersham). Membrane was blocked for 1 h in 5 % milk powder. After a short wash in PBS-Tween buffer, membrane was incubated with anti-Hsp27 (1:500, Stressgen, Cat No: SPA-803), anti-ApoA1 (1:250, Rockland, Cat. No: 600-101-196), and anti-β-actin (1:1,400, Sigma, Cat. No: A2103) antibodies overnight at 4 °C. After a brief wash, membranes were incubated with anti-goat (1:10,000, Jackson ImmunoResearch, Cat. No: 305-035-003) or anti-rabbit (1:35,000, Jackson ImmunoResearch, Cat. No: 111-035-003) secondary antibodies for 45 min at

room temperature. Signals were developed using Luminata Forte Western HRP Substrate (Millipore) according to the manufacturer's instructions. Signal intensities were quantified using ImageJ 1.45 software.

Behavioral tests

For behavioral testing, 7-month-old male mice were used. Before starting the tests, mice were handled daily for 2 weeks. Behavioral tests were performed between 9:00 a.m. and 4:00 p.m. Tests were recorded by video camera, and data were analyzed by EthoVision 2002 computer-controlled video tracking system (The Noldus Information Technology, The Netherlands).

Open field

Locomotor activity and anxiety-like behavior were studied using open field test. Each mouse was placed in the center of a plastic oval arena (50×70×30 cm) virtually divided into central and peripheral areas by the software. Total distance travelled (in centimeters), number of rearings, time spent with grooming, and time spent in the peripheral areas were measured. Data were collected for 5 min. All animals were tested once.

Barnes maze test

In the Barnes maze test, using a bright light, animals were reinforced to escape from the open platform surface to a small dark chamber located under the platform called "target box." The experiment was performed according to a technique published on the internet (Sunyer et al. 2007). The maze consisted of a circular platform (100 cm in diameter) with 20 holes (hole diameter, 5 cm) along the perimeter. The circular open field was elevated 90 cm from the floor. A black escape (target) box (19×6×7 cm) was located under one of the holes. The location of the target box was consistent for any given mouse, but was randomized across mice. The platform was surrounded by curtains and visual cues on it to help the mice with orientation. Mice were placed in the middle of the maze and were free to explore it. The trial ended after 3 min or when the mouse entered the escape box. When a mouse entered the escape box, it was allowed to stay in it for 1 min. If the mouse did not find the escape box, it was guided to it and left inside for 1 min. One trial per day was performed for five consecutive days. Time of latency to enter the target box was measured. On day 8, the probe trial was performed without the escape box to confirm that this spatial task was acquired based on navigation using distal-environment room cues. The trial ended after 2 min. Time of latency to reach the target hole was measured.

Morris water maze test

For the Morris water maze test, a circular pool (130 cm in diameter and 60 cm in height) was filled to a height of 45 cm with water. Water temperature was maintained between 22 °C and 23 °C, and it was made opaque by mixing milk in it. For the invisible platform test, a white colored platform (5 cm in diameter) was placed at the center in one of four quadrants of the pool (training quadrant) and submerged 1 cm below the water surface so that it was invisible at water level. The pool was surrounded by curtains, and different spatial cues were attached to the curtains to help the mice with orientation. The location of the platform was fixed at the same quadrant while the start position of swimming was varied. Mice were given two trials per day for five consecutive days, during which they were allowed to find the platform within 90 s. Once the mouse located the platform, it was permitted to stay on it for 10 s. If the mouse did not find the platform within 90 s, it was guided to the platform and placed on it for 10 s. The escape latency, the time required for the mouse to find and climb onto the platform, was recorded. The means of the data from each day were used for statistics. Some of the mice have shown floating behavior in the Morris water maze test. This means that mice remained motionless most of the time during the trial period. These mice were also very passive in the Barnes maze test. We counted the trials when mice showed floating/passive behavior and found that three mice were motionless almost in the 70 % of trials; therefore, these mice were excluded from the behavioral tests.

Immunohistochemistry

Subsequent to the behavioral tests, mice were sacrificed by cervical dislocation, and the brains were removed for further studies. Frozen sections (10 μm) of the brains were processed for immunohistochemistry. Sections were fixed in acetone for 3 min. After a brief wash with PBS, sections were incubated with 1.5 % H_2O_2 in methanol for 20 min then with 3 % H_2O_2 for 20 min to block endogenous peroxidases. PBS wash was followed by collagenase treatment using 0.1 mg/ml collagenase type II (Sigma) in a buffer containing 0.25 M NaCl, 50 mM Tris–HCl, pH 7.4, and 1 mM EDTA at 37 °C for 5 min. After a brief wash in PBS, sections were incubated in 5 % goat serum for 1 h at room temperature to prevent nonspecific binding of the antibody. Sections were then covered with anti-Aβ [1–42]

(1:60, Invitrogen, Cat No. 700254), anti-cleaved caspase-3 (1:50, Cell Signalling, Cat. No. Asp-175), or with anti-Hsp27 (1:50, Stressgen, Cat No: SPA-803) primary antibodies overnight at 4 °C. Slides were washed with PBS, then goat anti-rabbit secondary antibody (1:150, Jackson ImmunoResearch, Cat. No.111-035-003) was applied for 45 min. After washing, slides were incubated with developing reagent (10 % dimethyl formamide, 10 % imidazole, 0.02 % aminoethyl carbazole, 0.04 % H_2O_2) at room temperature until the brown color appeared (approximately 10 min). Sections were then counterstained with hematoxylin solution for 2 min at room temperature followed by washing with PBS, and they were covered with glycerol gelatin.

For Aβ [1–42] and cleaved capase-3 antibody stainings, 7-month-old transgenic (n=6) and wild type (n=6) animals were used. Stained sections were photographed using a light microscope equipped with CCD camera, and amyloid plaques or apoptotic cells were counted in 15 microscopic fields by two independent individuals.

Fluoro-Jade C staining

Brains were removed, and 10-μm acetone-fixed frozen sections were prepared. Slides were immersed into absolute ethanol for 5 min, then rinsed in 70 % ethanol for 2 min, in 30 % ethanol for an additional 2 min, and finally in distilled water for 2 min. Sections then were incubated in 0.06 % potassium permanganate solution for 30 min. Following a 2-min water rinse, sections were transferred into a 0.0004 % solution of Fluoro-Jade C (Chemicon) fluorescent dye dissolved in 0.1 % acetic acid. Slides were washed three times in distilled water for 1 min. After drying, sections were cleared in xylene for 2 min and coverslipped with DPX nonfluorescent mounting media. Degenerating neurons emitting green fluorescence were visualized under Nikon Eclipse E600 fluorescent microscope using a 520-nm fluorescein filter set. Six 7-month-old animals from each group were selected, and two slides of each animal's brain were stained using Fluoro-Jade C dye. On each slide, 15 digital photographs were taken, and degenerating neurons were counted by two independent individuals.

Hippocampal slice electrophysiology

Following standard procedures, 400-μm-thick transverse hippocampal slices were prepared from the brain of 7-month-old mice using a McIlwain tissue chopper (Campden Instruments, Loughborough, UK). Slices were incubated in standard artificial cerebrospinal fluid (ACSF) at ambient temperature for 60 min, which was constantly gassed with 95 % O_2–5 % CO_2. ACSF contained (millimolar) NaCl, 130; KCl, 3.5; $CaCl_2$, 2; $MgCl_2$, 2; NaH_2PO_4, 0.96; $NaHCO_3$, 24; D-glucose, 10 (pH 7.4). Individual slices were transferred to a 3D-MEA chip with 60 tip-shaped and 60-μm-high electrodes spaced by 200 μm (Ayanda Biosystems, S.A., Lausanne, Switzerland). The slice was continuously perfused with oxygenated ACSF (1.5 ml/min at 36 °C) during the whole recording session. Data were recorded by a standard, commercially available MEA setup (Multi-Channel Systems MCS GmbH, Reutlingen, Germany). The Schaffer collateral was stimulated by injecting a biphasic voltage waveform (−100/+100 μs) through one selected electrode at 0.033 Hz. Care was taken to choose the stimulating electrode in the same region from one slice to the other. The peak-to-peak amplitudes of fEPSPs at the stratum pyramidale and stratum radiatum of CA1 were analyzed. After a 30-min incubation period, the threshold and the maximum of stimulation intensity for evoked responses was determined. For evoking responses, 30 % of the maximal stimulation intensity was used.

Following a stable 10-min control sequence, the stimulus intensity was continuously increased from 400 to 3,200 mV with 400 mV steps (input/output curve). Paired-pulse protocol consisted of two stimulation pulses injected with interstimulus intervals of 20, 50, 200, and 500 ms with 0.033 Hz. Three data points were obtained at every stimulation intensity/interstimulus intervals at the input/output curve and paired-pulse protocol. LTP was induced using a theta-burst stimulation (TBS) pattern applied at the maximum stimulation intensity. TBS comprised a train with ten bursts given at 5 Hz per train and four pulses given at 100 Hz per burst.

RNA extraction and quantitative real-time PCR

For quantitative real-time PCR (Q-PCR), 6-month-old Hsp27 transgenic (n=3) and wild type mice (n=3) were used. Total RNA was extracted from whole brain using Trizol reagent (Invitrogen), according to the manufacturer's protocol. RNA samples then were cleaned using NucleoSpin RNA Clean-up columns (Macherey-Nagel) and treated with DNaseI (Macherey-Nagel) for 30 min. First-strand cDNA was synthesized by reverse transcription (5 μg RNA/30 μl reaction volume) using High-Capacity cDNA Archive kit (Applied Biosystems). The temperature profile of the reaction was as follows: 10 min at room temperature, 2 h at 37 °C, 5 min on ice, and finally, 10 min at 75 °C.

Q-PCR was performed on a RotorGene 3000 instrument (Corbett Research, Sydney, Australia) using gene-specific primers listed in Table 1. The cDNA was diluted 1:20, and 9 μl of this mix was used as a template in the reaction.

Table 1 List of Q-PCR primers used in this study

Gene	Forward primer	Reverse primer
Amyloid precursor protein (APP)	TACTGCATGGCGGTGTGT	CGTGGGAAGTTTATCAGGATCT
Apolipoprotein-AI (ApoAI)	TATGTGGATGCGGTCAAAGA	TGAACCCAGAGTGTCCCAGT
Apolipoprotein-D (ApoD)	GAAGGCTTTTACAGGCTCCA	GGCCAGGAACATCAGCAT
Apolipoprotein-E (ApoE)	CAATTGCGAAGATGAAGGCTC	TAATCCCAGAAGCGGTTCAG
Nitric oxide synthase-1 (Nos1)	CATCAGGCACCCCAAGTT	CAGCAGCATGTTGGACACA
Nitric oxide synthase-3 (Nos3)	CCAGTGCCCTGCTTCATC	GCAGGGCAAGTTAGGATCAG
LDL receptor-related protein-1 (Lrp1)	ACCACCATCGTGGAAAATG	GTCCCAGCCACGGTGATA
LDL receptor-related protein-2 (Lrp2)	GATGGATTAGCCGTGGACTG	TCCGTTGACTCTTAGCATCTGA
Low-density lipoprotein receptor (LDLr)	CAAGAGGCAGGGTCCAGA	CCAATCTGTCCAGTACATGAAGC
Heat shock protein 90 (Hsp90)	GTCTCGTGCGTGTTCATTCA	CATTAACTGGGCAATTTCTGC

Reactions were performed in a total volume of 20 μl containing 10 μl of FastStart SYBR Green Master mix (Roche) and 5 mM of each primer. The amplification was carried out with the following cycling parameters: 15 min heat activation at 95 °C, followed by 45 cycles denaturation at 95 °C for 25 s, annealing at 60 °C for 25 s, and extension at 72 °C for 20 s. Fluorescent signals were collected after each extension step at 72 °C. Relative expression ratios were normalized to β-actin.

Statistical analysis

All data obtained in this experiment were expressed as mean ± SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Fisher's post hoc test using OriginPro8 software. Electrophysiological data were analyzed by two-way ANOVA followed by Tukey's post hoc test. The level of statistical confidence was set at $p<0.05$.

Results

Generation of transgenic mice

Transgenic mice overexpressing the human Hsp27 protein were generated in our laboratory as described earlier (Tóth et al. 2010). By crossing the human Hsp27 overexpressing mice with the APPswe/PS1dE9 mouse strain, we generated a triple transgenic mouse strain. The APPswe/PS1dE9 strain (The Jackson Laboratory, Bar Harbor, ME, USA) is a widely used mouse model of AD (Jankowsky et al. 2004, Reiserer et al. 2007, Hamilton and Holscher 2012), and it shows several symptoms of the disease including impaired spatial learning, reduced long-term potentiation, widespread amyloid deposition, and mild neurodegeneration (The Jackson Laboratory (2013)) (http://jaxmice.jax.org/strain/004462.html). First, cerebral expression of the Hsp27 transgene was investigated. Using western blot analysis, robust expression of human Hsp27 was detected in the cortical and hippocampal tissues of Hsp27 and triple transgenic (AD/Hsp) brains, while no expression was found in wild type and APPswe/PS1dE9 (AD) littermates (Fig. 1a). Hsp27 expression was also confirmed in the brain of Hsp27 and APPswe/PS1dE9/Hsp27 animals using immunohistochemical staining (Fig. 1b). While Hsp27 antibody stained hippocampal tissues of Hsp27 transgenics homogeneously, it showed a spotted, plaque-like staining pattern in the hippocampal region of APPswe/PS1dE9/Hsp27 brains (Fig. 1c).

Behavioral tests

Seven-month-old male mice were divided into three experimental groups: an AD mouse model group (APPswe/PS1dE9, $n=11$), an APPswe/PS1dE9/Hsp27 triple transgenic group ($n=12$), and a wild type group ($n=9$).

Locomotor activity

First, locomotor activity (total distance travelled), exploratory behavior (number of rearings), and anxiety (time spent on the periphery, time spent with grooming) were recorded using open field test. There was no significant difference between groups in these behavioral features (total distance travelled, $p=0.25$; rearings, $p=0.34$; time spent on periphery, $p=0.33$; grooming, $p=0.64$).

Barnes maze test

Spatial learning and memory were tested using Barnes maze and Morris water maze tests. Both tests are based on spatial navigation, and the performance of the animals depends on hippocampal function (Kennard and Woodruff-Pak 2011). In Barnes maze test, APPswe/PS1dE9 mice spent more than 130 s with searching the escape box on

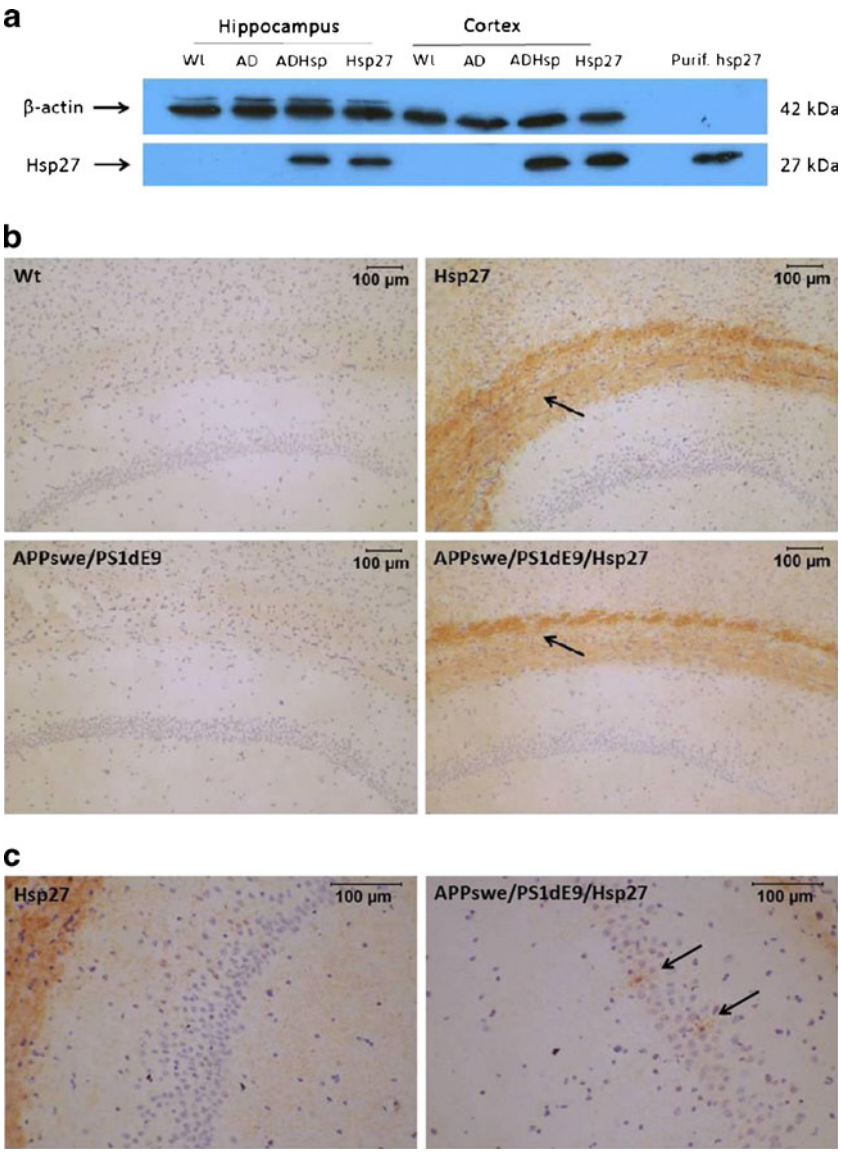

Fig. 1 **a** Detection of human Hsp27 expression in the hippocampus and cortex of wild type (Wt), APPswe/PS1dE9 (AD), APPswe/PS1dE9/Hsp27 (ADHsp), and Hsp27 transgenic mice (Hsp27) using western blot analysis. **b** Hsp27 immunostaining of brain sections of 7 months old wild type (Wt), Hsp27 transgenic (Hsp27), APPswe/PS1dE9, and APPswe/PS1dE9/Hsp27 mice. *Brown staining* indicates human Hsp27 immunoreactivity (*black arrows*). Nuclei were counterstained with hematoxylin. *Scale bar* indicates 100 μm. **c** Higher magnification of the hippocampal region of Hsp27 transgenic and APPswe/PS1dE9/Hsp27 mice stained by anti-Hsp27 antibody. *Black arrows* indicate a plaque-like staining of Hsp27. *Scale bar* represents 100 μm

each day of the training period. In contrast, wild type and APPswe/PS1dE9/Hsp27 mice learned soon to find the escape hole as indicated by the reduction of escape latency. On the fourth day, mice from the wild type group spent 76 s with searching and performed significantly better than APPswe/PS1dE9 mice (140 s; $p<0.05$; Fig. 2a). However, there was no significant difference between the performance of wild type and APPswe/PS1dE9/Hsp27 mice. On the fifth day, the escape latency of the wild type and APPswe/PS1dE9/Hsp27 groups were 92 and 72 s, respectively, performing significantly better then APPswe/PS1dE9 mice (169 s; $p<0.01$ and $p<0.001$), and again, there was no significant difference between APPswe/PS1dE9/Hsp27 and wild type groups (Fig. 2a). The probe trial was performed 3 days after the last training session. The result of the probe trial was similar to the previous trial; wild type and APPswe/PS1dE9/Hsp27 mice found the correct target hole within 50 and 41 s, respectively, and these were significantly shorter compared to APPswe/PS1dE9 mice (93 s; $p<0.05$ and $p<0.01$). No statistical difference was observed between wild type and APPswe/PS1dE9/Hsp27 groups (Fig. 2b).

Morris water maze test

Spatial learning was also tested using Morris water maze to confirm the result of Barnes maze test. On the third day, wild type mice find the escape platform in 41 s on average, and this was significantly shorter compared to APPswe/PS1dE9 group (76 s, $p<0.01$; Fig. 2c). On the fourth day, the escape latency of the wild type and APPswe/PS1dE9/Hsp27 groups was 52 and 50 s, respectively, performing significantly better than APPswe/PS1dE9 mice (74 s; $p<0.05$). There was no difference

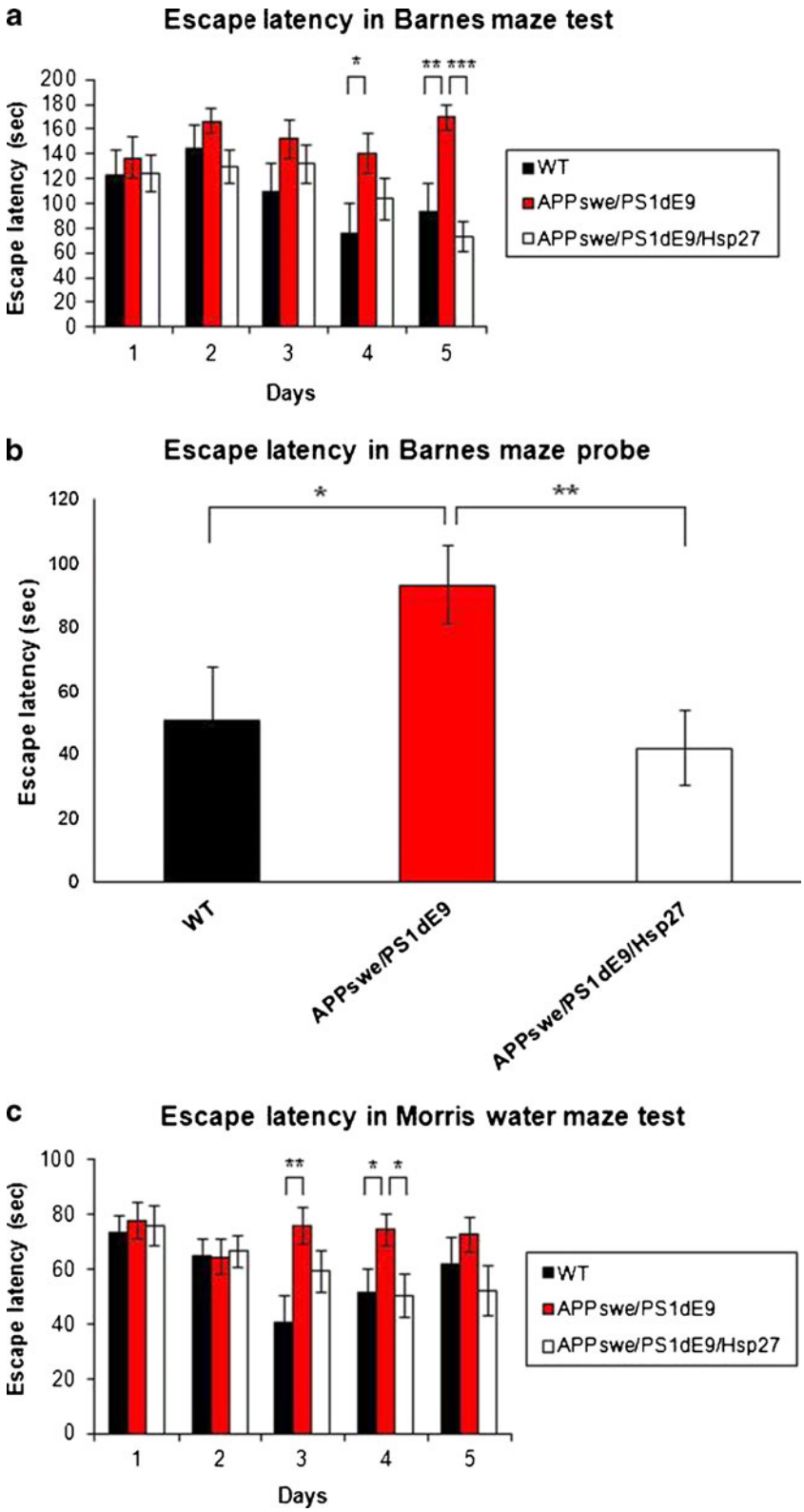

Fig. 2 Assessment of learning and memory using behavioral assays. Cognitive behavior of 7 months old wild type (WT, $n=9$), APPswe/PS1dE9 ($n=11$), and APPswe/PS1dE9/Hsp27 ($n=12$) transgenic mice were compared. Escape latency in seconds during training session (**a**) and probe test of the Barnes maze test (**b**). Escape latency in seconds in Morris water maze test (**c**). Mean ± SEM is shown; *$p<0.05$; **$p<0.01$; ***$p<0.001$

between the performance of APPswe/PS1dE9/Hsp27 and wild type groups (Fig. 2c). Interestingly, on the last day of training sessions, there was no statistical difference between groups, and the performance of most animals has declined.

Hippocampal slice electrophysiology

Hippocampal function was studied using paired-pulse facilitation (PPF) and long-term potentiation (LTP). Excitability

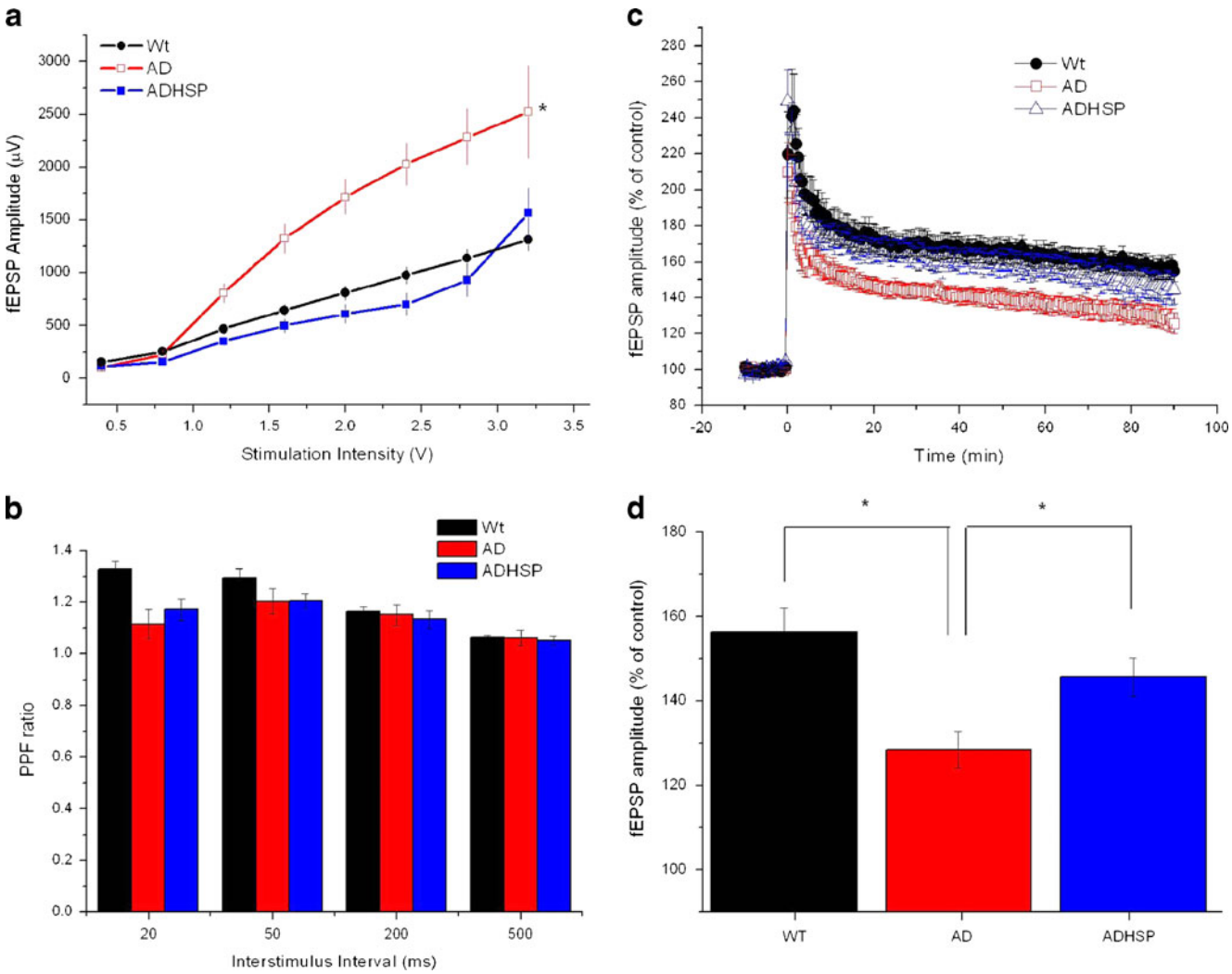

Fig. 3 Electrophysiological recordings on hippocampal slices from 7 months old mice. **a** Neuronal excitability of transgenic mice at different stimulation intensities. Data was analyzed by two-way ANOVA followed by Tukey's test. *Asterisk* denotes $p<0.05$. **b** Assessment of synaptic function in wild type (Wt, $n=8$), APPswe/PS1dE9 (AD, $n=6$), and APPswe/PS1dE9/Hsp27 (ADHSP, $n=7$) mice using PPF. There was no significant difference in PPF values of different genotypes. **c** TBS induced LTP in transgenic (AD and ADHSP) and wild type mice. **d** *Bar graph* shows the fEPSP amplitude means of the last 5 min of LTP. *Asterisk* denotes $p\leq0.05$ analyzed by ANOVA and post hoc Bonferroni test

of neurons was studied at stimulation intensity of 0.4, 0.8, 1.2, 1.6, 2.0, 2.4, 2.8, and 3.2 V. Slices prepared from brains of AD model (APPswe/PS1dE9) mice showed an elevated fEPSP increase at the same stimulus intensity compared to control, indicating an increased excitability of neurons ($p\leq$ 0.05, two-way ANOVA; Fig. 3a). This increased synaptic excitability was normalized in APPswe/PS1dE9/Hsp27 mice (Fig. 3a). However, paired-pulse facilitation did not differ significantly among the groups, indicating that presynaptic function was unaffected by the genotype (Fig. 3b).

LTP is a cellular correlate of learning and memory. We found that LTP was impaired in the APPswe/PS1dE9 group (125±6 % vs. 154±6 % in the wild type, $p\leq0.05$), but this was partially rescued in the APPswe/PS1dE9/Hsp27 genotype mice (144±8 %; Fig. 3c, d).

Amyloid staining

Amyloid plaque formation was studied by immunostaining of frozen brain sections of 7-month-old transgenic and wild type mice using anti-Aβ [1–42] antibody. Numerous amyloid deposits were observed in the hippocampus and cortex of APPswe/PS1dE9 transgenic animals (0.99/field of view and 1.08/field of view, respectively), in contrast to wild type animals (0.02/field of view in the hippocampus and 0.06/field of view in the cortex; Fig. 4a, b). Amyloid plaques were detected in APPswe/PS1dE9/Hsp27 brains as well (0.69/field of view in the hippocampus and 0.78/field of view in the cortex; Fig. 4a, b); however, the number of plaques was significantly less in both regions than in APPswe/PS1dE9 group ($p<0.05$; Fig. 4c), indicating that Hsp27 might prevent

amyloid aggregation and plaque formation, or it can facilitate amyloid clearance and degradation in the brain.

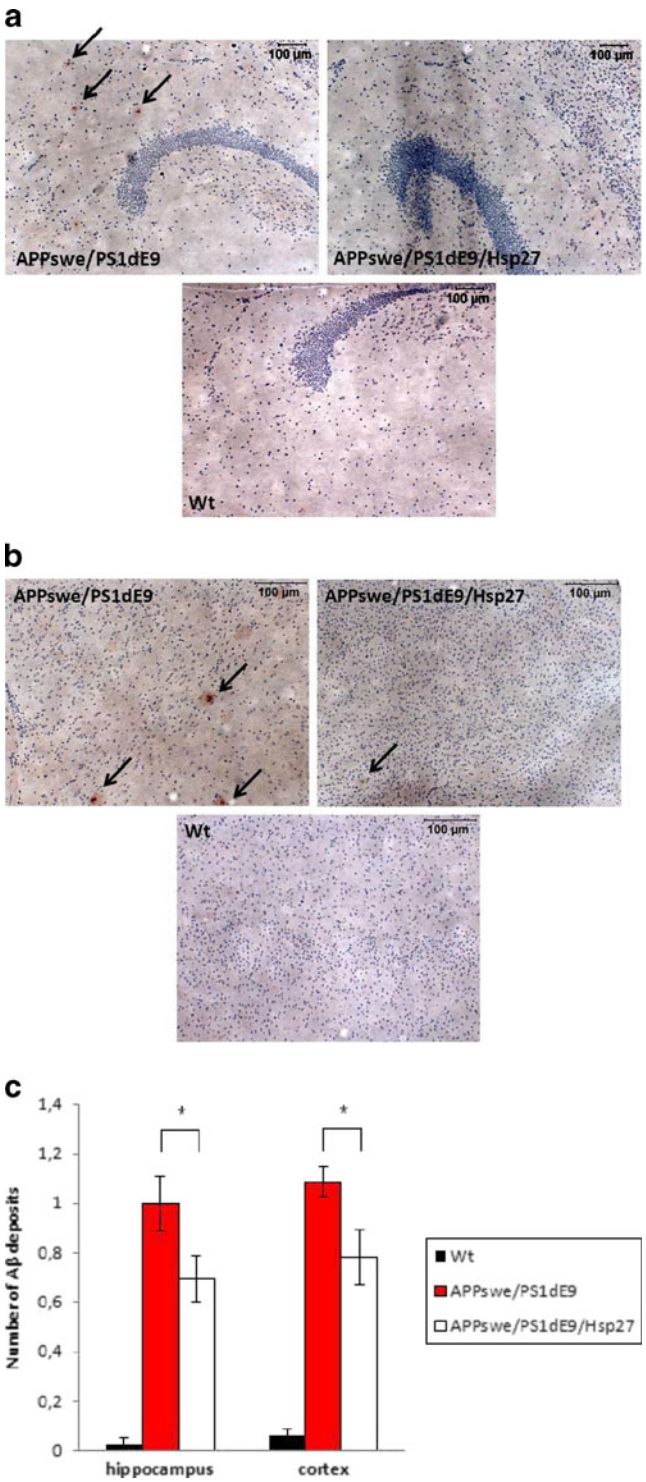

Fig. 4 Immunostaining of hippocampal (**a**) and cortical (**b**) amyloid plaques in 7 months old APPswe/PS1dE9, APPswe/PS1dE9/Hsp27 transgenic, and wild type (Wt) mice. *Arrows* indicate the presence of amyloid deposits. **c** Number of Aβ deposits/field of view in wild type (Wt, n=6), APPswe/PS1dE9 (n=6), and APPswe/PS1dE9/Hsp27 mice (n=6; mean ± SEM is shown; *p<0.05)

Neuronal apoptosis

We used anti-active caspase-3 antibody and Fluoro-Jade C stainings to monitor apoptosis and neurodegeneration in the brain of 7-month-old transgenic mice. Activation of effector caspase-3 molecules triggers the activation of caspase cascade, which eventually leads to apoptosis of the cells (Boatright and Salvesen 2003). Fluoro-Jade C is an anionic fluorochrome, which sensitively and specifically binds to degenerating neurons (Schmued et al. 2005). We detected significantly increased number of apoptotic cells (Fig. 5a) and degenerated neurons (Fig. 5b) in the hippocampal as well as cortical regions of APPswe/PS1dE9 and APPswe/PS1dE9/Hsp27 transgenic brains compared to wild type animals (p<0.001); however, there was no statistical difference between AD model mice and AD model mice expressing Hsp27.

Gene expression analysis of Hsp27 transgenic mice

Results obtained above indicated that Hsp27, as a molecular chaperone, might have a role in inhibition of amyloid

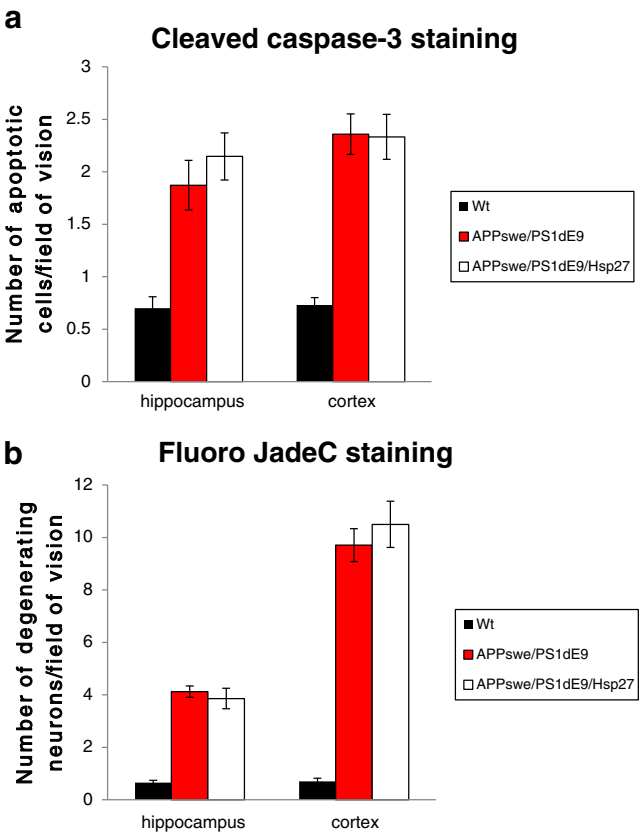

Fig. 5 Quantitative comparison of apoptotic cell (**a**) and degenerating neuron (**b**) numbers in the hippocampus and cortex of 7 months old wild type (Wt, n=6), APPswe/PS1dE9 (n=6), and APPswe/PS1dE9/Hsp27 (n=6) mice. Apoptotic cells were detected by anti-active caspase-3 antibody staining, while degenerating neurons were visualized using Fluoro-Jade C staining. Mean ± SEM is shown

accumulation/deposition or in enhanced amyloid clearing and degradation. In order to clarify its molecular role, we monitored the gene expression of several genes potentially involved in β-amyloid metabolism such as APP, ApoA1, ApoD, ApoE, LDLr, Lrp1, Lrp2, Hsp90, and neurodegeneration (NOS1 and NOS2) in the cortex of Hsp27 transgenic mice using Q-PCR. Primers used in this study are listed in Table 1. The expression level of ApoD and Lrp2 was slightly increased (128 % and 128 %, respectively), in the brain of Hsp27 transgenic mice compared to wild type controls (100 %), whereas there was no change in the mRNA level of APP, ApoE, LDLr, Lrp1, Hsp90, NOS1, and NOS3. Rather surprisingly, cortical expression of ApoA1 was reduced by half in Hsp27 transgenics versus wild type mice (Fig. 6a). Decreased ApoA1 expression in Hsp27 transgenic mice was further confirmed using western blotting. ApoA1 protein level was reduced in Hsp27 transgenic mice (61.1 %), but slightly elevated in AD model mice (126.7 %) compared to wild types (100 %). However, AD mice overexpressing human Hsp27 protein possessed similar ApoA1 protein level than wild type mice, indicating that Hsp27 influenced ApoA1 expression (Fig. 6b, c).

Discussion

In this study, we investigated the effects of small heat shock protein, Hsp27, on the development of AD-related phenotypes in transgenic mice. For this purpose, we used APPswe/PS1dE9 transgenic strain. These mice develop several AD-related phenotype including amyloid-beta deposits, abnormal spatial memory, axon degeneration, and synapse loss (The Jackson Laboratory (2013)) (http://jaxmice.jax.org/strain/004462.html). We overexpressed the human Hsp27 protein in AD model mice and monitored learning and memory, synaptic function, amyloid deposition, and neuronal apoptosis in the triple transgenic mice.

Decline of the short-term memory is among the primary symptoms in AD patients. We found that spatial learning was impaired in APPswe/PS1dE9 mice as the time required to find the escape hole in Barnes maze test, or the platform in Morris water maze test was significantly longer compared to wild type mice. However, the performance of APPswe/PS1dE9/Hsp27 group was similar to the wild type group in both tests, which suggests that overexpression of Hsp27 protein ameliorates learning and memory deficits.

We have also found that synaptic excitability was increased in AD model mice, but this was normalized by Hsp27 overexpression. This is in accordance with earlier findings of others who demonstrated that seizure susceptibility is increased in AD model mice (Westmark et al. 2008; Vogt et al. 2009), and heat shock proteins can moderate chemically induced seizures (Akbar et al. 2003; Ekimova et al. 2010). LTP, a cellular correlate of learning and memory (Bliss and Colingridge 1993), was also investigated on hippocampal slices. Previously, it was demonstrated that soluble oligomers of Aβ are able to inhibit LTP and cause learning and memory deficit (Haas and Selkoe 2007; Bozso et al. 2010). Our results have shown that LTP was impaired in APPswe/PS1dE9 mice indeed, but it was normalized upon Hsp27 overexpression. There is substantial evidence from transgenic mouse models that intracellular Aβ initiates cellular dysfunction, synaptic, electrophysiological, and behavioral changes before it accumulates in extracellular plaques (Moechars et al. 1999; Kumar-Singh et al. 2000; Mucke et al. 2000). The toxic Aβ molecules (either soluble oligomers or fibrillar forms) might initiate a cascade, which finally leads to neuronal dysfunction. Therefore, inhibition of Aβ oligomerization and deposition or enhanced clearance of Aβ might play a crucial role in the prevention and therapy of AD. We have found a significantly lower level of amyloid deposition in the brain of AD/Hsp27 animals compared to the AD group, indicating that Hsp27 is somehow involved in these processes. In AD, heat shock protein expression is associated with Aβ deposition and neurofibrillary tangles, and recent findings suggest that Hsps prevent the accumulation of Aβ (Abdul et al. 2006; Evans et al. 2006). Wilhelmus et al. (2006) demonstrated that Hsp20, Hsp27, and αB-crystallin bind strongly to Aβ and inhibit its aggregation. Furthermore, Hsp20, Hsp27, and αB-crystallin inhibited cerebrovascular Aβ toxicity, probably by reducing the accumulation of Aβ at the cell surface.

Aggregated forms of Aβ might lead to neurodegeneration through different pathways. Several studies have shown that chronic inflammatory response to deposited amyloid resulted in neurodegeneration (Akiyama et al. 2000; Wyss-Coray and Mucke 2002). Although heat shock proteins have protective effects in neurodegenerative disease models, the molecular basis of this protection is not fully understood. It is likely that heat shock proteins have effect at several levels, and their neuroprotective function might not be linked exclusively to their effect on protein aggregation (Muchowski and Wacker 2005). For example, Hsps are known to reduce oxidative stress and mediate apoptotic pathway. They can also facilitate proteasomal degradation, which might help in Aβ clearance. However, the exact molecular mechanisms of Aβ-induced neurotoxicity are not fully known. These mechanisms are very diverse, and probably include the increased production of reactive oxygen species (ROS), altered calcium homeostasis, and enzyme activities. Several lines of evidence are available suggesting that Aβ can interact with artificial and biological membranes altering their fluidity. Altered membrane fluidity can influence the function of other membrane components. Certain studies suggest that the insertion of Aβ into the plasma membrane triggers different events catalyzed by perturbation of the conformation of membrane proteins, which finally leads to neurotoxicity (Kanfer et al. 1999). This

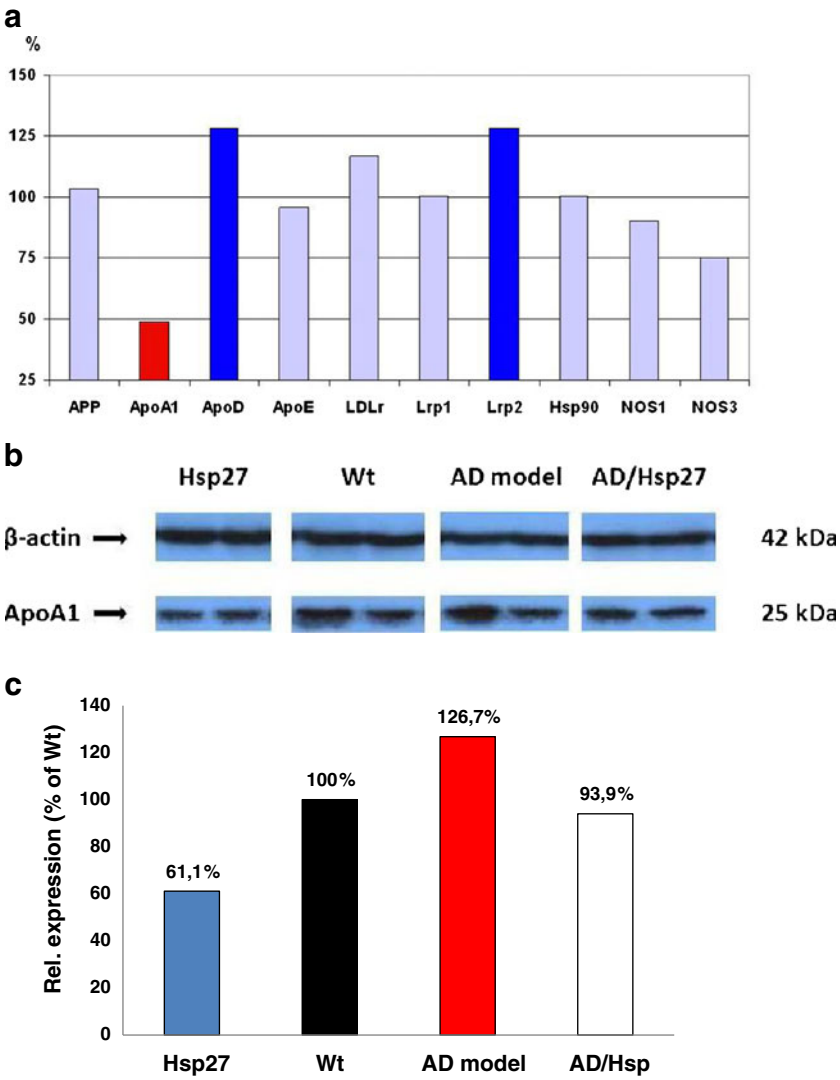

Fig. 6 Gene expression profiles of different genes involved in amyloid metabolism in Hsp27 transgenic mice using Q-PCR analysis (**a**). Data was normalized to the endogenous β-actin, then expressed as a percent of wild type expression (100 %). **b** Representative western blot of ApoA1 expression in Hsp27 transgenic (Hsp27), wild type (Wt), AD model (APPswe/PS1dE9), and AD/Hsp27 (APPswe/PS1dE9/Hsp27) brains. **c** Statistical analysis of western blot experiment. Hsp27 transgenic (n=5), wild type (Wt, n=6), AD model (n=5), and AD/Hsp triple transgenic mice (n=6) were compared

hypothesis suggests that the neurotoxic cascade of Aβ is initiated in the cell membrane. It is also important, that α-, β-, and γ-secretases are associated with cellular membranes; thereby, their function, such as APP processing, strongly depends on membrane fluidity. Peters et al. (2009) showed that oligomeric Aβ can reduce membrane fluidity, which in turn stimulates the amyloidogenic processing of APP. Several studies suggest that a subpopulation of Hsps is present either on the surface or within the cellular membranes, and they have been shown to modulate major attributes of the membrane lipid phase such as fluidity and permeability. By stabilizing the membrane, Hsps can restore membrane functionality during stress conditions (Horváth et al. 2008). We can suppose that Hsp27 also can interact with and stabilize cellular membranes; thereby, it can diminish the level of Aβ-induced neurotoxic cascade or, moreover, the production of Aβ. The generation of ROS is also a crucial factor in the molecular mechanisms of Aβ-induced neurotoxicity. Lipid peroxidation, and protein and DNA oxidation are remarkable in the brain of AD patients, and Aβ might have a central role in the induction of these processes (reviewed in Butterfield et al. 2001). Hsp27 has a protective effect in oxidative stress; thereby, Hsp27 might eliminate the neurotoxicity of Aβ at least in part due to this property.

To know more about the role of Hsp27 in amyloid metabolism—whether it inhibits APP production or enhances amyloid clearance/degradation—we performed gene expression analysis. No decrease in APP production was detected; however, a subtle elevation of ApoD and LRP1 protein levels in Hsp27 transgenic brains was observed. Although ApoD and LRP1 proteins have important roles in neuroprotection and Aβ clearance, respectively (Terrise at al. 1998; Kalman et al. 2000; Thomas et al. 2001; Muffat et al. 2008; Kang et al. 2000), we do not consider that the mild changes obtained in their expression would be sufficiently enough to lead to efficient reduction of amyloid plaque

numbers. Now it is widely accepted that ApoD is elevated in many neuropathological conditions, including Alzheimer's disease, Parkinson's disease, stroke, schizophrenia, and bipolar disorder. It was also demonstrated that in *Drosophila*, overexpressed ApoD had a protective effect against oxidative stress and heat shock (Muffat et al. 2008). In vitro LDL receptor-related protein 1 (LRP1) mediates the clearance of both Aβ 40 and Aβ 42 through a bona fide receptor-mediated uptake (Kang et al. 2000); therefore, LRP and its ligand ApoE have been proposed to be a clearance pathway for Aβ. At the same time, the role of ApoA1 in amyloid metabolism and AD pathology is controversial. Although it has been shown that ApoA1 inhibits Aβ aggregation in vitro (Koldamova et al. 2001), in vivo studies resulted different results. Crossing of ApoA1 null mice with AD model mice resulted no change in amyloid deposition in the brain (Fagan et al. 2004). Similarly, when ApoA1 overexpressing mice were crossed with AD model mice, no alteration in amyloid metabolism was observed (Lewis et al. 2010). Our result presented here shows that expression of ApoA1 was downregulated in Hsp27 transgenic mice, indicating that Hsp27 might have a role in the regulation (directly or indirectly) of ApoA1 expression.

Taken together, further investigations are necessary to reveal the exact molecular pathways involved in neuroprotective function of Hsp27.

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Conflicts of interest The authors declare no competing financial interests.

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