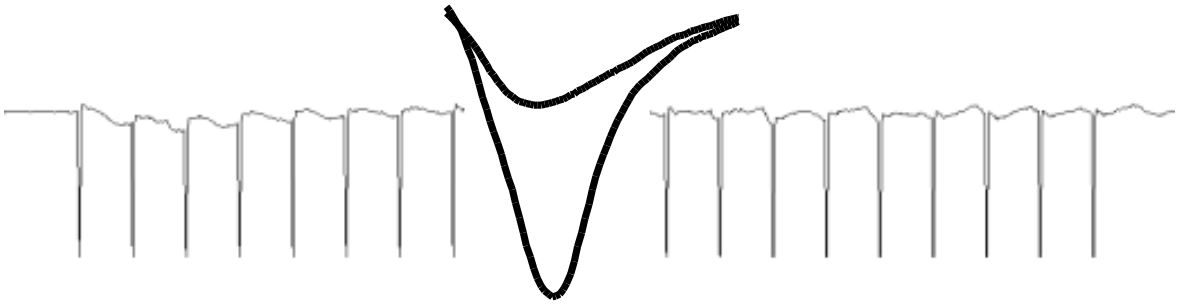
JULIUS-MAXIMILIANS-UNIVERSITÄT WÜRZBURG

Establishment and validation of hippocampal LTP for characterization of memory enhancing drugs as potential treatment of Alzheimer`s disease



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Submitted by

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from Nürnberg

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The thesis is based on the following manuscripts:

I. Kroker KS, Rosenbrock H, Rast G (2011) A multi-slice recording system for stable late phase hippocampal long-term potentiation experiments. *Journal of Neuroscience Method*s 194:394–401.
This manuscript is presented as chapter 2 in the thesis.

II. Kroker KS, Rast G, Rosenbrock H (2011) Differential effect of the mGlu5 receptor positive allosteric modulator ADX-47273 on early and late hippocampal LTP. *Neuropharmacology* 61(4):707-714.
This manuscript is presented as chapter 3 in the thesis.

III. Kroker KS, Rast G, Rosenbrock H. Differential effects of subtype-specific nicotinic acetylcholinergic receptor agonists on early and late hippocampal LTP. *European Journal of Pharmacology:* under minor revision.
This manuscript is presented as chapter 4 in the thesis.

IV. Kroker KS, Rast G, Giovannini R, Marti A, Dorner-Ciossek C, Rosenbrock H (2011) Inhibition of acetylcholinesterase and phosphodiesterase-9A has differential effects on hippocampal early and late LTP. *Neuropharmacology:* submitted.
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V. Kroker KS, Moreth J, Rast G, Kussmaul L, Rosenbrock H (2011) Effects of cognitive enhancing drugs on amyloid-beta oligomer induced impairment of hippocampal LTP. In preparation.
Parts of this manuscript are presented as chapter 6 in the thesis.

“Dissertation unter Einschluss mehrerer Manuskripte”

Erklärung zu Eigenanteilen an Publikationen und Zweitpuplikationsrechten

Puplikation (Vollständiges Zitat):
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Beteiligt an	**Autoren-Initialen, Verantwortlichkeit abnehmend von links nach rechts**				
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Puplikation (Vollständiges Zitat):
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ggf. Erläuterung: JM und LK: Bereitstellung / Charakterisierung der ADDLs

Für alle in dieser „kumulativen Dissertation“ verwendeten Manuskripte liegen die notwendigen Genehmigungen der Verlage und Co-Autoren für die Zweitpublikation vor.

Mit meiner Unterschrift bestätige ich die Kenntnisnahme und das Einverständnis meiner direkten Betreuer.

Katja Kroker, _____

Kandidat(in), Datum, Unterschrift

PRE-INDEX

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Summary

Alzheimer's disease (AD) is a progressive neurodegenerative disease of the brain. Today AD is the most common form of dementia in elderly people, accounting for around 50 – 60% of all cases of mental deterioration among persons over 65 years of age. It is clinically characterized by a progressive loss of memory and later on a decline in higher cognitive functions. The pathological hallmarks of AD, consistently demonstrated in brain tissue of patients, are extracellular amyloid-β (Aβ) plaques, intracellular neurofibrillary tangles of tau protein and a profound loss of mainly cholinergic and glutamatergic synapses and ultimatively neurons. Estimates foresee that more than 80 million individuals will be affected by the disease by 2040 due to population aging worldwide underlining the high medical need for this disease. In general, there exist two different potential approaches to treat AD patients: curative approaches, e.g. drugs inhibiting β-/γ-secretase and enhancing α-secretase as well as Aβ antibodies, and palliative approaches, e.g. memory enhancing drugs. In order to find suitable drugs for the treatment of AD, experimental model systems are utilized to explore potential drug candidates. Such an experimental system is hippocampal long-term potentiation (LTP), which is widely accepted as an *in vitro* model of cellular processes fundamentally involved in memory formation.

The present thesis focuses on the establishment and validation of LTP in rat hippocampal slices to characterize memory enhancing drugs as a potential treatment of AD. First, a multi-slice recording system was set up enabling stable measurements of LTP for up to seven hours from several slices simultaneously (chapter 2). Then, distinct protocols to induce early and late CA1 LTP, resembling short-term and long-term memory, were established. They were validated by addressing the hallmarks accepted for these forms of LTP: protein-synthesis independence and NMDA receptor dependence without contribution of L-VDCCs for early LTP, as opposed to protein-synthesis and NMDA / L-VDCCs dependence for late LTP (chapter 3).

As in AD patients a loss of mainly cholinergic and glutamatergic synapses is obvious, these validated forms of LTP were used to study drugs potentially being able to enhance cholinergic and/or glutamatergic neuronal functions. The effects of two drugs exclusively interfering with cholinergic function on LTP were tested: the α4β2 nicotinic acetylcholinergic receptor agonist TC-1827 (chapter 4) and the acetylcholine esterase inhibitor donepezil (chapter 5). Both drugs were found to increase early LTP, but to not

affect late LTP. Furthermore, two drugs exclusively interfering with glutamatergic function were analyzed: the metabotropic glutamate 5 receptor postive allosteric modulator ADX-47273 (chapter 3) and the phosphodiesterase (PDE) 9A inhibitor BAY 73-6691 (chapter 5). ADX-47273 increased late LTP, but had no effect on early LTP, whereas BAY 73-6691 showed enhancing effects on both early and late LTP and even transformed early into late LTP. The same effects like for the PDE9A inhibitor were observed for the α7 nicotinic acetylcholinergic receptor partial agonist SSR180711 (chapter 4), which interferes with both, cholinergic and glutamatergic function. Thus, drugs facilitating glutamatergic function or both glutamatergic and cholinergic function seem to be more efficacious in enhancing LTP than drugs facilitating solely cholinergic function.

To evaluate whether this finding also proves true for experimental circumstances mimicking decreased cognitive function together with pathophysiology in AD patients, the ability of the drugs to ameliorate LTP impaired by soluble Aβ oligomer was analyzed (chapter 6). Soluble Aβ oligomers, also referred to as amyloid-β derived diffusible ligands (ADDLs), are thought to a putative cause of AD. Here, they were demonstrated to impair early and late LTP to different extents by exclusively targeting NMDA receptors and/or their signaling. These results further contribute to the hypothesis that soluble Aβ oligomers cause synaptic dysfunction which might lead to cognitive decline seen in AD patients. Regarding drug effects, donepezil and TC-1827 slightly restored ADDLs induced impairment of early LTP, but had no effect on late LTP impaired by ADDLs. In contrast, both, SSR180711 and BAY 73-6691 completely rescued early as well as late LTP impaired by ADDLs. ADX-47273 had no restoring effect on ADDLs induced early LTP impairment, but partially restored late LTP impaired by ADDLs. Thus, the earlier finding of the present thesis was confirmed: drugs facilitating glutamatergic function not only seem to be more efficacious in enhancing LTP than drugs facilitating solely cholinergic function, but are also superior in ameliorating soluble Aβ oligomer induced LTP deficits.

Therefore, from a preclinical perspective and based on the results of the present thesis, drugs interfering with glutamatergic function seem to have a high therapeutic potential as alternative treatment concerning cognitive deficits. Probably, they represent more efficacious approaches for the symptomatic treatment of AD than current treatments solely facilitating cholinergic function.

Zusammenfassung

Die Alzheimer'sche Erkrankung ist eine neurodegenerative Erkrankung des Gehirns. Heute ist die Alzheimer'sche Erkrankung die am häufigsten auftretende Form von Demenz bei älteren Menschen und verantwortlich für 50 – 60% aller mentalen Beeinträchtigungen bei über 65-jährigen. Eine zunehmende Verschlechterung des Gedächtnisses und in späteren Stadien auch ein Rückgang höherer kognitiver Funktionen stellen den typischen Krankheitsverlauf dar. Charakteristisch für das Gehirngewebe von Alzheimer-Patienten sind extrazelluläre β-amyloide (Aβ) Plaques und aus intrazellulärem Tau-Protein bestehende neurofibrilläre Bündel sowie ein Verlust hauptsächlich von cholinergen und glutamatergen Neuronen. Aufgrund der alternden Bevölkerung wird für das Jahr 2040 die Zahl der Betroffenen weltweit auf mehr als 80 Millionen geschätzt. Somit wird es in der Zukunft einen noch größeren Bedarf an Medikamenten zur Behandlung der Alzheimer'schen Erkrankung geben. Prinzipiell lassen sich zwei verschiedene therapeutische Ansätze unterscheiden: Den Krankheitsverlauf beeinflussende Ansätze, z.B. β-/γ-Sekretase inhibierende und α-Sekretase aktivierende Substanzen sowie Aβ-Antikörper, und symptomatische Ansätze, z.B. gedächtnissteigernde Substanzen. Um geeignete Medikamente für die Behandlung der Alzheimer'schen Erkrankung zu finden, werden experimentelle Modellsysteme zur Erforschung von Substanzkandidaten verwendet. Ein solches experimentelles System ist die hippocampale Langzeitpotenzierung (LTP), welche ein anerkanntes *in vitro* Modell für die Erforschung der zugrundeliegenden zellulären Prozesse der Gedächtnisbildung ist.

Die vorliegende Arbeit beschäftigt sich mit der Etablierung und Validierung von LTP in hippocampalen Hirnschnitten der Ratte um gedächtnissteigernde Substanzen zur potentiellen Behandlung der Alzheimer'schen Erkrankung zu charakterisieren. Dazu wurde zunächst ein Messsystem zur parallelen Charakterisierung mehrerer Schnitte aufgebaut, das Messungen bis zu sieben Stunden erlaubt (Kapitel 2). Dann wurden unterschiedliche Protokolle etabliert um Früh- und Spätphasen-LTP zu generieren. Dabei würde Frühphasen-LTP konzeptionell eher mit dem Kurzzeitgedächtnis einhergehen, während Spätphasen-LTP dem Langzeitgedächtnis gleichkommen würde. Die Protokolle wurden so validiert, dass sie den literaturbasierten Definitionen für Früh- und Spätphasen-LTP entsprechen: Proteinsynthese-Unabhängigkeit und Abhängigkeit von NMDA Rezeptoren für Frühphasen-LTP, gegenüber Proteinsynthese-Abhängigkeit sowie additive Abhängigkeit von NMDA Rezeptoren und L-VDCCs für Spätphasen-LTP (Kapitel 3).

Da in Alzheimer-Patienten hauptsächlich ein Defizit cholinerger und glutamaterger Neurone vorliegt, wurden die validierten LTP Formen benutzt, um solche Substanzen zu analysieren, die potentiell cholinerge und/oder glutamaterge neuronale Funktion erhöhen. Die Effekte zweier ausschließlich cholinerge Funktion erhöhender Substanzen wurden analysiert: Der α4β2 nicotinische Acetylcholin-Rezeptor Agonist TC-1827 (Kapitel 4) und der Acetylcholinesterase-Inhibitor Donepezil (Kapitel 5). Beide Substanzen erhöhten Frühphasen-LTP, aber hatten keinen Effekt auf Spätphasen-LTP. Desweiteren wurden zwei Substanzen getestet, die ausschließlich mit glutamaterger Funktion interferieren: Der metabotrope Glutamatrezeptor 5 positiv allosterische Modulator ADX-47273 (Kapitel 3) und der Phosphodiesterase (PDE) 9A-Inhibitor BAY 73-6691 (Kapitel 5). ADX-47273 erhöhte Spätphasen-LTP, aber hatte keinen Effekt auf Frühphasen-LTP, wohingegen BAY 73-6691 eine erhöhende Wirkung auf beide LTP Formen aufwies und sogar Früh- in Spätphasen-LTP umwandelte. Die gleichen Effekte, wie bei dem PDE9A-Inhibitor, konnten auch mit dem partiellen α7 nicotinische Acetylcholin-Rezeptor Agonisten SSR180711 (Kapitel 4) demonstriert werden. SSR180711 wirkt sowohl auf cholinerge, als auch auf glutamaterge neuronale Funktion. Somit scheinen Substanzen, die glutamaterge Funktionen unterstützen, wirksamer im Bezug auf die Erhöhung von LTP zu sein als Substanzen, die ausschließlich cholinerge Funktionen fördern.

Um herauszufinden, ob diese Erkenntnisse auch auf experimentelle Umstände übertragbar sind, welche die beeinträchtigten kognitiven Funktionen in Zusammenhang mit pathologischen Veränderungen in Alzheimer-Patienten imitieren, wurde die Fähigkeit der Substanzen überprüft, durch lösliche Aβ Oligomere verschlechtertes LTP zu verbessern (Kapitel 6). Lösliche Aβ Oligomere, auch als *amyloid-β derived diffusible ligands* (ADDLs) bezeichnet, werden zurzeit als eine mutmaßliche Ursache der Alzheimer'schen Erkrankung angesehen. In der vorliegenden Arbeit wurde gezeigt, dass ADDLs Früh- und Spätphasen-LTP in verschiedenem Ausmaß vermindern, indem sie ausschließlich auf NMDA Rezeptoren und/oder ihre Signalkaskaden einwirken. Diese Ergebnisse leisten einen weiteren Beitrag zu der Bestätigung der Hypothese, dass lösliche Aβ Oligomere eine synaptische Dysfunktion verursachen, die zum Leistungsrückgang der kognitiven Fähigkeiten in Alzheimer-Patienten beiträgt. Donepezil und TC-1827 konnten die durch ADDLs induzierten Defizite bei Frühphasen-LTP geringfügig wiederherstellen, aber sie hatten keinen Einfluss auf das durch ADDLs verschlechterte Spätphasen-LTP. Im Gegensatz dazu, konnten sowohl SSR180711 als auch BAY 73-6691 ein durch ADDLs verschlechtertes Früh- und Spätphasen-LTP komplett wiederherstellen. ADX-47273 hatte

keinen positiven Effekt auf Frühphasen-LTP, welches durch ADDLs verschlechtert worden war, konnte aber ein durch ADDLs verschlechtertes Spätphasen-LTP teilweise wiederherstellen. Somit wurde der vorherige Befund der Arbeit bestätigt: Substanzen, welche die glutamaterge Funktion verbessern, scheinen nicht nur wirksamer im Bezug auf LTP-Erhöhung zu sein als Substanzen die ausschließlich cholinerge Funktion erhöhen, sondern sie sind auch in der Lage, durch lösliche Aβ Oligomere verursachte Defizite bei LTP zu verbessern.

Aus einem präklinischen Blickwinkel und basierend auf den Ergebnissen der vorliegenden Arbeit weisen demnach Substanzen, die glutamaterge Funktionen verbessern, ein hohes therapeutisches Potential als alternative Ansätze bezüglich kognitiver Defizite auf. Möglicherweise könnten sie sogar wirksamere Ansätze für die symptomatische Behandlung der Alzheimer'schen Erkrankung darstellen, als derzeitige Behandlungen, die ausschließlich cholinerge Funktion verbessern.

CHAPTER 1

General introduction

1.1. Alzheimer's disease

Alzheimer's disease (AD) was first described in 1907 by the German psychiatrist and neuropathologist Alois Alzheimer (Alzheimer, 1907 reviewed by Vishal et al., 2011). It is a progressive, irreversible neurodegenerative disease of the brain (Sonkusare et al., 2005) and today the most common form of dementia in elderly people, accounting for around 50–60% of all cases of mental deterioration among persons over 65 years of age (Blennow et al., 2006; Francis et al., 1999; Kar et al., 2004). It is clinically characterized by a loss of memory, beginning early in the disease process, and a decline in higher cognitive functions (Arehart-Treichel, 2011; Boyle et al., 2006). As the disease progresses, other cognitive dysfunctions appear - e.g. disorientation, confusion and problems with reasoning – along with behavioral/emotional disturbances - e.g. agitation, anxiety, delusion, depression and insomnia – impairing functions in activities of daily living (Kar et al., 2004; Schifilliti et al., 2010; Waldemar et al., 2007). The time between onset of clinical symptoms and death of AD patients is around 8.5 years, but the time course of the disease is variable (Ashford and Schmitt, 2001; Francis et al., 1999). The number of dementia cases worldwide in 2010 was estimated to be about 35.6 million. Over the next decades, a fourfold increase in the prevalence of AD is expected due to population aging worldwide. Estimates foresee that worldwide more than 80 million individuals will be affected by the disease by 2040 – alone in the U.S. about 11 million (Fig.1; Alzheimer's Association, 2011; Brookmeyer et al., 1998, 2007; Forlenza et al., 2010; Scatena et al., 2007). Both genetic and environmental factors are considered to contribute to the development of AD. The minority of cases has an obvious genetic origin with linkage studies indicating predominantly a missense mutation in the amyloid precursor protein or Presenilin-1 or -2 gene (Bertram and Tanzi, 2005; Cai et al., 1993; Citron et al., 1992, 1994; Goate et al., 1991; Haass and De Strooper, 1999; Holmes, 2002; Levy-Lahad et al., 1995; Selkoe, 2001; Selkoe et al., 2002; Sherrington et al., 1995), but also other candidate genes were identified. One example is the ε4 allele of the apolipoprotein E gene, which is associated with a significant increase of up to 70% for the risk of developing AD during aging (Corder et a., 1993; Holmes, 2002; Mahley, 1988; Masters and Beyreuther, 1998; Strittmatter et al., 1993).

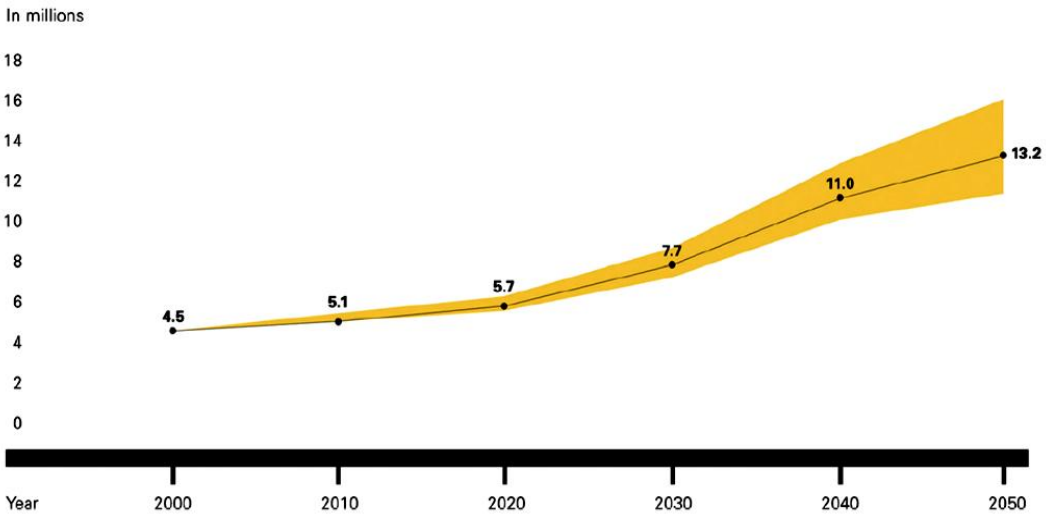


Figure 1: Estimates concerning the number of people with AD in the U.S..
Projected numbers of people aged 65 and over in the U.S. population with AD (in million) using the U.S. Census Bureau estimates of population growth. Numbers indicate middle estimates per decade. Shaded areas indicate low and high estimates per decade. From Alzheimer`s Association, 2011.

In its early stages, AD is often unrecognized or is misdiagnosed, because most of the early symptoms of AD are similar to the consequences of aging. There is no single test that accurately diagnoses AD. Hence, a variety of assessments and laboratory measurements are used for diagnosis, including urine and blood tests, cerebrospinal fluid markers, genotyping as well as positron emission tomography, magnetic resonance imaging and computerized tomography scans (Ballard et al., 2011; Cummings, 2004). A definitive diagnosis of AD is only possible by necropsy. Systematic post-mortem investigations of the brain tissue of patients with AD have consistently demonstrated the occurrence of extracellular plaques and neurofibrillary tangles (Gouras et al., 2005; LaFerla and Oddo, 2005; Querfurth and LaFerla, 2010), a profound loss of synapses (Scheff et al., 2006, 2007) and cortical gray matter (Rusinek et al., 1991; Thompson et al., 2003) as well as a ventricle enlargement (Nestor et al., 2008).

1.1.1. Amyloid hypothesis

The brains of patients with AD are histopathologically characterized by two hallmark lesions: extracellular senile amyloid-β (Aβ) plaques and intracellular neurofibrillary tangles (Fig. 2; Gouras et al., 2005; Nelson et al., 2009; Querfurth and LaFerla, 2010).

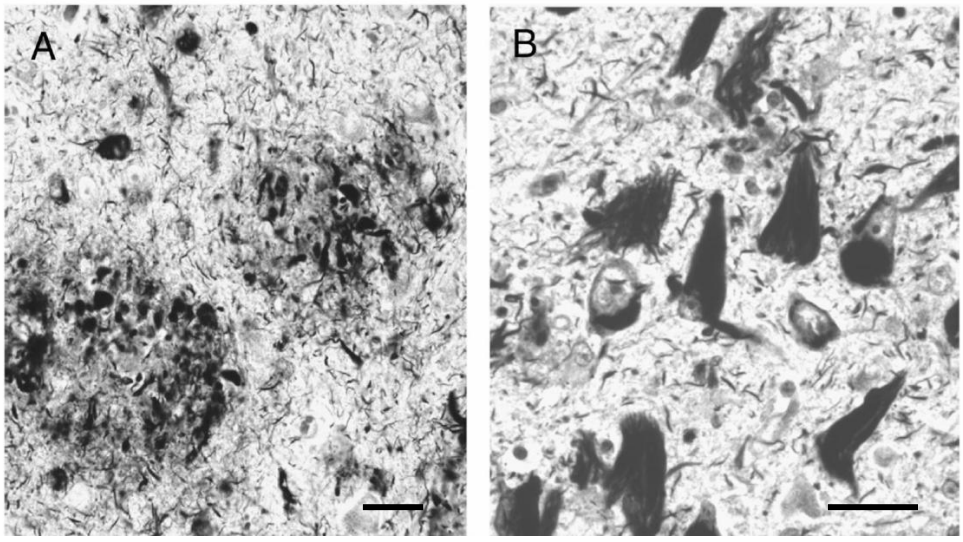


Figure 2: Histopathological hallmarks of AD.
Hallmarks are demonstrated with Bielschowsky silver impregnation. (A) Neuritic plaques are extracellular fibrillary amyloid deposits, surrounded by swollen, degenerating, argyrophilic neurites. (B) Neurofibrillary tangles are composed of intracellular, insoluble and protease-resistant fibrillary polymers of tau protein. In both panels, there are wispy argyrophilic neuropil threads. Scale bars = 25 µm. Adopted from Nelson et al., 2009.

Neuritic plaques are extracellular fibrillary Aβ deposits surrounded by dystrophic neurites, activated microglia and reactive astrocytes (Gouras et al., 2005; Kar et al., 2004). These plaques are most prominent in the entorhinal cortex, hippocampus and association cortices (Nelson et al., 2009; Price et al., 1991; Selkoe, 2001; Wisniewski et al., 1997). Their number does not appear to be associated with the severity of dementia, although a clinical correlation between elevated level of Aβ peptides in the brain and cognitive decline has been reported (Näslund et al., 2000). The predominant Aβ peptide forms in senile plaques consist of 40 (Aβ40) and 42 (Aβ42) amino acids (Iwatsubo et al., 1994) and were found to be a metabolite generated also in healthy persons. The Aβ forms are a proteolytic product of the transmembrane amyloid precursor protein (APP), which is thought to be involved in neuronal growth, survival and postinjury repair (Hardy and Selkoe, 2002; Priller et al.,

2006; Sabo et al., 2003). Aβ is generated through sequential cleavage of APP by α-, β-(BACE1) and γ-secretase (LaFerla et al., 2007; Selkoe, 2001; Sisodia et al., 2002). As a result of aging and AD, subtle changes in Aβ generation and/or its metabolism, can occur causing an increased production of the Aβ42 peptide leading to its aggregation and deposition (Fig. 3; Borchelt et al., 1996; Duff et al., 1996; Hardy and Selkoe, 2002; Haass et al., 1992; Lee et al., 2010).

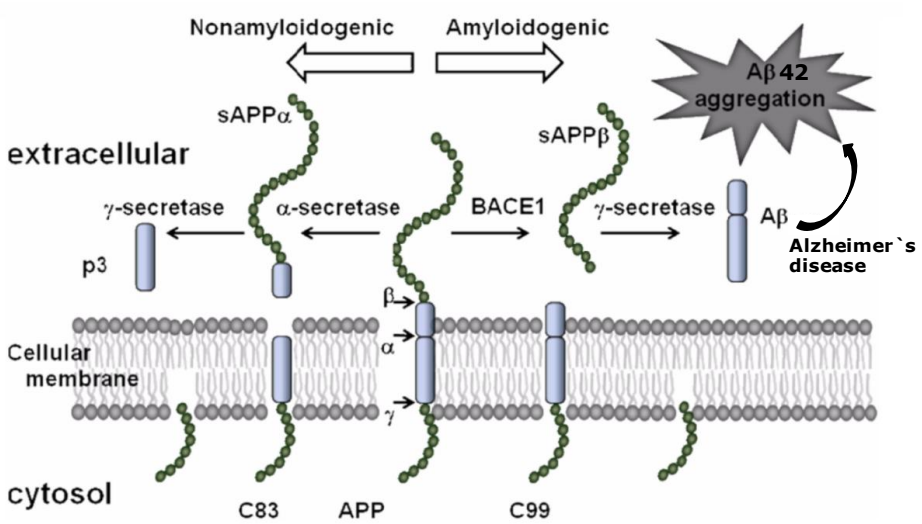


Figure 3: Schematic representation of APP processing and Aβ aggregation.
Mature APP is metabolized by two competing pathways, the α-secretease pathway that generates $sAPP_{\alpha}$ and C83, and the β-secretase pathway that generates $sAPP_{\beta}$ and C99. C99 is a substrate for γ-secretase, generating Aβ. In AD patients increased production of the Aβ42 peptide leads to its aggregation into small multimers (dimers, trimers, etc.) known as oligomers. Adopted from Lee et al., 2010.

Besides Aβ plaques, neurofibrillary tangles are also a pathological hallmark of AD. These tangles are composed of paired helical filaments containing an abnormal hyperphosphorylated form of the microtubule-associated protein tau (Grundke-Iqbal et al., 1986; Iqbal et al., 2009; Selkoe, 2001). The tau protein normally stabilizes microtubules in neurons, but hyperphosphorylated tau abnormally accumulates as paired helical filaments inside neurons and their branching projections such as axons and dendrites (Kosik and Finch, 1987). The formation of paired helical filaments reduces the ability of tau to stabilize microtubules, leading to disruption of neuronal transport and eventually to the death of affected neurons (Billingsley and Kincaid, 1997; Mandelkow et al., 2003; Roy et al., 2005; Trojanowski et al., 2005). Neurofibrillary tangles are particularly abundant in the entorhinal cortex, hippocampus, amygdala, association cortices of the frontal, temporal and

parietal lobes and certain subcortical nuclei that project to these regions (Haroutunian et al., 1999; Schifilliti et al., 2010; Selkoe, 2001). The extent of neurofibrillary pathology, and particularly the number of cortical neurofibrillary tangles, correlates positively with the severity of dementia, and thus has been used to classify the stage of the disease (Arriagada et al., 1992; Bierer et al., 1995; Braak and Braak, 1997).

Regarding the relationship between Aβ and tau in AD, experimental evidence indicates that Aβ can induce an increased phosphorylation of tau at the disease-relevant sites (Busciglio et al., 1995; De Felice et al., 2008; Greenberg and Kosik, 1995; Rank et al., 2002; Zheng et al., 2002). Indeed, studies carried out in transgenic mice suggest a modulatory link between Aβ and tau. APP/tau double-mutant mice and tau transgenic mice intracranially injected with synthetic Aβ developed enhanced neurofibrillary pathology as compared to the single mutant tau mice (Götz et al., 2001; Lewis et al., 2001). Additionally, tau pathology in three fold transgenic AD mice (Oddo et al., 2003a,b) can be diminished by clearing Aβ due to passive immunotherapy (Oddo et al., 2004). These studies indicate that the development of tau pathology is a downstream consequence of the Aβ pathology (Hardy and Selkoe, 2002; Oddo et al., 2008; Tseng et al., 2008). Moreover, as other types of neurodegenerative diseases besides AD also exhibit neurofibrillary tangles (Hong et al., 1998; Mailliot et al., 2000), the formation of tau filaments seems to be a more general event in neurodegenerative diseases leading to neuronal death, whereas Aβ seems to be the specific initiator in AD.

As to the apparent involvement of Aβ in AD, the “amyloid cascade hypothesis” was postulated, which originally links the pathological process of AD and neuronal cell death to aggregation and deposition of Aβ (Hardy and Higgins, 1992). This hypothesis has led to a huge number of studies concerning the effects of Aβ onto neurons and has received abundant verification (Fig. 4; Armstrong, 2011; Forlenza et al., 2010; Pimplikar, 2009). Aβ has both neurotrophic and neurotoxic effects, depending on the duration of exposure, the dose and the degree of aggregation of the peptide (Kowall et al., 1991; May et al., 1992; Youssef et al., 2008). Investigations in animal models and human brain samples have put a special emphasis on soluble Aβ forms (Dahlgren et al., 2002; Klein, 2002; Lue et al., 1999; Tabaton and Piccini, 2005; Walsh et al., 1999). In fact, it was demonstrated that acute injection of soluble Aβ into the brain of rats and mice impairs learning and memory (Balducci et al., 2010; Poling et al., 2008; Youssef et al., 2008). Hence, several lines of evidence suggest that accumulation of Aβ peptide in the brain may initiate and/or contribute to the pathogenesis of AD. Overproduction and/or reduced clearance of Aβ

peptides are likely key to amyloid aggregation, which in turn contributes to the development of senile plaques and neurofibrillary tangles (Hardy and Higgins, 1992; Forlenza et al., 2010; Selkoe, 2001).

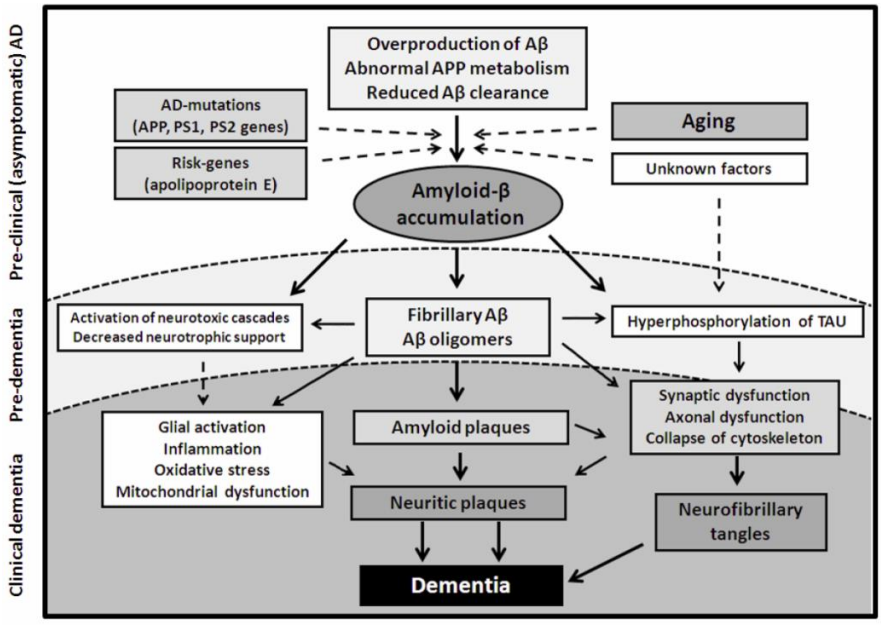


Figure 4: The amyloid cascade hypothesis.
Accumulation of Aβ (pre-clinical AD) contributes to the development of fibrillary Aβ and Aβ oligomers (pre-dementia) and eventually to development of neuritic plaques and neurofibrillary tangles (clinical dementia). (Dotted arrows indicate possible or secondary mechanisms affecting core pathological processes within the amyloid cascade; PS1/2 = presenilin 1/2) From Forlenza et al., 2010.

Due to the amyloid cascade hypothesis, approaches for a disease modifying treatment have focused on methods to reduce Aβ through selective Aβ-lowering agents. Several active and passive vaccines are under clinical investigation for their efficacy in reducing the level of Aβ in the plasma and brain of subjects with mild-to-moderately advanced disease state (Okura et al., 2006). Furthermore, having identified all the components required for secretase function, the development of selective β- and/or γ-secretase inhibitors or approaches for α-secretase enhancement are being pursued in order to lower Aβ production in the brain (Ganjei, 2010; Geling et al., 2002; Hong et al., 2000; Luo and Yan, 2010; Vassar, 2001). An alternative approach would be to use small molecules to bind Aβ monomers and prevent their assembly into cytotoxic oligomers (Ganjei, 2010, Woo et al., 2011).

1.1.2. Synaptic and neuronal dysfunction

As a result of the Aβ pathology, AD is correlated with a profound loss of synapses and with neuronal dysfunction (Fig. 5; Scheff et al., 2006, 2007; Selkoe, 2002). Degenerating neurons and synapses in the brains of individuals with AD are located predominantly within regions that project to or from areas that display high densities of plaques and tangles. Biochemical investigations from biopsy and autopsy of tissues from AD patients indicate that various neurotransmitters and modulators including acetylcholine, serotonin, noradrenalin, somatostatin and glutamate are differentially altered in the brains of individuals with AD (Francis et al., 1993, 1999; Mann and Yates, 1986). The loss of synapses and neuronal function are supposed to be the reason for cognitive decline in patients (Larson et al., 1992; Terry et al., 1991). In particular, pronounced dysfunction of the cholinergic and the glutamatergic system can be observed in AD patients and will be discussed in the next paragraphs.

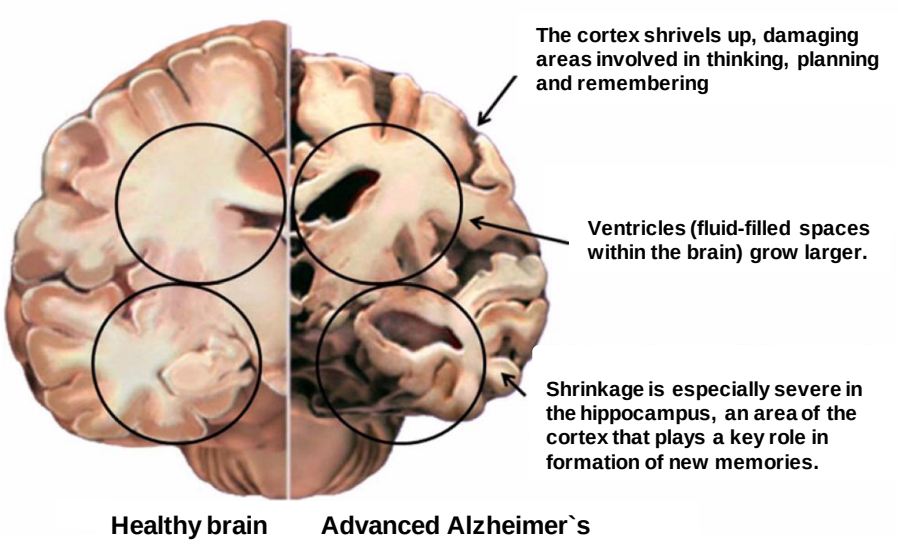


Figure 5: Schematic representation of massive cell loss in advanced AD.
From Lee et al., 2010.

1.1.2.1. Cholinergic dysfunction

Cholinergic neurons release the neurotransmitter acetylcholine (ACh), which can bind two different receptor subtypes: the nicotinic acetylcholine receptors (nAChRs) and the muscarinic acetylcholine receptors (mAChRs). The involvement of the cholinergic system (Fig. 6) in cognitive functions is widely documented in animal and human research. Disruption of the cholinergic system causes a decline in cognitive functions (Elrod and Buccafusco, 1991; Newhouse et al., 1994; Vitiello et al., 1997), whereas its activation causes improvement of cognitive functions (Fodale et al., 2006; Leiser et al., 2009; Rezvani and Levin, 2001).

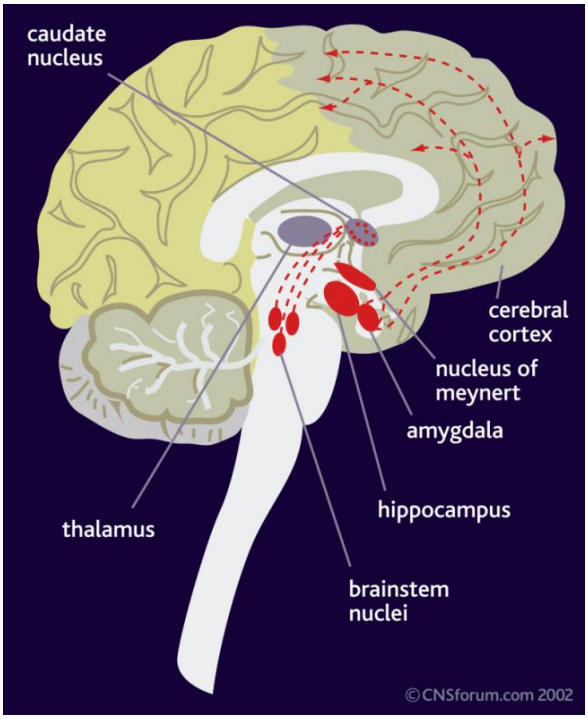


Figure 6: The acetylcholine pathway.
Brain ACh projections are illustrated from their origins in major nuclei in the basal forebrain and the pons. Of particular relevance to AD are the projections from the medial septal nucleus to the hippocampus and from the nucleus basalis of Meynert to frontal cortex.
From www.cnsforum.com.

The most consistently reproduced finding in brain tissues from AD patients is a profound reduction in the activity of the ACh synthesizing enzyme, choline acetyltransferase (ChAT), in the neocortex, which correlates positively with the severity of dementia (Francis et al., 1999; Perry et al., 1978; Ladner and Lee, 1998; Neary et al., 1986). Reduced choline uptake (Slotkin et al., 1990), ACh release (Gil-Bea et al., 2004; Nilsson et al., 1968) and loss of cholinergic neurons from the basal forebrain region (Gil-Bea et al., 2004; Whitehouse et al., 1982) further indicate a selective cholinergic deficit in brains of individuals with AD. The loss of cholinergic fibers in AD patients appears particularly pronounced in areas dedicated to memory and cognition such as hippocampus and cortex (Blusztajn and Berse, 2000; Dournaud et al., 1995; Francis et al., 1999; Geula and Mesulam, 1996; Kar et al., 2004; Whitehouse et al., 1982). Studies on cholinergic receptors demonstrated that mAChRs are reduced in the brains of individuals with AD,

with the M2 mAChRs being mainly affected (Kar et al., 2004; Mash et al., 1985; Tsang et al., 2006). Additionally, nAChRs are markedly reduced in the hippocampus and cortex of post-mortem brains of individuals with AD, an observation which has been confirmed by *in vivo* positron emission tomography (Francis et al., 1999; Nordberg et al., 1995). Thereby, it was demonstrated that mainly α4β2 and α7 nAChRs subtypes are affected (Burghaus et al., 2000; Court et al., 2001; Martin- Ruiz et al., 1999).

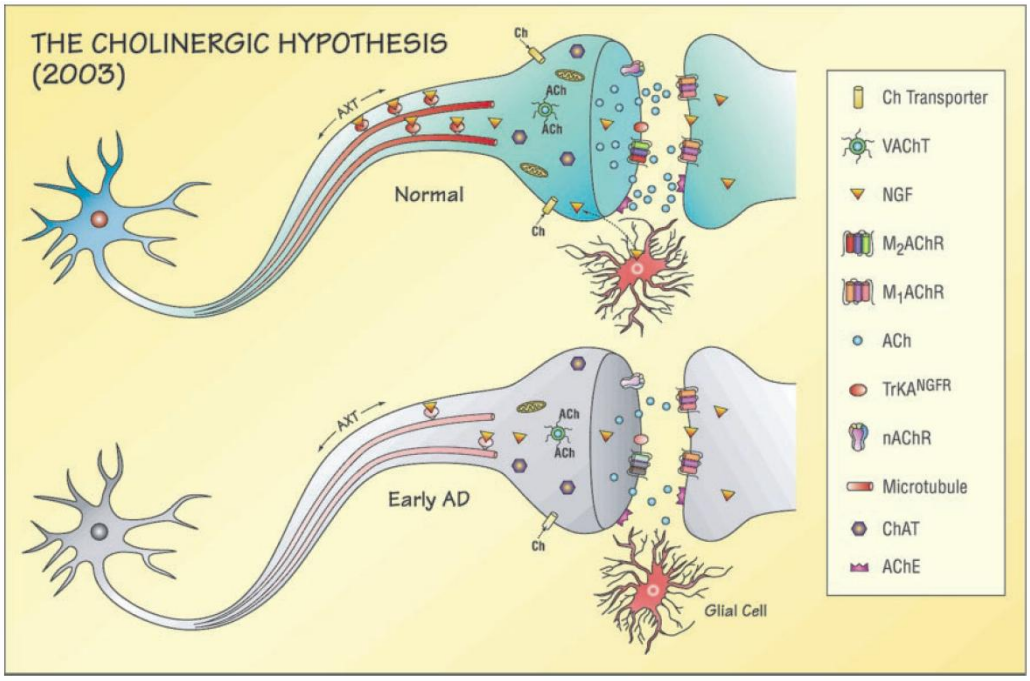


Figure 7: The cholinergic hypothesis.
Schematic representation of known and proposed changes in cholinergic neurons that occur in the aged and early AD brain compared with healthy young persons. Alternations in high-affinity choline uptake, impaired achetylcholine release, deficits in the expression of nicotinic and muscarinic receptors, dysfunctional neurotrophin support (i.e., NGF receptor TrkA), and deficits in axonal transport are represented in the early AD neuron either by a decrease in the number of symbols presented or by reduced color intensity. From Terry et al., 2003.

In consequence to these obvious changes in the cholinergic system of brain tissue from AD patients the so-called "cholinergic hypothesis" of AD was proposed by Bartus et al. in 1982. It states that a loss of cholinergic function in the central nervous system contributes significantly to the cognitive decline associated with advanced age and AD (Fig. 7; Bartus et al., 1982; Bartus, 2000; Francis et al., 1999; Perry et al., 1978; Terry and Buccafusco,

2003). Several *in vivo* imaging studies conducted in AD patients support the cholinergic hypothesis. For example, positron emission tomography studies indicate that nAChR deficits are an early phenomenon in AD, which significantly correlate with the level of cognitive impairment (Nordberg, 2001). Other positron emission tomography studies indicate both age- and AD-related decreases in the binding of nonselective muscarinic ligands in neocortical regions (Zubieta et al., 2001). Moreover, single-photon emission computed tomography studies indicate that the vesicular acetylcholine transporter is reduced in early onset AD patients (Efange et al., 1997).

Recent studies demonstrated that Aβ causes the cholinergic hypofunction in AD patients (Fig. 8). For example, several steps of ACh synthesis and release are reduced by Aβ and there is a significant increase in the rate of high-affinity choline uptake (Auld et al., 1998; Bales et al., 2006; Harkany et al., 1995a,b; Kar et al., 1998; Kristofiková et al., 2001; Pedersen et al., 1996; Pedersen and Blusztajn, 1997). Additionally, it was found that Aβ reduces the loading of ACh into vesicles by inhibiting the fast axonal transport of the vesicular acetylcholine transporter in the sciatic nerve of the rat (Kasa et al., 2000, 2004). Moreover, a redistribution of the acetylcholine esterase (AChE) within the cells is caused by Aβ42 (Kasa et al., 1998) supporting the suggestion that neuronal degeneration is initiated by a failure in axonal transport. Furthermore, in cortical cultures, the G-protein coupling of mAChR activation is impaired by Aβ (Kelly et al., 1996). Similarly, in rats, the density of total mAChRs was dose dependently decreased through repeated *in vivo* intracerebroventricular administration of Aβ (Pavia et al., 2000). Moreover, there is further evidence that neuronal nAChRs interact with Aβ42 (Nagele et al., 2002; Oddo et al., 2005; Wang et al., 2000b). Aβ42 can bind with high affinity to the α7 nAChR and with lower affinity to the α4β2 nAChR and hence might block the functional interaction of nicotinic agonists with their receptors (Wang et al., 2000a). It has been suggested that the high-affinity binding of Aβ42 to α7 nAChRs might cause the internalization and gradual accumulation of Aβ42 in the neurons of AD brains. This suggestion is supported by a study which demonstrated a substantial intraneuronal Aβ42 accumulation only in cells that express relatively high levels of the α7 nAChR (Nagele et al., 2002). These data might provide a plausible explanation for the selective vulnerability of neurons expressing the α7 nAChR in AD brains.

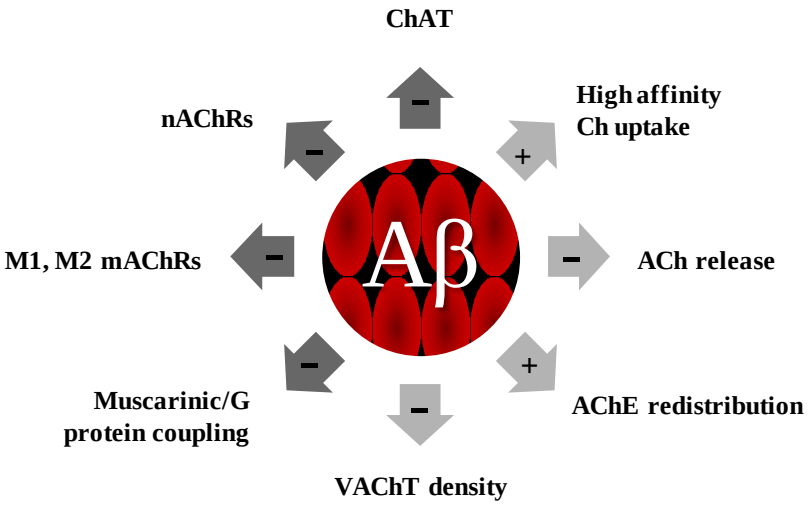


Figure 8: Effect of Aβ on cholinergic neurons.
Schematic representation of the known changes in cholinergic neurons due to activation of Aβ.

In the light of the cholinergic hypothesis, drugs that enhance cholinergic transmission, i.e. acetylcholine esterase (AChE) inhibitors, were developed as therapy of AD patients. These drugs increase and prolong the synaptic levels of available ACh by preventing its degradation and are currently the most widely prescribed drugs for the treatment of AD. They include tacrine hydrochloride (*Cognex*®; approved in 1993), donepezil hydrochloride (*Aricept*®; approved in 1996; current gold standard therapy), rivastigmine tartrate (*Exelon*®; approved in 2000) and galantamine hydrobromide (*Razadyne*®/*Reminyl*®; approved in 2001) (Birks and Harvey, 2003; Geerts and Grossberg, 2006; Kaduszkiewicz et al., 2005; Trinh et al., 2003). AChE inhibitors are therapeutically used for patients diagnosed with mild-to-moderate AD, but their efficacy in patients is only moderate as demonstrated in a recent meta-analysis (Courtney et al., 2004; Raschetti et al., 2007). Moreover, this treatment only alleviates symptoms of the disease, but is not curative (Raschetti et al., 2007; Rountree et al., 2009). Alternative ways to target the cholinergic system for memory improvement in dementia by highly selective nicotinic or muscarinic agonism are still in early stages of clinical testing (Dunbar et al., 2007; Geerts, 2006).

1.1.2.2. Glutamatergic dysfunction

Glutamate is the primary excitatory neurotransmitter of the central nervous system (Fig. 9). It is used by approximately two-thirds of synapses in the neocortex and hippocampus, while ACh is found in approximately 5% of the synapses/ neurons (Fonnum, 1984). Glutamate is involved in most aspects of cognition and higher mental function (Danbolt, 2001; Fonnum, 1984). Thus, it is not surprising that loss of cortical neurons, especially the large pyramidal neurons in the hippocampus utilizing glutamate for neurotransmission, correlate closely with cognitive impairment and progression of AD (Braak and Braak, 1997; Francis et al., 1993).

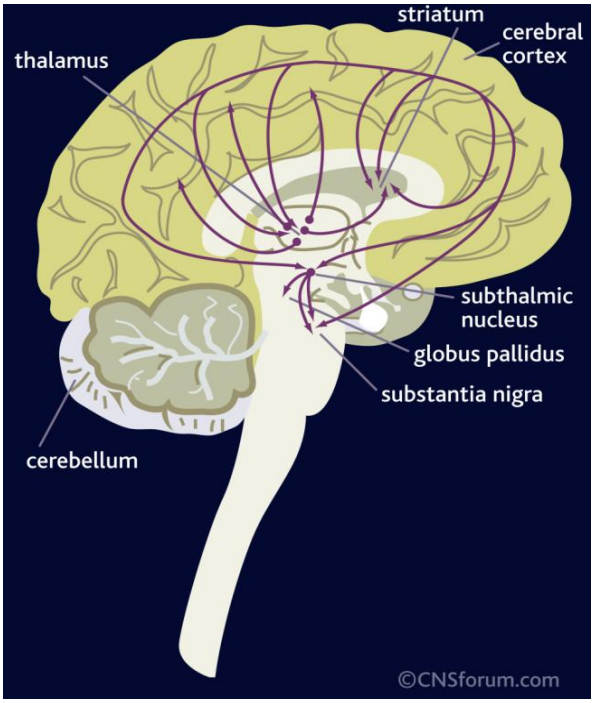


Figure 9: The glutamate pathways.
In the normal brain the prominent glutamatergic pathways are the cortico-cortical pathways (thalamus - cortex) and the extrapyramidal pathway (cortex - striatum). Other glutamate projections exist between the cortex, substantia nigra, subthalmic nucleus and pallidum. Glutamate-containing neuronal terminals are ubiquitous in the central nervous system. From Carlsson, 1995.

The most convincing evidence to date of a glutamatergic dysfunction in AD are histological findings of both cell and synapse loss of presumed glutamatergic neurons (Francis 2003; Morrison and Hof, 1997; Terry et al., 1991). Moreover, glutamatergic transmission is severely altered by early degeneration of corticocortical connections and hippocampal projections in AD (Francis et al., 1993). In AD patients, numerous studies have implicated reduced concentration of glutamate (Antuono et al., 2001; Rupsingh et al., 2011; Procter et al., 1988), decreased binding to receptors (Cross et al., 1987; Greenamyre et al., 1985, 1987; Maragos et al., 1987) and reduced glutamate uptake into vesicles of synaptosome preparations (Procter et al., 1988; Scott et al., 2011). Furthermore, a large decrease in glutamate receptors has been observed in the cortex and hippocampus (Greenamyre et al., 1987; Procter et al., 1989). In general, two types of glutamate receptors can be distinguished: ionotropic glutamate receptors, which form an

ion channel pore opening upon activation, and metabotropic glutamate receptors, which are G-protein coupled receptors indirectly activating ion-channels in the plasma membrane through a signaling cascade. Ionotropic glutamate receptors are sub-classified into α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate and N-Methyl-D-Aspartate (NMDA) receptors. Concerning AMPA receptors, especially their GluR1 and GluR2/3 subunits are reduced in AD patients and this reduction seems to be correlated with neurofibrillary tangle formation (Ikonomovic et al., 1995; Wakabayashi et al., 1999; Yasuda et al., 1995). Only few studies regarding changes of kainate receptors in AD exist, which show that kainate binding is significantly reduced in the parahippocampal gyrus (Dewar et al., 1991). Concerning NMDA receptors and their changes in AD patients, there is still an ongoing debate. Most reports indicated a reduction of NMDA receptors in AD patients (Hynd et al., 2001, 2004; Panegyres et al., 2002; Penney et al., 1990; Ulas and Cotman, 1997; Wang et al., 2000c) and thus it was proposed that the selective decrease of NMDA receptors and/or their hypoactivity may affect the memory dysfunction in AD. Controversially, Ikonomovic et al. (1999) reported that the NMDAR subunit NR1 is markedly increased in vulnerable neurons of AD and thus excitotoxicity was proposed to be responsible for neuronal loss. Excitotoxic cell death may result from excessive activation of NMDA receptors, leading to raised intracellular calcium levels and subsequent activation of a cascade of enzymes, resulting in cell death by necrosis and apoptosis (Lipton, 1999). Despite the controversies in the literature, it seems to be more likely that NMDA receptors may contribute significantly to the pathophysiology in AD via degeneration of synaptic activity rather than cell death via excitotoxicity. However, more research has to be done to clarify this issue (Lee et al., 2002). In comparison to ionotropic glutamate receptors, there are much less data on the role and expression of metabotropic glutamate receptors (mGluRs) in AD; however, dysfunction of metabotropic glutamate receptors was demonstrated to be associated with abnormal tau phosphorylation (Boldyrev et al., 2004).

In consequence to these obvious changes in the glutamatergic system of brain tissue from AD patients the so-called “glutamatergic hypothesis” of AD was proposed by Maragos et al. in 1987. It states that a loss of glutamatergic function in the central nervous system contributes significantly to the cognitive decline associated with advanced age and AD (Fig. 10; Cross et al., 1987; Francis et al., 1993; Maragosa et al., 1987; Palmer and Gershon, 1990).

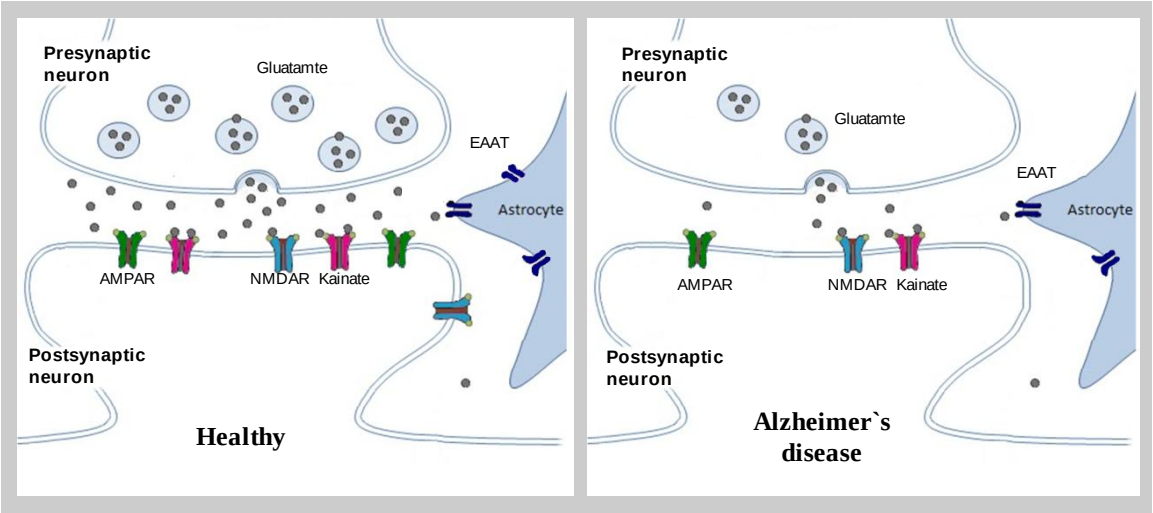


Figure 10: The glutamatergic hypothesis.
Schematic representation of known and proposed changes in glutamatergic neurons that occur in the aged and early AD brain compared with healthy young persons. Alterations in glutamate transmission, glutamate concentration, glutamate uptake, decreased binding of glutamate to receptors and a decrease in receptors are represented in the AD neuron by a decrease in the number of symbols.

Glutamatergic dysfunction seems to be a downstream effect of the Aβ cascade (Fig. 11). Aβ is reported to alter kinetics of synaptic AMPA receptors (Parameshwaran et al., 2007) and inhibition of AMPA receptor stabilization as well as insertion to postsynaptic membranes (Roselli et al., 2005). Furthermore, Aβ is known to increase AMPA receptor endocytosis and receptor desensitization (Almeida et al., 2005; Gu et al., 2009; Hsieh et al., 2006; Li et al., 2009; Zhao et al., 2004). Concerning kainate receptors, Aβ is supposed to increase their sensitivity (Morimoto et al., 2003). Supporting the hypothesis of hypoactivity, Aβ was demonstrated to account for reduction of postsynaptic surface NMDA receptors (Goto et al., 2006; Johansson et al., 2006; Snyder et al., 2005) and impairment of NMDA receptor function (Snyder et al., 2005; Tyszkiewicz and Yan, 2005). Additionally, studies have implicated Aβ interfering with several major signaling pathways downstream of the NMDA receptor (Kelly et al., 2005; Puzzo et al., 2005; Snyder et al., 2005) including the calcium-dependent protein phosphatase calcineurin (Chen et al., 2002), CaMKII (Zhao et al., 2004) and protein phosphatase 1 (Knobloch et al., 2007) and CREB phosphorylation (Chen et al., 2002). Nevertheless, there also exist few data supporting the hypothesis of excitotoxicity by demonstrating that Aβ increases NMDA receptor sensitivity (Morimoto and Oda, 2003). Not much is known about mGluRs and Aβ, apart from a report on agonists of group II and III protecting primary neurons from Aβ peptide toxicity (Copani et al., 1995).

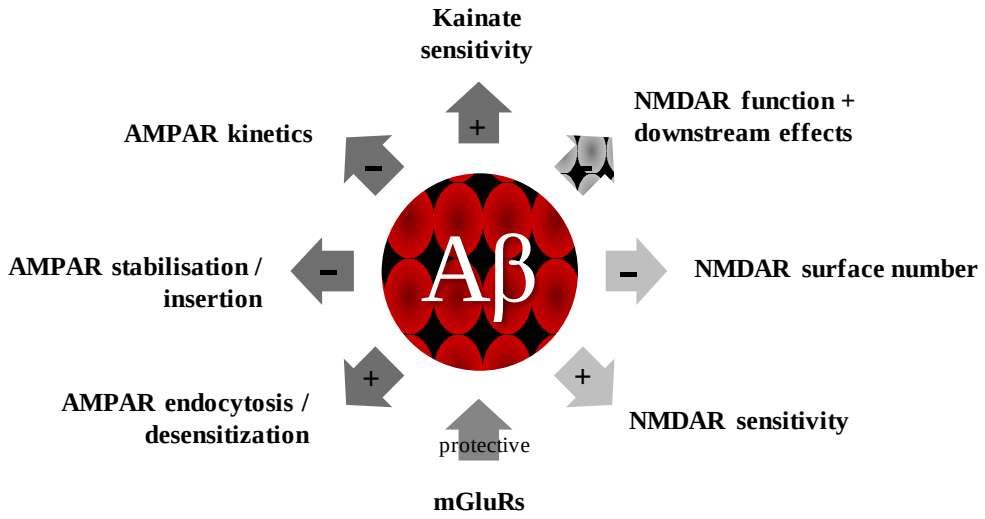


Figure 11: Effect of Aβ on glutamatergic neurons.
Schematic representation of the known changes in glutamatergic neurons due to activation of Aβ.

There exist several approaches to correct glutamatergic dysfunction in AD. For example, positive modulation of AMPA receptors by so-called AMPAkines, which are considered to act by increasing the sensitivity of these receptors, are in clinical trials for mild cognitive impairments (Johnson and Simmon, 2002). Compounds that target AMPA receptors have already shown encouraging results in AD patients (Lynch, 2002). Following the hypothesis of NMDA receptor hypoactivity, positive modulation of the NMDA receptor has been attempted via its glycine co-agonist site in preclinical and clinical studies showing that the partial glycine-site agonist D-cycloserine improves cognitive function (Myhrer and Paulsen, 1997; Tsai et al., 1998). Following the hypothesis of NMDA receptor excitotoxicity, negative modulation of the NMDA receptor has been approached via the weak NMDA receptor antagonist memantine providing some therapeutic benefits in patients suffering from moderate-to-severe AD (Herrmann et al., 2011; Thomas and Grossberg, 2009). Currently, metabotropic glutamate receptors are being intensively studied as therapeutic targets for neurodegenerative diseases since their unique functions allow modulation of glutamate transmission via G-protein dependent pathways which may modulate neuronal functions more effectively than ionotropic glutamate receptors (Bruno et al., 2001; Copani et al., 1995; Nicoletti et al., 2011).

1.2. Hippocampal learning and memory

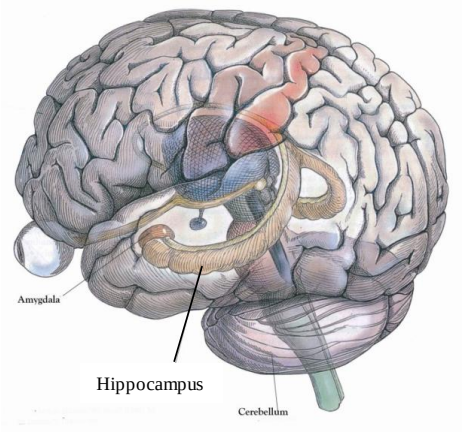


Figure 12: Schematic representation of the hippocampus in the brain.
From www.macalester.edu/psychology

The hippocampus is a major component of the mammalian brain. It is a paired structure, with mirror-image halves in the left and right hemispheres of the brain being located inside the so-called medial temporal lobe (Fig. 12). The hippocampus belongs to the limbic system also consisting of amygdala, anterior thalamic nuclei, septum, limbic cortex and fornix. In 1957 Scoville and Milner showed that bilateral hippocampal removal, as a treatment for epilepsy suffered by patient H.M., resulted in anterograde amnesia. Thus, for the first time, the importance of the hippocampus and temporal lobe structures for memory was identified. Since then, studies in humans and animals (Jarrard, 1993; Squire et al., 1984) have consolidated the essential finding of that study. More recently, noninvasive methods using direct brain imaging techniques, characterized blood flow and oxygen consumption identified fluctuations in these parameters during learning tasks in the hippocampus (Squire, 1992; Squire et al., 1990). Specifically, the hippocampus is involved in episodic memory, i.e. memory for personally experienced events set in a spatio-temporal context, and in spatial or topographical memory (Burgess et al., 2002; Smith and Mizumori, 2006). In AD, the hippocampus is one of the first regions of the brain to be affected causing first symptoms in patients, including memory problems and disorientation (Burgess et al., 2002; Frisoni et al., 2008). Thus, improvement of hippocampal learning and memory is an attractive approach to influence cognitive symptoms of the disease. The research of learning and memory requires experimental model systems that can be utilized both to characterize the underlying mechanisms associated with memory and to explore drug candidates for the treatment of memory deficits. Although genetically engineered mouse models for AD-like pathology exist, the following discussion will be limited to hippocampal rodent model systems with wildtype animals, because the studies described in this work utilized wildtype rats. In fact, cognitive enhancing drugs are able to improve memory performance even in wildtype animals, thereby addressing those memory domains and brain regions known to be affected in AD (Buccafusco et al., 2004; Prickaerts et al., 2005).

1.2.1. Behavioral rodent models

A variety of experimental paradigms are available for the investigation of learning and memory in behavioral studies. Some of these cognition tasks are mainly or even exclusively hippocampus-dependent and therefore represent attractive experimental approaches for AD research.

The Morris water maze task is the perhaps most commonly used model for hippocampal spatial learning. In this test an animal's capacity to remember spatial cues is required to locate a hidden submerged platform (Morris et al., 1982; Morris, 1984). Using this test, numerous studies have proven an essential role for the hippocampus in spatial learning. In addition, several studies have built on the original observation of O'Keefe which identified the involvement of specific hippocampal pyramidal cells in encoding information about the location of an animal in a particular space (O'Keefe, 1979). Apparently the key role of the hippocampus in spatial learning consists in constructing and storing spatial maps (Teng and Squire, 1999).

Another widely used model to investigate hippocampal learning is the T-maze task, which is a behavioral test to assess spatial working memory performance of animals. This test is based on the natural willingness of rodents to explore a new environment. The natural tendency of rats and mice in a T-maze is to alternate their choice of the goal arm. The response on each trial varies according to what they have previously experienced (Hudon et al., 2002). Typical alternation rates are around 90% (Deacon et al., 2003). The T-maze is a suitable test to investigate hippocampal (dys)function, because full lesions of the hippocampus (Deacon and Rawlins, 2005; Lalonde, 2002) and already deletions of AMPA receptor subunits (Reisel et al., 2002) impair performance of rodents in this task.

Another behavioral task for hippocampal learning and memory is contextual fear conditioning, which is based on the capacity of mammals, including rodents, to associate environmental cues with an aversive stimulus. In this model, conditioned emotional responses are elicited by placing an animal in a chamber in which an aversive experience has previously been made (Blanchard and Blanchard, 1972). There is good evidence indicating that contextual fear conditioning is mostly hippocampus-dependent. This evidence is based on lesion studies, selective infusion of pharmacological agents into the hippocampus and animals genetically engineered to have hippocampal deficits (Chen et al., 1996; Kim et al., 1993; Saxe et al., 2006).

1.2.2. Long-term potentiation

A long time of research has been spent to find a model system for the cellular and molecular mechanisms of learning and memory. Originally, it was hypothesized that information storage relies on changes in strength of synaptic connections between neurons that are active (Cajal, 1912 reviewed by Stahnisch and Nitsch, 2002). This hypothesis was supported by Hebb in 1949, who proposed that if two neurons are active at the same time, their synaptic efficiency onto a common target neuron will be strengthened (Hebb, 1949 reviewed by Cooper, 2005; Morris and Hebb, 1999). In artificial neuronal networks, such as attention–language interactions and learning-input correlations, Hebbian learning was proven to be successful (Garagnani et al., 2008; Gütig et al., 2003). An enormous effort has been put into understanding the mechanism by which strengthening of synaptic connections can be achieved and into finding a model for this process. This research has led to the model of long-term potentiation (LTP) which was first published in 1973 (Bliss and Lomo, 1973). They reported that trains of high frequency stimulation to the rabbit perforant path caused a sustained increase in efficiency of synaptic transmission in the granule cells of the dentate gyrus. This report, and others which followed during the 1970s, confirmed the Hebbian nature of this form of synaptic plasticity. Cooperativity (Lee et al., 1983; McNaughton, 2003), associativity (Barrionuevo and Brown, 1983; Levy and Steward, 1979) and input specificity (Dunwiddie and Lynch 1978; Nishiyama et al., 2000), being the characteristics of LTP, as well as the durability of LTP (Abraham et al., 1995; Reymann et al., 1985) support the hypothesis that LTP may be a biological mechanism for at least some forms of memory. LTP has not only been found in the hippocampus (Bliss and Lomo, 1973; Dunwiddie and Lynch, 1978), but in many brain regions, including piriform (Larson et al., 2005), entorhinal (Alonso et al., 1990) and prefrontal cortices (Auclair et al., 2000), the septum (Garcia et al., 1997) and superior cervical ganglia (Brown and McAfee, 1982) as well as in the spinal cord (Pedersen and Gjerstad, 2007). Furthermore, LTP is not limited to the mammalian brain, but has been described in other vertebrates such as the goldfish (Schmidt, 1990), bullfrog (Koyano et al., 1985), bird (Scott and Bennett, 1993) and even in some invertebrates (Antonov et al., 2003; Glanzman, 2008; Menzel and Manz, 2005; Oleskevich et al., 1997; Walters and Byrne, 1985). From the mechanistic point of view, the opposing process to LTP is long-term depression (LTD) described firstly in the cerebellar cortex (Ito, 1989). It is defined as an activity-dependent reduction in the efficacy of neuronal synapses which can - like LTP - last for hours

(Collingridge et al., 2010). LTD selectively weakens specific synapses in order to make constructive use of synaptic strengthening caused by LTP. This is necessary because, if allowed to continue increasing in strength, synapses would ultimately reach a ceiling level of efficiency, which would inhibit the encoding of new information. Therefore, LTD itself is also believed to be involved in learning and memory processes (Ito, 1989). However, there are still remaining questions to be addressed regarding its exact contribution in learning and memory *in vitro* and *in vivo*. Thus, in this work only LTP will be discussed, because it is a well established and widely accepted *in vitro* model of synaptic plasticity, which can even be used for profiling putative memory enhancing substances.

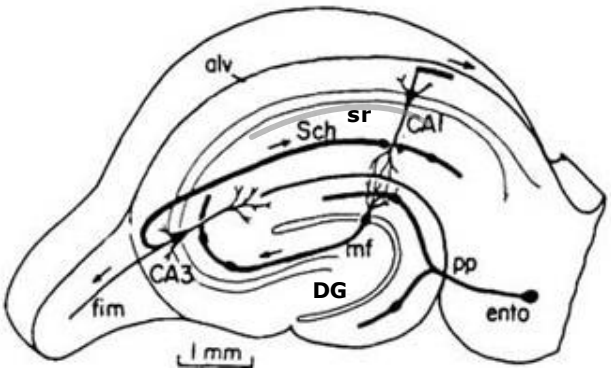


Figure 13: The pathways of the hippocampus. Section of hippocampal formation enlarged to show main neuronal elements. DG, dentate gyrus; ento, fibers from entorhinal cortex; pp, perforating pathway; mf, mossy fibers; Sch, Schaffer axon collaterals; CA1, CA3, pyramidal cells; fim, fimbria; alv, alveus; sr, stratum radiatum. From Andersen et al, 1971.

As already mentioned above, the hippocampus is specifically attractive for studying learning and memory. Therefore, in this work, LTP measurements were exclusively made in hippocampal slices. As first shown by Per Anderson, the hippocampus has three major pathways: the perforant pathway, the mossy fiber pathway and the Schaffer collateral pathway (Fig. 13; Anderson et al., 1971 reviewed by Amaral and Witter, 1989). The mechanisms underlying LTP are not the same in all three pathways. In the following chapter only the mechanism of LTP in the CA1 region of the hippocampus will be discussed, as all experiments concerning this work were undertaken in the Schaffer collaterals. The reason to focus on LTP in the CA1 region in the hippocampus is based on the fact that the mechanisms of LTP and its pharmacological manipulation are best described in this region. Also, the neuroanatomy of the CA1 region is well known and comparatively less complex, and thus, it is experimentally well amenable. Therefore, CA1 LTP is considered the gold-standard model for testing potential memory influencing drugs. In the CA1 region, LTP consists of distinct phases involving different molecular mechanisms. The early phase, which lasts one to three hours, is independent of protein-synthesis, while more persistent late LTP, which lasts several hours, requires synthesis of new proteins (Huang, 1998).

1.2.2.1. Mechanisms underlying early and late LTP in the CA1 region

In Fig.14, a model of the mechanisms underlying early and late LTP in the CA1 region of the hippocampus is described. The Schaffer collateral pathway connects the pyramidal neurons of the CA3 region of the hippocampus with those of the CA1 region and employs glutamate as transmitter. Its axons originate ipsi- and contralaterally and their terminals form synapses with the dendrites of CA1 pyramidal neurons in the stratum radiatum (see hippocampal anatomy in Fig. 13). During normal, low-frequency synaptic transmission, glutamate is released from the presynaptic terminal and acts on two types of glutamate receptors, i.e. NMDA and AMPA receptors, which are embedded in the postsynaptic membrane. NMDA receptors are blocked by magnesium ions and glutamate binding without a simultaneous depolarization of the post-synaptic neuron is insufficient to open them. However, glutamate binding to AMPA receptors, which are responsible for most of the rapid, moment-to-moment excitatory activity, triggers the influx of positively charged sodium ions into the postsynaptic cell, causing a transient depolarization called excitatory postsynaptic potential.

A weak stimulation pattern can induce **early LTP**, which lasts one to three hours and is protein-synthesis independent (Lynch, 2004). A stimulation pattern, e.g. weak high frequency (Bashir et al., 1991) or weak theta stimulation (Schröder et al., 2004), causes a simultaneous presnaptic release of glutamate and postsynaptic depolarization leading to the release of the magnesium blockage of the NMDA receptor. Thus, the NMDA receptor is dually regulated by ligand and voltage and thereby acts as a coincidence detector (Coan and Collingridge, 1987; Cotman et al., 1988). Consistent with its important role in early LTP induction, there exist numerous studies demonstrating that inhibition of NMDA receptor activation blocks early LTP (Collingridge et al., 1983; Harris et al., 1984). After release of the magnesium ion, calcium can enter through the NMDA channel into the postsynaptic cell (Collingridge et al., 1983; Harris et al., 1984; Jahr and Stevens, 1987). The influx of calcium ions into the postsynaptic spine can be blocked by injection of calcium chelators causing abolishment of LTP (Lynch et al., 1983; Malenka et al., 1988). Similarly, LTP induction occurs when the postsynaptic cell is loaded with calcium (Malenka et al., 1988). The rise in calcium triggers calcium dependent kinases, the calcium/calmodulin-dependent protein kinase II (CaMKII; Fukunaga et al., 1993; Ouyang et al., 1997; Sweatt, 1999) and protein kinase C (PKC; Sweatt, 1999). Inhibitors of CaMKII block LTP in CA1 (Malenka et al., 1989; Malinow et al., 1989) and injection of a

constitutively active form of CaMKII induces LTP (Lledo et al., 1995; Pettit et al., 1994). Similarly, antagonists of PKC block the induction of LTP (Malinow et al., 1989). CaMKII and PKC phosphorylate existing AMPA and NMDA receptors to increase their conductance (Derkach et al., 1999; Lau and Zukin, 2007; Malenka and Bear, 2004; Rebola et al., 2010; Soderling and Derkach, 2000). Furthermore, they increase the number of AMPA and NMDA receptors in the plasma membrane at synapses via activity-dependent changes in synaptic trafficking (Lau and Zukin, 2007; Malenka and Bear, 2004; Malinow, 2003; Shi et al., 1999, 2001; Lu et al., 2001). Thus, by increasing the efficiency and number of AMPA receptors at the synapse, future excitatory stimuli generate larger postsynaptic responses.

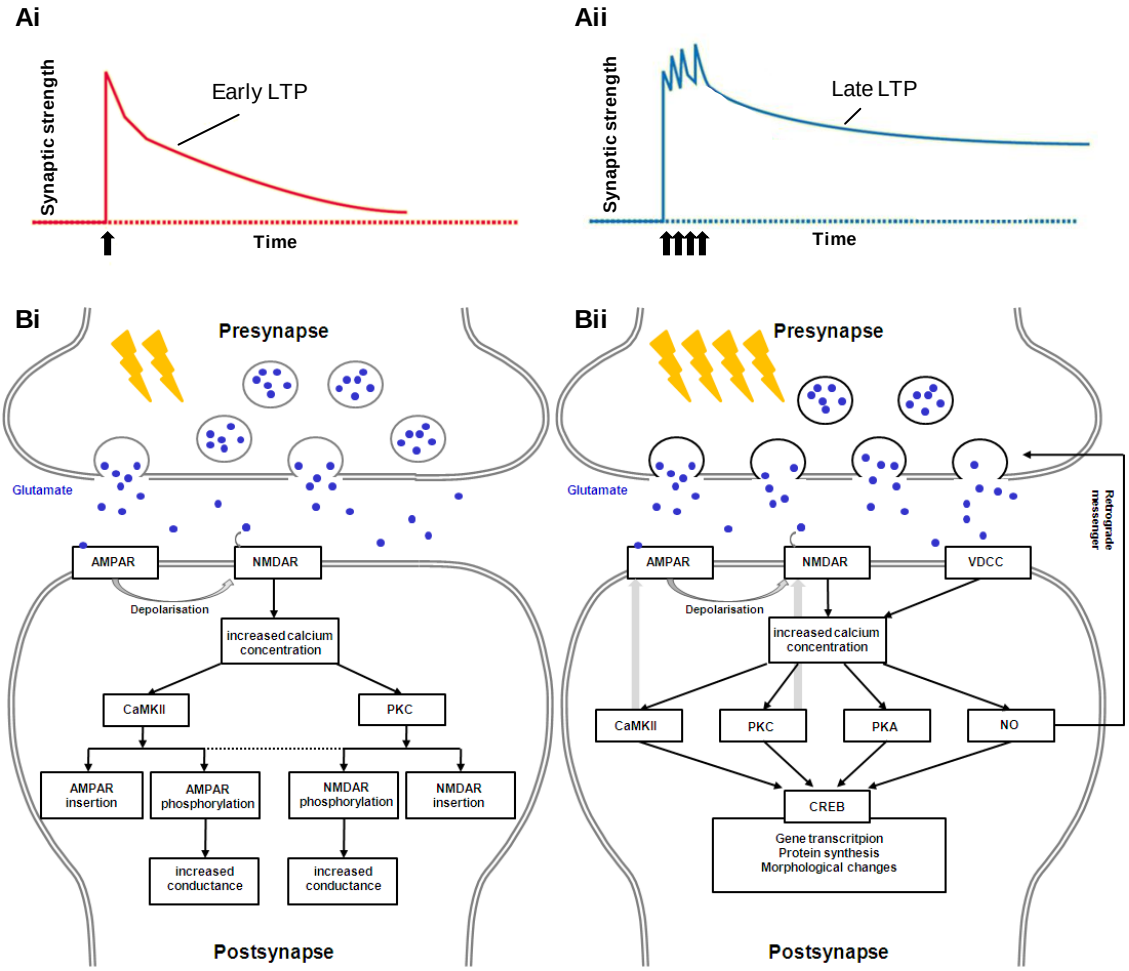


Figure 14: Mechanisms underlying early and late LTP in the CA1 region.
(A) The durations of early (i) and late (ii) LTP are shown schematically. Arrow/s indicate/s the different stimulation protocols. Adopted from Huang, 1998. (B) Schematic drawing of the mechanisms of early (i) and late (ii) LTP.

The use of a stronger stimulation pattern, e.g. repeated high frequency stimulation (Lu et al., 1999) or strong theta burst stimulation (Rönicke et al., 2009), causes the induction of a more persistent phase of LTP, namely **late LTP**. Late LTP is the natural extension of early LTP being defined as lasting longer than three hours and being protein-synthesis dependent (Frey et al., 1988, 1996; Lu et al., 1999). Due to stronger stimulation and thus stronger depolarization, besides NMDA receptors, also voltage-dependent calcium channels (VDCCs), which are embedded in the postsynaptic membrane, may reach the open state (Grover and Tyler, 1990; Kullman et al., 1992). Thus, even more calcium enters the cell not only activating CaMKII and PKC, but also the NO-sGC-cGMP (nitric oxide - soluble guanylate cyclase - cyclic guanosine monophosphate) pathway and the cAMP-PKA-MAPK-CREB (cyclic adenosine monophosphate - protein kinase A - mitogen-activated protein kinase - cAMP response element-binding) pathway. Calcium causes NO production by stimulating calcium/calmodulin-dependent neuronal NO synthases (Arancio et al., 2001; Christopherson et al., 1999; Son et al., 1998; Zhuo et al., 1994). The diffusible NO is believed to act as a retrograde messenger (Böhme et al., 1991; O'Dell et al., 1991; Schuman and Madison, 1991), which can cause changes in the presynaptic neurotransmitter release machinery (Feil and Kleppisch, 2008). Yet, reports about NO acting postsynaptically also exist (Garthwaite, 2008; Ko and Kelly, 1999). Most postsynaptically effects of NO are mediated by NO-GCs, NO-sensitive enzymes that form the second messenger cGMP and whose blockade abolishes late LTP (Boulton et al., 1995; Son et al., 1998; Zhuo et al., 1999). The generated cGMP causes an induction/activation of effector molecules and/or transcription factors, e.g. protein kinases, ion channels, phosphodiesterases, and/or CREB (Garthwaite, 2008). Beneath the NO-sGC-cGMP pathway, several studies have indicated that late LTP is also dependent on the cAMP-PKA-MAPK-CREB pathway. Among the downstream consequences of an increase in cAMP concentration is the activation of PKA (Huang and Kandel, 1994; Frey et al., 1993), whose inhibition blocks LTP (Huang and Kandel, 1994), and MAPK (also called ERK) (Impey et al., 1998; Roberson et al., 1999). The important role of MAPK in expression of LTP was first underlined by the finding that its inhibition resulted in suppression of late LTP in CA1 (English and Sweatt, 1997; Impey et al., 1998). The downstream consequences of MAPK activation are wide ranging including phosphorylation of cytoskeletal proteins, signaling proteins and nuclear proteins/transcription factors (Jovanovic et al., 1996; Lynch, 2004; Matsubara et al., 1996) with CREB being the most important. In fact, mutant mice lacking CREB isoforms α and δ exhibit attenuated late LTP

(Bourtchuladze et al., 1994). Remarkably, not only activation of the MAPK pathway is able to activate CREB by its phosphorylation, but also PKA, PKC, CaMKII as well as other kinases are involved in CREB activation. CREB activation due to its phosphorylation is the first step in gene transcription (Alberini et al., 2005; Silva et al., 1998) resulting in translation of immediate early genes, which can also act as transcription factors to induce late-response genes. Increased protein levels of certain immediate early genes, like *zif268/Erg1*, *Egr2*, *Egr3*, *AP-1*, *c-jun*, *jun-B*, *Arc* and *junD* (Abraham et al., 1991; Cheval et al., 2011; Poirier et al., 2008; Yin et al., 2002), are associated with late LTP in the way that all these gene products are thought to lead to functional and structural changes of the synapse. In the case of *zif268* one gene product was identified as a nerve growth factor response gene product, which has been shown to stimulate cell growth and differentiation (Milbrandt, 1987). Results from a study using *zif268* knockout mice demonstrated that this gene is required for late LTP (Cheval et al., 2011; Poirier et al., 2008). Recent attention has focused on analysis of changes in *Arc* (activity-regulated cytoskeleton-associated protein; also called as *Arg3.1*) in LTP (Lyford et al., 1995; Yin et al., 2002; Yilmaz-Rastoder et al., 2010). Because *Arc* protein binds to actin, it has been proposed that it participates in cytoskeletal remodeling after synaptic activation. Consistent with the observed LTP-associated increase in *Arc* expression, disruption of *Arc* inhibits late LTP (Bramham et al., 2008). It is likely that the increase in protein-synthesis that accompanies late LTP contributes to the establishment of the morphological changes that have been reported, for example, the increases in postsynaptic surface area (Desmond and Levy, 1990), spine number (Lee et al., 1980) and spine area (Fifkova and Vanharreveld, 1977). Late LTP has also been shown to increase the number of large spines (Desmond and Levy, 1990), axospinous perforated synapses (Geinisman et al., 1991) and perforated synaptic densities with larger apposition zones between pre- and postsynaptic structures (Buchs and Muller, 1996). Changes in distribution (Applegate et al., 1987) and numbers (Meshul and Hopkins, 1990) of synaptic vesicles and changes in synaptic curvature (Desmond and Levy, 1990) have also been reported. So far however, it is unclear whether protein-synthesis takes place in the postsynaptic cell body or in its dendrites (Kang and Schuman, 1996; Kelleher et al., 2004; Steward and Worley, 2001).

1.2.2.2. Natural modulation of LTP

As glutamate is the primary excitatory neurotransmitter of the central nervous system, the backbone of LTP is based on the glutamatergic system. Animals with alterations in the glutamatergic system, for example the NMDA receptors (Single et al., 2000), will not survive either being stillborn or die right after birth. Even though the glutamatergic system is ubiquitary essential for LTP, there are many other factors which are necessary to regulate the induction and maintenance of LTP. For example neurotransmitters, like D-serine (Henneberger et al., 2010; Yang et al., 2003), γ-aminobutyric acid (Davies et al., 1991; Fujii et al., 2000), glycine (Martina et al., 2004), norepinephrine (Katsuki et al., 1997), epinephrine (Korol and Gold, 2008), histamine (Brown et al., 1995; Luo and Leung, 2010), serotonine (Corradetti et al., 1992), acetylcholine (Leung et al., 2003; Ovsepian et al., 2004), dopamine (Li et al., 2003; Swant and Wagner, 2006) and adenosine (Asztely et al., 1994; Huang et al., 1999), and even hormones, like insulin (Stranahan et al., 2008; van der Heide et al., 2005) or peptides like brain-derived neurotrophic factor (Lu et al., 2008), are known to influence LTP. Thus, many different types of neuronal transmitter systems can modulate LTP making it flexible to allow reaction to environmental changes. However, this metaplasticity makes LTP also vulnerable to any changes concerning its modulators, e.g. to a loss of a specific neuron type. In general, alterations of modulators are mainly responsible for changes in LTP making them interesting targets for treatment of memory deficits. Nevertheless, caution is required in generalizing across species, stimulation paradigms or brain regions with regard to specific molecular processes that occur following LTP induction (Steward et al., 2007). For example, a stronger stimulation protocol is needed to induce LTP in mouse hippocampal slices than in rat hippocampal slices.

1.2.2.3. LTP and memory

The concept, that LTP is a cellular model for learning and memory, is supported by a wide variety of evidence. These include: phases, strengthening by repetition, saturation, rhythms of the hippocampus during learning and memory tasks, inhibition, biochemical changes and correlations of LTP decay with the time course associated with natural forgetting. These links between LTP and memory will be discussed in the following paragraphs.

Memory and LTP have similar phases. There is short-term memory, which endures for a few hours, and long-term memory, which persists for several days and often much longer (Goelet et al., 1986). At the cellular level, the storage of long-term memory is associated with gene expression, *de novo* protein-synthesis and formation of new synaptic connections (Costa-Mattioli et al., 2009; Goelet et al., 1986; Izquierdo et al., 2002; Pang and Lu, 2004). Consistently, protein-synthesis inhibitors can block persistent memory but leave short-term memory unaffected (Alkon et al., 2005; Goelet et al., 1986; Grecksch and Matthies, 1980). Thus, there is an obvious parallel between memory and LTP, since LTP also consists of distinct phases involving different molecular mechanisms (Huang, 1998). The early phase, which lasts one to three hours, is independent of protein-synthesis, while more persistent late LTP, which lasts several hours *in vitro* and weeks *in vivo*, requires *de novo* protein-synthesis (Fazeli et al., 1993; Frey et al., 1988; Huang, 1998; Malenka and Nicoll, 1999; Nguyen et al., 1994; Reyman and Frey, 2007).

Although memory induction can certainly be complete within a single trial (Rock, 1956), there are numerous instances in which memory is strengthened by repeated exposure to the learning event (Lee et al., 1991). Thus, if LTP is involved in memory formation, it should be strengthened through repetition. Indeed, synaptic efficacy can be strengthened through repeated exposure to the tetanizing stimulus. For example, whereas a single 100 Hz train leads to early LTP lasting about one to three hours, three trains of 100 Hz stimulation induce late LTP lasting longer than three hours (Albensi et al., 2006; Huang, 1998).

If it is argued that the same set of synapses is activated and modified in the same way by LTP and spatial learning, then one could conclude that saturation of LTP would impair learning. Indeed, some studies showed that saturating LTP impaired spatial learning (Castro et al., 1989; Moser et al., 1998). The authors suggested that LTP and spatial learning relay on the same cellular mechanisms.

The two most often used LTP stimulation protocols, high frequency stimulation and theta burst stimulation (Albensi et al., 2006), are both known to naturally occur in living animals and to be associated with learning and memory. Theta burst activity can be observed in the hippocampus during episodes of learning and memory in animals (Buzsáki, 2002; Hasselmo, 2005; Lubenov and Siapas, 2009; Mitchell et al., 1982; Winson, 1978). High frequency oscillations, referred to as sharp wave/ripples, occur within the hippocampus, are associated with synchronous discharge of a large neuronal population in multiple hippocampal sites (Jackson et al., 2006; Ylinen et al., 1995) and are known to be important

for consolidation of associative memory (Behrens et al., 2005; Ramadan et al., 2009; Ylinen et al., 1995).

A major focus of several groups has been to identify the cell signaling cascades and biochemical changes that might be involved in consolidation of memory and/or expression of late LTP. In a study by Whitlock et al. (2006) it was found that one-trial inhibitory avoidance learning in rats produced the same changes in hippocampal glutamate receptors as did LTP. A consensus has emerged indicating that both late LTP and spatial learning are dependent on increased glutamate release (Böhme et al., 1991; Daisley et al., 1998; Feil and Kleppisch, 2008; O'Dell et al., 1991; Richter-Levin et al., 1991; Schuman and Madison, 1991) and subsequent activation of NMDA receptors (Coan and Collingridge 1987; Collingridge et al., 1983; Harris et al., 1984; Morris et al., 1986; Sakimura et al., 1995; Tsien et al., 1996) causing influx of calcium, which recruits intracellular pathways. In fact, it is known that CaMKII (Fukunaga et al., 1993; Frankland et al., 2001; Giese et al., 1998; Malenka et al., 1989; Malinow et al., 1989; Ouyang et al., 1997; Silva et al., 1992a,b; Sweatt, 1999; Weeber et al., 2000), PKA (Abel et al., 1997; Frey et al., 1993; Huang and Kandel, 1994; Schafe et al., 2001), MAPK (Atkins et al., 1998; Blum et al., 1999; English and Sweatt, 1997; Impey et al., 1998; Schafe et al., 2000; Selcher et al., 1999) and PKMζ activation (Pastalkova et al., 2006) are involved in late LTP as well as in spatial memory performance. Activation of immediate early genes (*zif268*: Bozon et al., 2002; Jones et al., 2001; *Arc*: Guzowski et al., 2000), and transcription factors (CREB: Bourtchuladze et al., 1994; Guzowski and McGaugh, 1997; Impey et al., 1996; Lamprecht et al., 1997; Nguyen et al., 1994; Nguyen and Kandel, 1997; Silva, 1998) as well as increased protein-synthesis (Alkon et al., 2005; Fazeli et al., 1993; Frey et al., 1988; Goelet et al., 1986; Grecksch and Matthies, 1980; Huang, 1998; Malenka and Nicoll, 1999; Nguyen et al., 1994; Reyman and Frey, 2007) have also been demonstrated.

Last, but not least, decay of LTP correlates with the time course associated with natural forgetting in animals (Huang, 1998; Otto et al., 1991).

Taken together, all this evidence supports the hypothesis that LTP can be considered as a biological substrate for at least some forms of memory and thus, that it can be readily used as an *in vitro* model for learning and memory.

1.3. Thesis Outline

My PhD-project focuses on the establishment and validation of CA1 LTP in rat hippocampal slices to characterize memory enhancing drugs acting on specific molecular targets as potential treatment options of AD. As the brains of AD patients are characterized by a profound loss of mainly cholinergic and glutamatergic synapses/neurons, I focused on testing drugs potentially being able to enhance cholinergic and glutamatergic neuronal functions. The idea was to compare these drugs based on their effects on LTP in order to elucidate whether glutamatergic or cholinergic drugs might be more efficacious in enhancing learning and memory. Furthermore, I analyzed the potential of some of these drugs to rescue LTP being impaired by Amyloid-beta derived diffusible ligands. Amyloid-beta derived diffusible ligands – also described more generally as Aβ-oligomers - are believed to be the direct or indirect cause for formation of senile plaques, neurofibrillary tangles and synaptic loss leading to cognitive dysfunction in AD patients.

In detail, I focused on the following aspects (Fig. 15):

1. A system to measure LTP was established. Therefore, a commercially available multi-slice recording system was set up and improved by two stacked grids enabling absolutely stable measurements of LTP for up to seven hours from several slices simultaneously. The software Notocord® was included for on-line data acquisition and a Pumped Perfusion System was incorporated allowing recycling of drug solutions applied to the slice.

2. In order to analyze the effect of a certain drug on LTP precisely, it is mandatory to use well defined and standardized protocols. Therefore, clearly defined and validated protocols were established for the induction of early and late LTP. The established protocols were validated for involvement of NMDA receptors and L-VDCCs by using MK-801 and nifedipine, respectively. Then the protocols were used to elucidate the effect of a mGlu5 receptor positive allosteric modulator (ADX-47273) on early and late LTP (Fig. 15, shown in bluish).

3. Standard of care treatment of AD patients are acetylcholine esterase (AChE) inhibitors, which aim to increase the neurotransmitter acetylcholine. Here, an alternative and more direct approach to increase cholinergic neurotransmission was

investigated. Thus, the effect of direct activation of specific cholinergic receptors on early and late LTP was analyzed using selective α4β2 and α7 nAChR agonists, TC-1827 and SSR180711, respectively (Fig. 15, shown in greenish).

4. Clinical experience demonstrates only moderate efficacy for donepezil, the current standard AChE inhibitor prescribed to treat AD patients. Candidates for alternative treatments of cognitive dysfunction might be phosphodiesterase (PDE) inhibitors, which influence the glutamatergic system. Thus, the effects of donepezil and a PDE9A inhibitor (BAY 73-6691) on early and late LTP were analyzed and compared (Fig. 15, shown in reddish).

5. Amyloid-beta derived diffusible ligands (ADDLs) are believed to be the putative cause of AD. Therefore, the effects of synthetic ADDLs on early and late LTP were analyzed (Fig. 15, shown in yellowish). Then, the potential of TC-1827, SSR180711, BAY 73-6691 and donepezil to rescue ADDLs impaired LTP were evaluated.

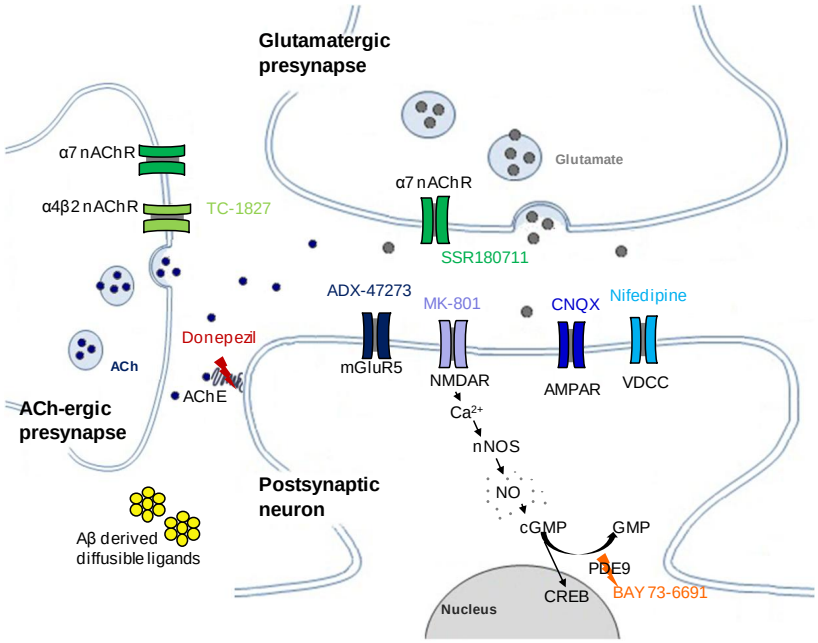


Figure 15: Outline.

Shown in blue: AMPAR, NMDAR and VDCC as well as their respective blockers/antagonists CNQX, MK-801 and nifedipine. The mGlu5 receptor and its positive allosteric modulator ADX-47273.

Shown in green: The α4β2 nAChR and α7 nAChR as well as their respective (partial) agonists TC-1827 and SSR180711.

Shown in red: AChE and PDE9 as well as their respective inhibitors donepezil and BAY73-6691.

Shown in yellow: Amyloid-beta derived diffusible ligands.

CHAPTER 2

A multi-slice recording system for stable hippocampal LTP experiments

Abstract

A major challenge in neuroscience is identifying the cellular and molecular processes underlying learning and memory formation. In the past decades, significant progress has been made in understanding cellular and synaptic mechanisms underlying hippocampal learning and memory using long-term potentiation (LTP) experiments in brain slices as a model system. To expedite LTP measurements it is helpful to further optimize such recording systems. Here, we describe a modification of a multi-slice recording system (SliceMaster, Scientifica Limited, East Sussex, UK) that allows absolutely stable measurements of field excitatory postsynaptic potentials (fEPSPs) for up to eight hours in up to eight slices simultaneously. The software Notocord® was used for on-line data acquisition and to control the digital pattern generator which can generate different patterns for slice stimulation, inducing different types of LTP. Moreover, in contrast to common gravity-driven perfusion systems, a Pumped Perfusion System was employed to recycle drug solutions applied to the hippocampal slice. In addition, slices were positioned on two stacked grids for optimal recording of fEPSPs. These two stacked grids were placed in the measuring chambers allowing recordings for several hours without any perturbances. In summary, this modified slice-recording system improves throughput and allows for better statistical design, increases number of used slices per animal and enables very robust LTP measurements for up to seven hours. Hence, this system is suitable not only to investigate molecular mechanisms underlying the late phase of LTP, but also to screen candidate compounds in the context of drug discovery.

2.1. Introduction

One of the most prominent features of the brain is its ability to acquire and store information. Although substantial progress has been made in the past few decades in understanding certain forms of acquisition and recall, the phenomenon of learning and memory is still not fully understood. Mammalian brains are flexible, being able to change their neuronal function and microstructures in response to internal and external stimuli. These forms of neuronal plasticity correspond to long-lasting changes in the strengths of synapses between neurons. Especially the hippocampus, a brain region crucial for the formation of episodic memory, is the preferred system to study changes of synaptic and neuronal plasticity. *In vivo*, hippocampal learning and memory can be assessed with certain cognition tests, for example the Morris water-maze (Morris, 1984) or the T-maze (Gerlai, 1998). *In vitro*, cellular and synaptic mechanisms underlying hippocampal learning and memory can be investigated with long-term potentiation (LTP) experiments in brain slices. In the last decades, *in vitro* studies, especially in hippocampal slices, gained increasing popularity as they allow more detailed investigations of physiology and pharmacology on the cellular and molecular level as compared to *in vivo* experiments.

LTP is the increase of the strength of synapses between neurons for prolonged periods following brief but intense synaptic activation (Bliss and Gradner-Medwin, 1973; Bliss and Lomo, 1973). Currently, the majority of LTP measurements are performed in the CA1 region of hippocampal slices. Although LTP has been extensively studied in the past years, the major hurdles for establishing LTP measurements are the following. First, LTP experiments are highly time-consuming resulting in a low throughput. Second, although researchers are committed to reduce the number of animals, not all prepared brain slices (i.e. about eight hippocampal slices of 400 µm thickness from one rat brain) can be used for experiments, since commonly used recording systems only permit measurements from one slice at a time (Haas et al., 1979). Third, it is difficult to robustly measure LTP over a longer period of time which is required for measuring late phase LTP, an equivalent of synaptic plasticity underlying long-term memory. This difficulty might be due to the decreasing vitality of the slice or, more often, due to movements of the slice or the electrodes caused by mechanical instability of the recording system. The first two points were already addressed by Stopps et al. (2004) who established a system allowing electrophysiological recordings from up to eight brain slices simultaneously and hence increasing throughput and optimizing tissue use from animals. In this study, mechanical

instability compromising recording quality especially over a longer period of time is addressed.

Here, we describe the improvement of the measuring chambers of an already existing multi-slice recording system with two stacked grids enabling absolutely stable measurements of LTP for up to seven hours from several slices simultaneously. Further modification includes the software Notocord® used for on-line data acquisition and a Pumped Perfusion System allowing recycling of drug solutions applied to the slice.

2.2. Materials and methods

2.2.1. Multi brain slice system

The SliceMaster multi-slice recording system (Scientifica Limited, East Sussex, UK) was used to record field excitatory postsynaptic potentials (fEPSPs). The system is semi-automated allowing one operator to record simultaneously from an assembly of up to eight brain slices. The system has been described in detail by Stopps et al. (2004). Briefly, the SliceMaster used for this report consists of three temperature-controlled integrated brain slice chambers and can be upgraded with five more. Each chamber is equipped with one stimulation electrode, one recording electrode and a camera system allowing a visualisation of each brain slice on a shared video monitor. A central control panel makes the positioning of all electrodes and cameras possible, allowing a single operator to run all experiments simultaneously.

2.2.2. Perfusion

A separate inflow and outflow for each integrated brain slice chamber allows individual perfusion of each brain slice. Each integrated brain slice chamber has independent reservoirs for buffer and drug solutions. These independent reservoirs allow random assignment of treatments across slices. The tube lengths are minimal and identical for each integrated brain slice chamber. A suction pump discards the used liquid via the outflow into a waste box or recycles it back to the original reservoir. Unlike the gravity-fed system described previously (Stopps et al., 2004), a Pumped Perfusion System (PPS; Scientifica Limited, East Sussex, UK) was used in this study. Gassing and recirculation of solutions is possible and a flow rate between 0.1 and 20 mL min^{-1} can be chosen. The PPS combines full computer control of eight two-way, two three-way pinch valves and a peristaltic pump. It allows easy configuration of a series of valve functions and was modified to enable four two-way valves being controlled simultaneously (Fig. 16). There are two sets of valves, each equipped with four two-way valves: valve set one and valve set two. All tubes connected with valve set one arise from the buffer reservoirs, whereas all tubes coming from the different drug solutions are linked to valve set two. As soon as an experiment is started, valve set one is opened and automatically valve set two is closed. Hence, from

three different buffer reservoirs artificial cerebrospinal fluid (ACSF) passes the open valve set one, the peristaltic pump and is applied directly to the individual integrated brain slice chamber. The valve set two can be opened by software control, simultaneously closing valve set one, applying the different drug solutions to each of the individual integrated brain slice chambers. The remaining two three-way pinch valves can be used to recycle drug solutions at two individual chambers, with 20 mL being the minimum recycle volume. For documentation, the system can be configured to send a TTL pulse for each opening event. Furthermore, for each experiment a data log is created for all parameters of the valves and the peristaltic pump.

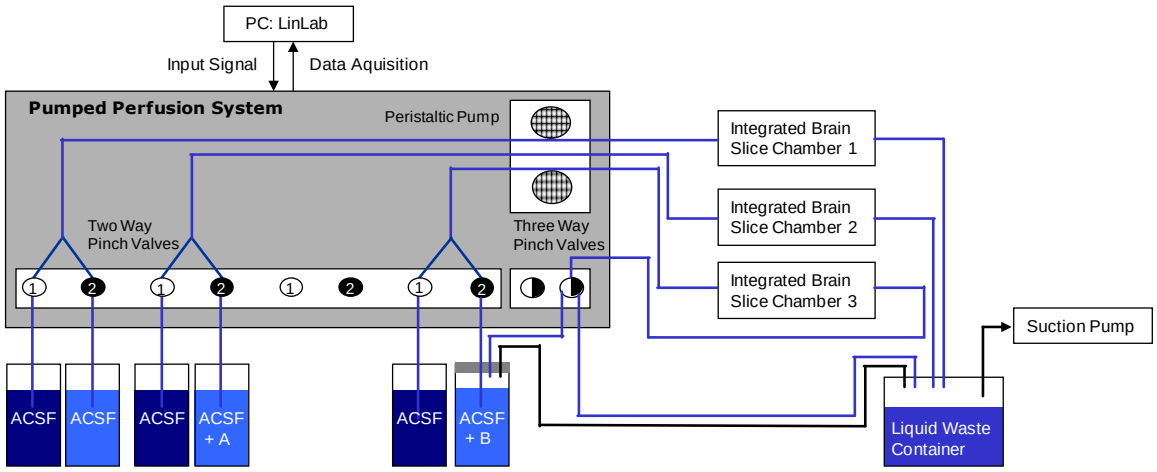


Figure 16: Pumped Perfusion System.
Schematic drawing showing the flow of ACSF and drug solutions (ACSF + A and ACSF + B) from the independent reservoirs (ACSF shown in dark blue, control and drug solutions shown in light blue) via the two-way pinch valves and the peristaltic pump to the single integrated brain slice chambers. After passing the integrated brain slice chambers the fluid is discarded by a suction pump (shown for the integrated slice chambers 1 and 2) or recycled via a three-way pinch valve (shown in black-white, shown for the integrated slice chamber 3). Opening of valve set 1, consisting of 4 two-way pinch valves (shown in white), results in application of ACSF. Two different drug solutions and ACSF, as control, are applied to the individual integrated brain slice chambers as soon as valve set 2 is opened, which consists of 4 two-way pinch valves (shown in black). The eight two-way pinch valves, the two three-way pinch valves and the peristaltic pump are controlled logged by the software LinLab. This figure shows the perfusion of 3 chambers as in this study 3 chambers were used. However, this Pumped Perfusion System can perfuse up to 5 chambers without any dismounting as one two-way pinch and one three-way pinch valves are free to use.

2.2.3. Minimization of fluidic turbulences

As all reservoirs for buffer and drug solutions have to be saturated with 95% O_2 and 5% CO_2, they need to be constantly bubbled with carbogen. This causes small bubbles being transported via the PPS to the integrated brain slice chambers. Bubbles in the PPS cause movements of the grid supporting the slice and result in unstable recordings. To minimize the movement of the slice, stacked grids were integrated. The original single grid was replaced by a very fine mesh (20 µm mesh size, polyamide; VWR International GmbH, Darmstadt, Germany) almost impenetrable for bubbles. On top of this mesh a spacer with a second rigid and coarse grid (500 µm mesh size, polypropylene; VWR International GmbH, Darmstadt, Germany) was attached supporting the slice (Fig. 17). The spacer between the upper and the lower grid was optimized in a way that bubbles moving the lower grid leave the upper grid unaffected (1.2 mm spacing). In the rare event of a bubble passing through the lower grid, wide openings at the edges of the upper grid allow these bubbles to pass. The movement of the passing bubble is minimized by the rigidity of the upper grid.

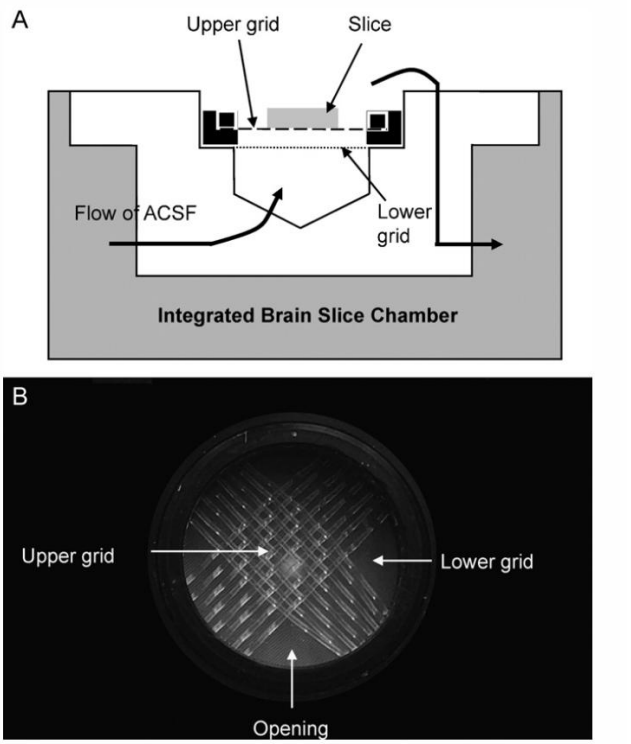


Figure 17: Principle of the stacked grids.
(A) The stacked grids in the integrated brain slice chamber are shown schematically. The lower grid has a very fine mesh (20 µm mesh size, polyamide) making it impenetrable for bubbles. The upper grid is rigid and coarse (500 µm mesh size, polypropylene) supporting the slice. (B) Photograph showing the two grids mounted into the integrated brain slice chamber from up above. The four openings at the edges of the upper grid allow remaining bubbles to pass the slice without destabilizing it.

To demonstrate the impact of this improvement of recording stability on statistical power a sub-maximal concentration of MK-801 (0.1 µM; (−)-MK-801 hydrogen maleate, Sigma–Aldrich Corporation, St. Louis, USA; Frankiewicz et al., 1996) was used to slightly reduce LTP magnitude (Fig. 20B).

2.2.4. Preparation of brain slices

Procedures involving animals and their care were conducted in conformity with institutional and European Union guidelines (EEC Council Directive 86/609) and were approved by the Ethical Committee of the respective regional councils. Male Wistar rats (Janvier, Le Genest Saint Isle, France) aged six to seven weeks were shortly anaesthetized with isoflurane and sacrificed by decapitation. The brains were quickly removed and immersed in ice-cold ACSF (containing in mM NaCl 124, KCl 4.9, $MgSO_4$ 8, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25.6, D-glucose 10, pH 7.4, saturated with 95% O_2 and 5% CO_2). Transverse hippocampal brain slices (400 μm thickness) were cut using a Vibratome (Vibratome 3000 Deluxe, Hugo Sachs Elektronik – Harvard Apparatus GmbH, March-Hugstetten, Germany). Both hemispheres were prepared resulting in approximately eight slices per brain. Slices were allowed to recover in a holding chamber containing ACSF bubbled with 95% O_2 and 5% CO_2 at room temperature (approximately 20 °C) for at least one hour. After recovering in the holding chamber, one slice was transferred to each integrated brain slice chamber, placed on the upper grid, immobilized with a platinum wire and continuously superfused (at a flow rate of 2.5 mL min^{-1}, 25 ± 0.2 °C) with the same ACSF composition used before except for 0.5 mM $MgSO_4$. Prior to doing any electrophysiological recordings, the slices were allowed to equilibrate for at least 30 minutes.

2.2.5. Extracellular recording and stimulation

fEPSPs were elicited in the CA1 region by stimulation of the Schaffer collateral–commissural fibres in the stratum radiatum using glass microelectrodes (borosilicate glass, wall thickness 0.225 mm, Ø outside 1.5 mm; Hilgenberg GmbH, Malsfeld, Germany; pulled with a DMZ-Universal Puller, Zeitz-Instruments GmbH, Müchen, Germany) with broken tips (filled with ACSF). For recording, the same glass microelectrodes (with intact tips, 2–6 MΩ, filled with ACSF) were placed in the apical dendritic layer. The amplitudes of fEPSPs were used as the parameter of interest (Collingridge et al., 1983). To generate fEPSPs at a constant sub-maximal stimulus, as in previous studies (Seabrook et al., 1997), the stimulus strength of the pulses was adjusted to 20–30% of the fEPSP maximum and this voltage was used for the experiment. During baseline recording each slice was

stimulated every 30 seconds for at least one hour. LTP was induced by different stimulation protocols. A "weak theta burst stimulation" consistent of four paired pulses with an interpulse interval of 10 ms that were applied at the theta frequency of 5 Hz (Schröder et al., 2004). A "strong theta burst stimulation" composed of ten times four pulses with an interpulse interval of ten milliseconds and an interval between bursts of 200 milliseconds, repeated two times in ten minutes intervals (Rönicke et al., 2009). A "weak high frequency stimulation" made up of 20 pulses at the frequency of 100 Hz (Bashir et al., 1991). An "intermediate high frequency stimulation" consisting of 50 pulses at the frequency of 100 Hz (Sastry et al., 1987). A "strong high frequency stimulation" composed of 100 pulses at the frequency of 100 Hz (Lu et al., 1999) and a "repeated high frequency stimulation" consisting of 100 pulses at the frequency of 100 Hz, repeated two times in five minutes intervals (Lu et al., 1999).

2.2.6. Recording and data acquisition

A modular electrophysiology system, supplied by npi electronic GmbH (Tamm, Germany), conducts the low noise recordings of extracellular signals. AC coupled signals are amplified 1000× and internally filtered with a five kHz low-pass filter as well as a three Hz high-pass filter. In our study, a customized system (programmable pattern generator) was used allowing simultaneous stimulation of all slices with either a single stimulus or a specific stimulus pattern. The pattern generator is built with a Single-Board Remote Input/Output (board: NI sbRIO-9631, National Instruments, TX, USA; http://sine.ni.com/nips/cds/view/p/lang/en/nid/205894) and programmed with LabVIEW (National Instruments, TX, USA). Triggering and timing of each pulse is done in the FPGA (Field-Programmable Gate Arrays). The accuracy is one microsecond. A customized webpage is used to configure the pattern generator via its built-in ftp server. A simultaneous sampling rate stimulation is created by the pattern generator, if it is triggered either manually or by the Digital Output One of the software Notocord® (NOTOCORD Systems, Croissy Sur Seine, France). If the pattern generator is triggered by the Digital Output Two of Notocord®, it creates a stimulation pattern. The programmable pattern generator allows the creation of customized stimulation patterns (high frequency stimulation, strong and weak theta burst stimulation, etc.). The stimulus isolators (10 V maximum voltage output, SLICE-ISO-01, npi electronic GmbH, Tamm, Germany) receive

all information from the pattern generator and stimulate the slices accordingly. Furthermore, individual slices can be stimulated with a single stimulus generated by manually triggering each individual stimulus isolator. The triggers of all stimulation events are fed into the data acquisition of Notocord® (Fig. 18).

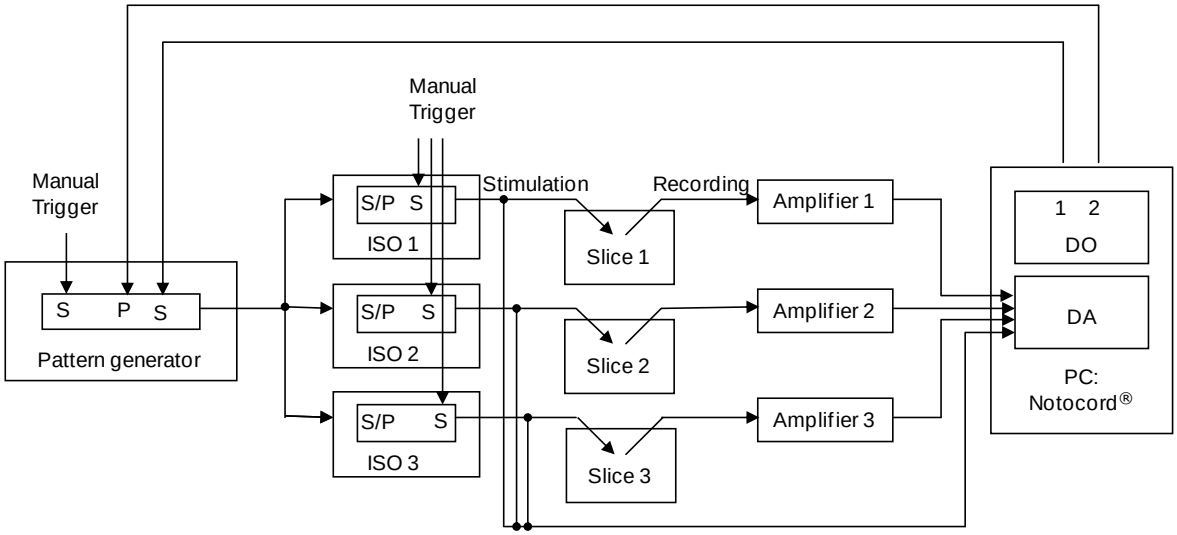


Figure 18: Recording and data acquisition.
Schematic drawing of the modular electrophysiology system used for recording and data acquisition. The pattern generator accomplishes simultaneous stimulation (S) of all three slices if it is either triggered manually or by the Digital Output 1 (DO 1) of the software Notocord®. If the pattern generator is triggered by the Digital Output 2 (DO 2) of Notocord®, it creates a patterned stimulation (P). The stimulus isolators receive all information from the pattern generator and stimulate the slices with a ground-free voltage or current pulse whose amplitude and duration can be individually set for each slice. To stimulate an individual slice with a single pulse (S), each individual stimulus isolator can be manually triggered. Along with the amplified recordings, the triggers of all stimulation events are fed into the Data Acquisition (DA) of Notocord®.

2.2.7. Software

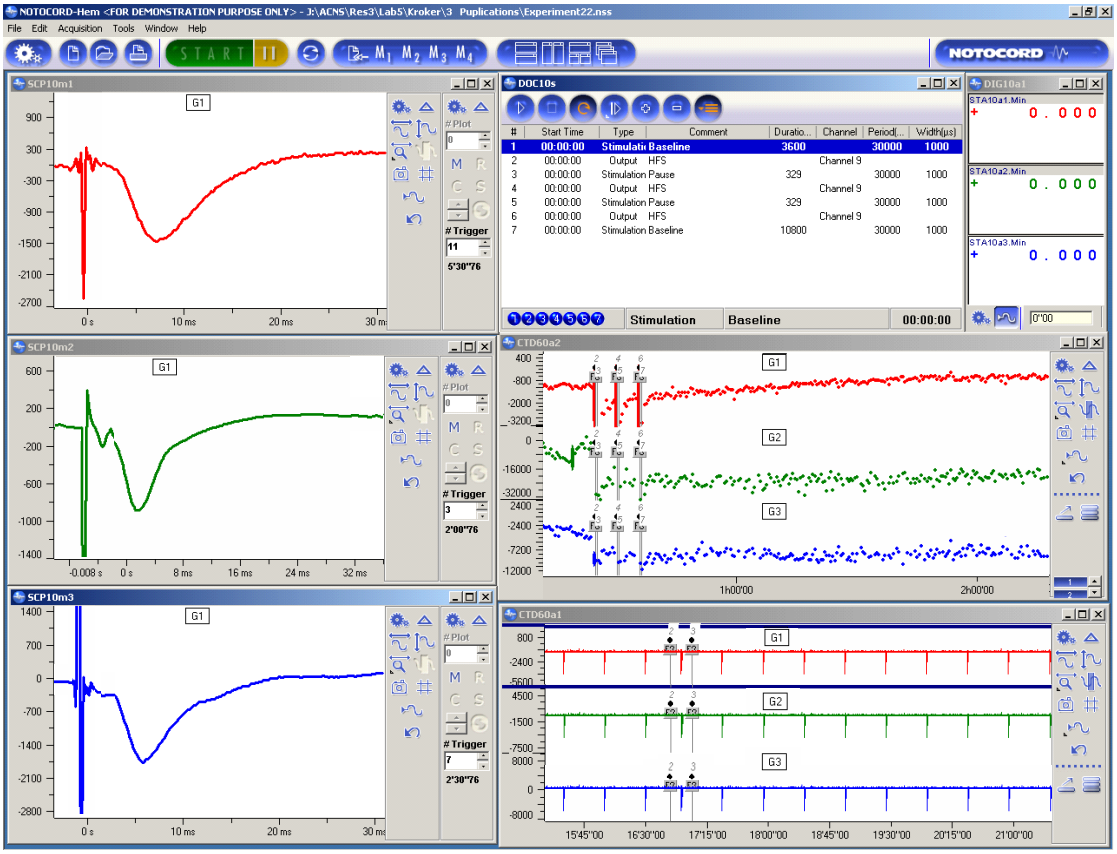


Figure 19: On-line data analysis of synaptic events.
Using Notocord® data acquisition and analysis may be performed on-line. The user interface is divided into five sections. On the left the fEPSPs of the individual slices are visualized. On the right top the stimulation protocol and the minimum of each individual fEPSP is shown. Below the minima of each recorded fEPSP are plotted against time and additionally the time series recording of each slice is shown. Event markers can be used to indicate stimulation with a specific stimulation pattern, application of a specific compound, etc.

For digital output of triggers, data acquisition and analysis the software Notocord® was used (Fig. 19). It is a modular software application allowing real-time acquisition (sampling frequency: 10 kHz), interactive display of the analyzed data and access to data from Microsoft Excel® (Microsoft Office) using Notocord®'s add-in. The Digital Output Control interface allows programming a series of events as they should occur in the framework for the experimental protocol. According to this protocol the Digital Output sends out control commands to the pattern generator via the A/D card DT 301 (Data Translation Inc., MA, USA; http://shop.datatranslation.eu/frontend/katalog.php) generating single pulse stimulation or stimulation with an arbitrary pattern. For each slice a

continuous display is shown and additional modules allow display of fEPSPs in a triggered oscilloscope-like manner. A digital filter removes spikes or high frequency noise. The minimum of the fEPSPs is analyzed in real-time and shown in a digital window as well as plotted on a graph.

2.2.8. UV-spectrometry and drug exposure

To assess the effect of a drug on LTP, the knowledge whether the drug reaches the slices in its intended concentration is indispensable, especially in pharmacological research. In this study, UV-spectrometry (at a wavelength of 290 nm) was used to verify exposure to an agent reducing the AMPA response (1 μM CNQX, Sigma–Aldrich Corporation, St. Louis, USA). Directly after leaving each integrated brain slice chamber the perfusion solution was sampled and analyzed in a UV spectrophotometer (Smartline 2500, Knauer Advanced Scientific Instruments, Berlin, Germany).

2.3. Results

2.3.1. Effects of the stacked grids

Extracellular recordings of evoked synaptic activity in the CA1 region of rat hippocampal slices were utilized to assess whether the two stacked grids in the integrated brain slice chambers enhance the stability of the system making recordings for several hours possible. For comparison, fEPSPs were elicited from slices supported by the original single grid and from slices positioned on the two stacked grids. Fig. 20A shows recordings from three slices for each condition. fEPSP recordings from slices positioned on the stacked grids were found to be much more stable than the ones from slices positioned on the original single grid. The recordings were terminated after seven hours, although the fEPSPs were still found to be of consistent amplitude. Statistical analysis comparing the average squared deviations of both groups to the averages of the baselines, shows that the average squared deviation from slices recorded on stacked grids is significantly lower than the one from slices recorded on single grids ($p < 0.001$).

To determine whether this low variability of the system allows detection of small differences between two groups and hence provides a high statistical power for experiments, LTP recordings (stimulation protocol: 1 s 100 Hz) of slices positioned either on the original single grid or on the two stacked grids were made. For each condition one group of slices ($n = 6$) was recorded for control and the other group ($n = 6$) was recorded under the influence of 0.1 µM of the NMDA-receptor antagonist MK-801 (Fig. 20B). Comparison of the two groups recorded on the original single grid (Fig. 20Bi) shows no significant effect (2 way-ANOVA, Dunnett's Test: not significant), whereas comparison of the two groups recorded on the two stacked grids (Fig. 20Bii) shows a significant effect (2 way-ANOVA, Dunnett's Test: $*p < 0.05$). These results can be reproduced by a statistical analysis using two group *t*-test (group 1 mean = μ_1; group 2 mean = μ_2; test significance level $\alpha = 0.05$, two sided test) of equal means (equal *n*‘s). This analysis indicates, that with the two stacked grids used in this study, a significant difference in means of 20% can be detected with recordings of only six slices (difference in means $(\mu_1 - \mu_1) = 20$, common standard deviation $(\sigma) = 10$, effect size $(\delta = |\mu_1 - \mu_2|/\sigma) = 2.0$, power (%) = 80; *n* per group = 6). Hence, due to the two stacked grids data variability is reduced and therefore a smaller number of slices is required to achieve statistical significance.

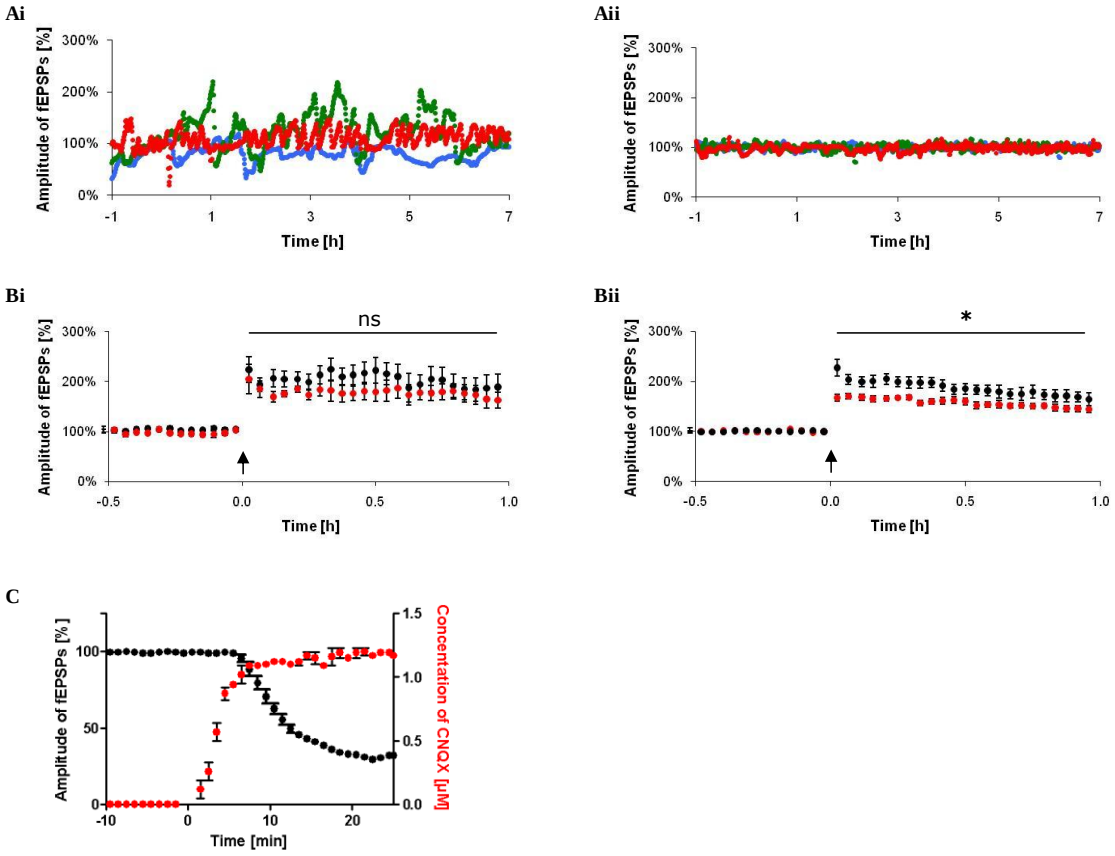


Figure 20: Influence of the two stacked grids on fEPSPs / LTP recordings and drug exchange.
(A) Comparison of fEPSP recordings in the CA1 region of hippocampal rat slices either positioned on the original single grid or on the two stacked grids. 3 slices (n = 3) were recorded for each condition. The recordings were terminated after 8 h. (i) fEPSP recordings of slices supported by the original single grid. (ii) fEPSP recordings of slices supported by the two stacked grids. (B) Comparison of LTP recordings in the CA1 region of hippocampal rat slices either positioned on the original single grid or on the two stacked grids. For each condition 6 slices (n = 6) were recorded for control and 6 (n = 6) slices were recorded under the influence of 0.1 µM MK-801. The recordings were terminated 1 h after LTP induction (1s 100 Hz). (i) Comparison of the control slices and MK-801 treated slices supported by the original single grid (2way-ANOVA, Dunnett Test: not significant). (ii) Comparison of the control slices and MK-801 treated slices supported by the two stacked grids (2way-ANOVA, Dunnett Test: *p < 0.05). (C) Analysis of the influence of the two stacked grids on the exchange of drug containing solutions. A time course for equilibration of a sub-maximal drug effect of CNQX (nominally 1 µM) is shown.

To address the question whether the stacked grids have an influence on the exchange of drug containing solutions, a time course for equilibration of a sub-maximal block of fEPSPs with CNQX (Andreasen et al., 1989) was performed (Fig. 20C). UV-spectrometry measurements show that the target concentration of CNQX (nominally 1 µM) was achieved in the integrated brain slice chamber within six minutes after CNQX perfusion

had started. Interestingly, the decrease of fEPSPs shows a five minutes delay after CNQX containing ACSF starts to perfuse the slice with respect to drug concentration and the equilibration is reached 15 minutes later. Therefore, the rate-limiting step is the penetration of the slice and the pharmacological action and not the perfusion itself.

2.3.2. Long-term potentiation

To address whether this customized system, upgraded with the PPS, the two stacked grids and the pattern generator, allows investigations of synaptic plasticity, LTP was generated in the CA1 region of rat hippocampal slices. As a baseline recording, fEPSPs were elicited at an intensity evoking 20–30% of the maximum response for one hour. Subsequently, different stimulation protocols created by the pattern generator were used to induce LTP: a weak theta burst stimulation, a strong theta burst stimulation, a weak high frequency stimulation, an intermediate high frequency stimulation, a strong high frequency stimulations and a repeated high frequency stimulation. All stimulation protocols used were found to potentiate fEPSP (Fig. 21). After weak theta burst stimulation, synaptic transmission increased up to a maximum of 150% and lasted about one hour (early phase LTP; see Reymann and Frey, 2007) (Fig. 21A). Strong theta burst stimulation potentiated fEPSPs up to a maximum of 200% lasting about 3.5 hours (late phase LTP; see Reymann and Frey, 2007) (Fig. 21B). Synaptic transmission was enhanced to a maximum of 180% lasting about two hours after weak high frequency stimulation (early phase LTP) (Fig. 21C). Intermediate high frequency stimulation induced fEPSPs potentiation of a maximal effect of 190% lasting for about three hours (presumably late phase LTP) (Fig. 21D). The conventional strong high frequency stimulation caused an enhancement of synaptic transmission up to 200% lasting longer than four hours (late phase LTP) (Fig. 21E). The strongest fEPSP potentiation was generated by the repeated high frequency stimulation having a maximal effect of 300% and lasting longer than seven hours (late phase LTP) (Fig. 21F). For all protocols used, only little variability in the fEPSPs was observed. In summary, very robust LTP may be induced by stimulation with a specific pattern created by the pattern generator and maintained using the PPS in combination with the two stacked grids.

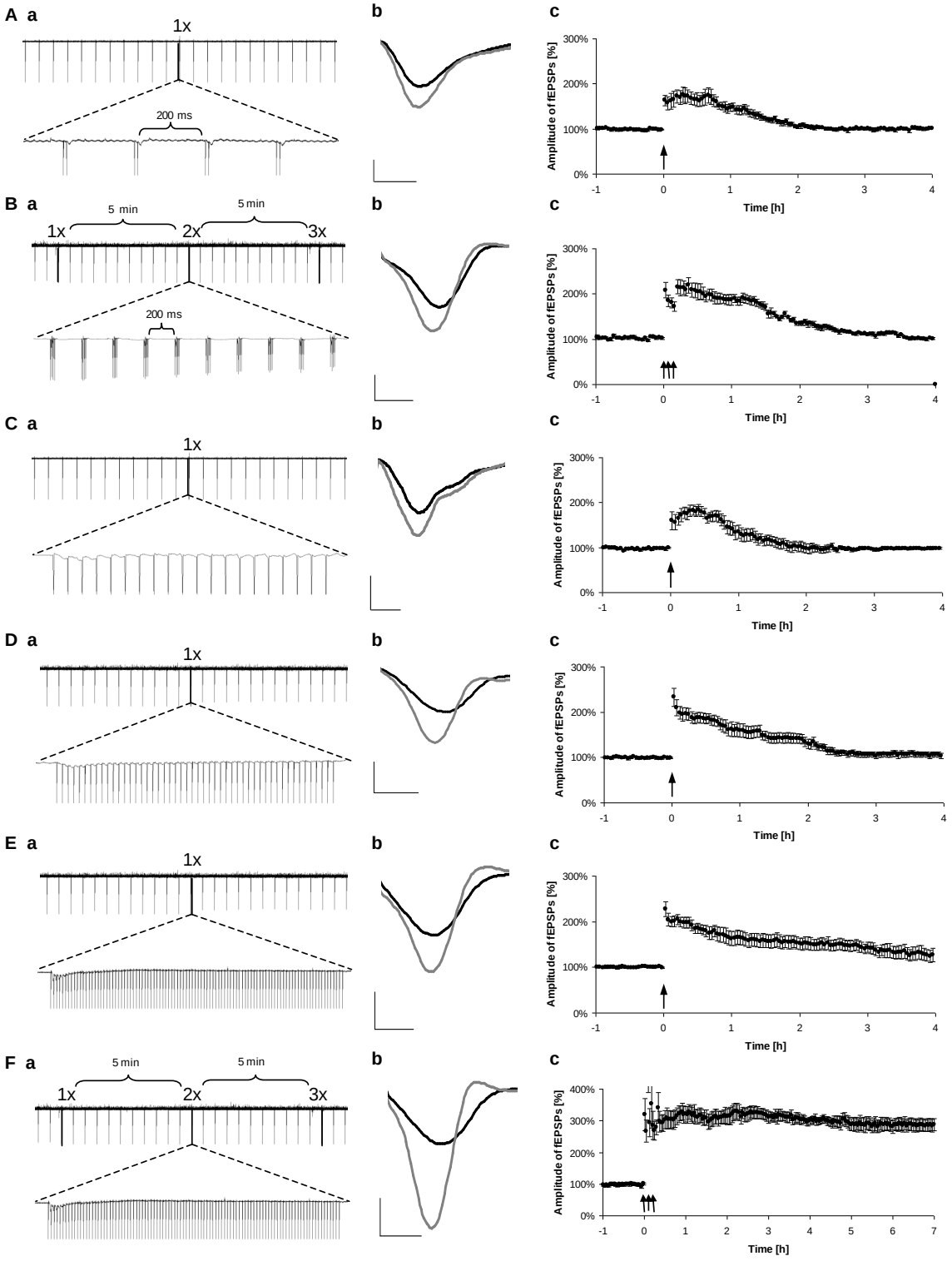


Figure 21: Robust LTP measurements.

Different LTP recordings are shown generated by different stimulation protocols in the CA1 region of rat hippocampal slices. Pictures (a) show the individual stimulation protocols used to induce LTP, (b) show individual fEPSPs before (black) and one after (grey) stimulation by each protocol and (c) show the LTP induced by each protocol (the arrow/s indicate/s the time of the LTP stimulation). (A) Early phase LTP induced by a weak theta burst stimulation (4 paired pulses with an interpulse interval of 10 ms that were applied at the theta frequency of 5 Hz). 5 slices (n = 5) were recorded for 5 h. (B) Late

continued:
phase LTP induced by a strong theta burst stimulation (10 times 4 pulses with an interpulse interval of 10 ms and an interval between bursts of 200 ms, repeated 2 times in 10 min intervals). 5 slices (n = 5) were recorded for 5 h. (C) Early phase LTP induced by a weak high frequency stimulation (20 pulses at the frequency of 100 Hz). 5 slices (n = 5) were recorded for 5 h. (D) Presumably late phase LTP induced by an intermediate high frequency stimulation (50 pulses at the frequency of 100 Hz). 5 slices (n = 5) were recorded for 5 h. (E) Late phase LTP induced by the conventional strong high frequency stimulation (100 pulses at the frequency of 100 Hz). 5 slices (n = 5) were recorded for 5 h. (F) Late phase LTP induced by a repeated high frequency stimulation (100 pulses at the frequency of 100 Hz, repeated 2 times in 5 min intervals). 5 slices (n = 5) were measured for 8 h.

2.4. Discussion

In this study, we describe the modification of a multi-slice recording system that allows absolutely stable measurements of fEPSPs, enabling recording of LTP for up to seven hours in up to eight slices simultaneously. This system overcomes the main hurdles for efficient routine LTP measurements: (1) system instability, (2) low throughput and (3) incomplete use of slices per animal.

A multi-slice recording system (SliceMaster, Scientifica Limited, East Sussex, UK) was modified in several ways. Firstly, the software Notocord® was employed. So far, in combination with the SliceMaster only Spike2 (Stopps et al., 2004; West et al., 2009; www.ced.co.uk) had been used. Among these software other programs can be utilized for LTP experiments (for an overview see Bortolotto et al., 2001) including the ones which need no programming - for example Molecular Devices pClamp (www.moleculardevices.com/pages/software/pclamp.html), Heka's Patch-Master (www.heka.com/physio/acquisition/patchmaster.html), AxoGraph (www.axographx.com), NClamp (/www.neuromatic.thinkrandom.com), Theta Burst's NAC Gather (www.thetaburst.com), the Strathclyde Electrophysiology Software program WinWCP (spider.science.strath.ac.uk/sipbs/page.php?show=software_winWCP), the LTP Program (Anderson and Collingridge, 2001; www.winltp.com/Ltp24/indexLtp24.htm) and WinLTP (Anderson and Collingridge, 2007; www.winltp.com) - and the ones which are customized - for example written with Wave-Metrics' Igor (www.wavemetrics.com) or National Instruments' LabView (www.ni.com). In this study, Notocord® was used, since it may be individually configured to acquire a multitude of biological signals, analyze them and create a report quickly. Furthermore, it is often employed in industry, because it is able to generate a protocol of the users' actions. In this report, Notocord® was not only used to visualize generated data files on-line or later, but also to control a digital pattern generator which generates different patterns for slice stimulation inducing different types of LTP. Secondly, a Pumped Perfusion System (PPS) was used. In contrast to gravity-fed systems (Stopps et al., 2004; Easter et al., 2007), where flow is a subject to changes in solution height, the PPS controls the flow precisely over the whole course of the experiment. The very low noise electronics of the PPS allows its placing inside the Faraday cage and hence results in a reduction of solution dead volumes. Additionally, the PPS can be used to recycle drug solutions minimizing amount of drugs applied to the slices. Thirdly, the original single grids in the isolated slice chambers were replaced by two stacked grids. The

two stacked grids were shown not to delay the pharmacological effect of CNQX and make the flow of ACSF very stable, hence allowing fEPSP recordings for several hours without any perturbances. This stability enables very robust LTP measurements for up to seven hours depending on the stimulation protocol used. Furthermore, it allows detection of a statistically significant differences of 20% between two groups using only six slices (*n* per group = 6), whereas as described in literature an average of ten slices (*n* per group = 10) is necessary (Schröder et al., 2008; van der Staay et al., 2008; Rosenbrock et al., 2010).

This modified multi-slice system not only allows measuring LTP for up to seven hours and improves statistical design, but also increases throughput and the rate of used slices per animal. The throughput is increased as up to eight slices per animal may be studied simultaneously permitting numerous controls or drug treatments to be performed in one experimental run. Conventional brain slice systems only permit recording from one or two brain slices at a time (Haas et al., 1979), even though it is often possible to prepare eight hippocampal slices (of 400 μm thickness) from a single rat. Hence, six or seven slices are not used for experiments. Since eight slices can be studied simultaneously with the multi-slice system, the number of unused slices is decreased and thereby the number of animals necessary to conduct a certain number of experiments is reduced. Therefore, this modified system helps researchers comply with the "three Rs" (Flecknell, 2002), requested by many sides with increasing intensity.

In conclusion, as the modified multi-slice system used in this study allows LTP recordings for up to seven hours in up to eight slices simultaneously, it is an attractive approach for routine late phase LTP measurements when high statistical power is needed. Thus, this system is both suitable to investigate molecular mechanisms underlying the late phase of LTP and it can be utilized to screen candidate compounds in the context of drug discovery.

CHAPTER 3

Validation of early and late LTP and the effect of the mGlu5 receptor positive allosteric modulator ADX-47273

Abstract

Conflicting findings are reported in the literature about the involvement of the mGlu5 receptor in hippocampal long-term potentiation (LTP), which might be a consequence of different sub-types of LTP induced by the investigators due to the specific experimental conditions used. A comparable controversy came up in the past concerning the influence of different experimental conditions on the involvement of L-type voltage dependent calcium channels (L-VDCCs) and NMDA receptors in hippocampal LTP. In this study, two stimulation protocols under identical conditions were used to probe modulatory effects of mGlu5 receptor activation in NMDA receptor and L-VDCCs dependent CA1 LTP: weak high frequency stimulation (20 stimuli at 100 Hz) to induce early LTP and repeated strong high frequency stimulation (3 times 100 stimuli at 100 Hz with 5 minutes interval) to induce late LTP, which - in contrast to early LTP - was shown to be protein-synthesis dependent. Using the NMDA receptor antagonist MK-801 and the L-type calcium channel blocker nifedipine, early LTP was shown to be dependent on NMDA receptors only, whereas late LTP was demonstrated to be dependent on NMDA receptors and L-VDCCs in about equal parts. Moreover, late LTP, but not early LTP, was increased by the mGlu5 receptor positive allosteric modulator ADX-47273, indicating that artificial augmentation of mGlu5 receptor activation by endogenous glutamate may boost the protein-synthesis dependent form of LTP but not the protein-synthesis independent form.

3.1. Introduction

Metabotropic glutamate 5 (mGlu5) receptors are expressed throughout the CNS including the cortex and the hippocampus (Abe et al., 1992; Kerner et al., 1997; Lujan et al., 1996; Mannaioni et al., 2001; Shigemoto et al., 1997; Spooren et al., 2003), brain structures known to be intrinsically involved in learning and memory. The role of the mGlu5 receptor in learning and memory has been investigated in several studies addressing long-term potentiation (LTP) of synaptic transmission (Anwyl, 2009), which is a fundamental experimental model of mammalian learning and memory (Bliss and Collingridge, 1993). However, conflicting reports exist about the role of the mGlu5 receptor in hippocampal LTP even to the point that some studies indicate that mGlu5 receptors are not important for LTP at all (Anwyl, 1999). For example, studies in mutant mice showing that CA1 LTP could be readily induced in mGlu5 receptor (−/+), mGlu5 receptor (−/−) or mGlu1/mGlu5 receptor double knockout mice (Bortolotto et al., 2005). In contrast, other studies have demonstrated a prominent role of the mGlu5 receptor in LTP. Indeed, the mGlu5 receptor antagonist MPEP was found to block LTP in the CA1 region of hippocampal slices (Francesconi et al., 2004; Shalin et al., 2006) as well as to lead to an impairment in hippocampal LTP *in vivo* (Manahan-Vaughan and Braunewell, 2005), whereas the mGlu1/5 receptor agonist DHPG was shown to facilitate induction of CA1 LTP (Cohen et al., 1998; Raymond et al., 2000). Likewise, mGlu5 receptor knockout mice showed an impairment of LTP in the CA1 region and exhibited deficits in memory tasks (Jia et al., 1998; Lu et al., 1997). It was suggested that these apparently controversial findings regarding mGlu5 receptor's involvement in hippocampal CA1 LTP were due to the examination of different types of LTP employed by the experimenters (Anwyl, 2009). Interestingly, in the past a similar reasoning was made regarding the role of L-type voltage dependent calcium channels (L-VDCCs) and NMDARs in LTP (Grover and Teyler, 1990; Morgan and Teyler, 2001).

In the CA1 region of the hippocampus, a general differentiation can be made between an early phase of LTP ('early LTP') being induced by a 'weak' stimulation and a late phase of LTP ('late LTP') being induced by a 'strong' stimulation (Albensi et al., 2007). Early LTP has been found to be independent of protein-synthesis, lasts between one and three hours and it was shown to rely on post-translational modifications of preexisting proteins (Huang, 1998; Reymann and Frey, 2007). Late LTP, on the other hand, has been found to be protein-synthesis dependent as demonstrated by use of inhibitors of protein transcription

as well as translation. This type of LTP lasts longer than three hours (Huang, 1998; Reymann and Frey, 2007). However, in late LTP protein-synthesis already contributes to potentiation at a point in time at which protein-synthesis independent potentiation is still present in an early LTP paradigm (Frey et al., 1988; Krug et al., 1984).

Clearly defined and validated protocols for the induction of early and late LTP on a single experimental platform under identical conditions are essential to test whether a certain receptor might have differential roles concerning the two types of LTP. Therefore, two protocols were examined in detail: weak high frequency stimulation (weak HFS; 20 stimuli at 100 Hz) to induce early LTP and repeated strong high frequency stimulation (repeated strong HFS; 3 times 100 stimuli at 100 Hz with 5 minutes interval) to induce late LTP. We analyzed not only the duration and the protein-synthesis dependency, but also examined the role of NMDA receptors and L-VDCCs on early and late LTP using their specific antagonists MK-801 and nifedipine, respectively. The mGlu5 receptor positive allosteric modulator ADX-47273 was used to test whether artificial augmentation of mGlu5 receptor activation by endogenous glutamate during LTP induction may influence the expression of the two basic forms of CA1 LTP.

3.2. Materials and methods

3.2.1. Preparation of brain slices

Procedures involving animals and their care were conducted in conformity with institutional and European Union guidelines (EEC Council Directive 86/609) and were approved by the Ethical Committee of the responsible regional council (Tübingen). Male Wistar rats (Janvier, Le Genest Saint Isle, France) aged six to seven weeks were shortly anaesthetized with isoflurane and sacrificed by decapitation. The brains were quickly removed and immersed in ice-cold ACSF (containing in mM NaCl 124, KCl 4.9, $MgSO_4$ 8, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25.6, D-glucose 10, pH 7.4, saturated with 95% O_2 and 5% CO_2). Transverse hippocampal brain slices (400 μm thickness) were cut using a Vibratome. Slices were allowed to recover in a holding chamber containing ACSF bubbled with 95% O_2 and 5% CO_2 at room temperature for at least one hour. Slices were then transferred to integrated brain slice chambers and continuously superfused (at a flow rate of 2.5 mL min^{-1}, 25 ± 0.2 °C) with the same ACSF composition used before except for 0.5 mM $MgSO_4$. Prior to doing any electrophysiological recordings, the slices were allowed to equilibrate for at least 30 minutes.

3.2.2. Multi brain slice recording

The SliceMaster multi-slice recording system (Scientifica Limited, East Sussex, UK) was used to record field excitatory postsynaptic potentials (fEPSPs). The system is semi-automated allowing one operator to record simultaneously from an assembly of up to eight brain slices. For the present study, an optimized system described by Kroker et al. (2011) was used which enables absolutely stable fEPSPs measurements for up to eight hours. Briefly, the SliceMaster system consists of three temperature-controlled integrated brain slice chambers and can be upgraded with five more. Each chamber is equipped with one stimulation electrode, one recording electrode and a camera system allowing a visualization of each brain slice on a shared video monitor. A central control panel makes the positioning of all electrodes and cameras possible, allowing a single operator to run all experiments simultaneously. A separate inflow using a Pumped Perfusion System (Scientifica Limited, East Sussex, UK) and separate outflow for each integrated brain slice

chamber allows individual perfusion of each brain slice. To minimize movement of the slices, stacked grids were integrated in each brain slice chamber.

fEPSPs were elicited in the CA1 region by stimulation of the Schaffer collateral–commissural fibres in the stratum radiatum using glass electrodes with broken tips (filled with ACSF). For recording, glass electrodes (2–6 MΩ, filled with ACSF) were placed in the apical dendritic layer. The amplitudes of fEPSPs were used as the parameter of interest (Collingridge et al., 1983). To generate fEPSPs at a constant sub-threshold stimulus, as in previous studies (Seabrook et al., 1997), the stimulus strength of the pulses was adjusted to 20–30% of the fEPSP maximum and this voltage was used for the experiment. During baseline recording each slice was stimulated every 30 seconds for at least one hour. Early LTP was induced by weak HFS made up of 20 pulses at the frequency of 100 Hz (Bashir et al., 1991; Kroker et al., 2011), whereas late LTP was induced by repeated strong HFS consistent of 100 pulses at the frequency of 100 Hz, repeated two times in 5 minutes intervals (Kroker et al., 2011; Lu et al., 1999).

3.2.3. Data acquisition, software and analysis

A modular electrophysiology system, supplied by npi electronic GmbH (Tamm, Germany), conducts the low noise recordings of extracellular signals. AC coupled signals are amplified 1000-fold and internally filtered with a five kHz low-pass filter as well as a three Hz high-pass filter. In our study, a customized system (programmable pattern generator) was used allowing simultaneous stimulation of all slices either with a single stimulus or a specific stimulus pattern (Kroker et al., 2011). For data acquisition and analysis the software Notocord® was used.

Data are shown as mean percent (± SEM) of the baseline fEPSP amplitude. Data were analyzed using either *t* tests to compare two conditions or one-way ANOVA (post-hoc test: Dunnett-Test) to compare multiple conditions. In both cases a p-value of ≤ 0.05 was considered significant. Histograms show the mean amplitude (± SEM) of EPSPs measured between 50 and 60 minutes after weak or repeated strong HFS according to Jia et al. (2010).

3.2.4. Drug solutions and application

The following drugs were used: 6-Cyano-7-nitroquinoxaline-2,3-dione (= CNQX), (5S,10R)-(–)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate (= MK-801), (2R,3S,4S)-2-(4-Methoxybenzyl)-3,4-pyrrolidinediol-3-acetate, 2-[(4-Methoxyphenyl)methyl]-3,4-pyrrolidinediol 3-acetate (= anisomycin) and 1,4-Dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester (= nifedipine) were purchased from Sigma-Aldrich Corporation (St. Louis, USA). The compound S-(4-fluoro-phenyl)-{3-[3-8fluorophenyl)-[1,2,4]-oxadiazol-5-yl]-piperidin-1-yl}-methanone (= ADX-47273) was synthesized in the Department of Chemical Research of Boehringer Ingelheim Pharma GmbH & Co KG.

The drugs were prepared as stock solutions and diluted in ACSF immediately before application. Nifedipine and ADX-47273 were prepared in DMSO (the final concentration of DMSO was 0.1%). All drugs, except MK-801, were applied 30 minutes before LTP stimulation and remained until 30 minutes after LTP stimulation. MK-801 was used in such a way that, corresponding to Frankiewicz et al. (1996), true equilibrium blockade of NMDA receptors can be expected: MK-801 was present immediately after preparing slices, thereafter for an average of 6.8 ± 0.4 h prior to LTP stimulation and remained until 30 minutes after LTP stimulation.

3.3. Results

3.3.1 fEPSPs, duration and protein-synthesis dependency of early and late LTP

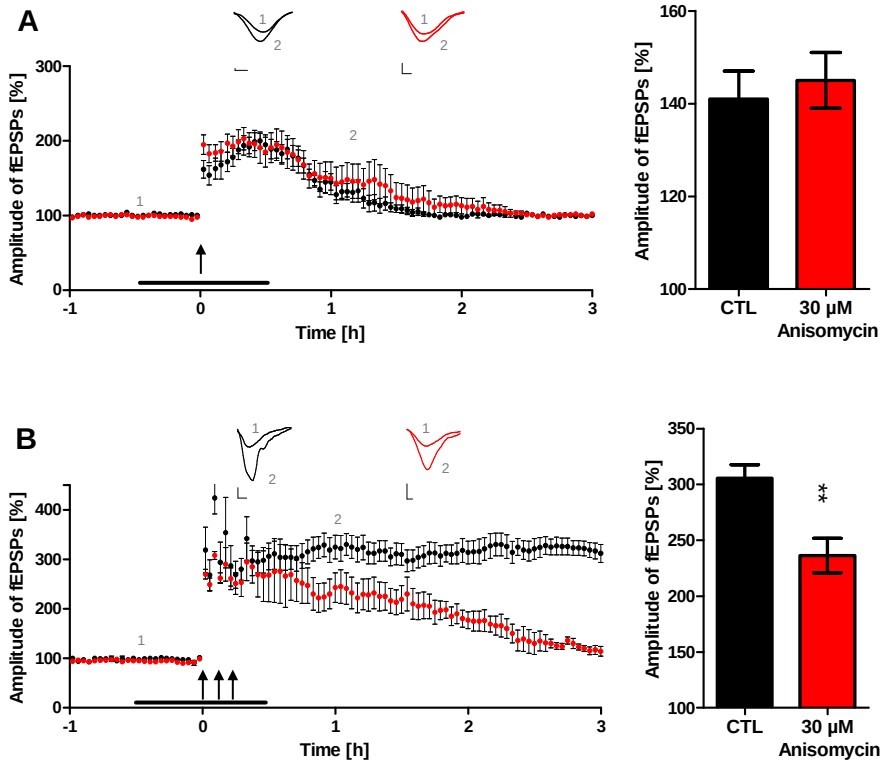


Figure 22: Duration and protein-dependency of LTP induced by weak HFS and repeated strong HFS.

Duration and protein-synthesis dependency of LTP induced by two different protocols: weak HFS (20 stimuli at 100 Hz) and repeated strong HFS (3 times 100 stimuli at 100 Hz with 5 min interval). Anisomycin (30 µM), a protein-synthesis inhibitor, was applied 30 min before LTP stimulation and remained until 30 min after LTP stimulation (= bar). Individual fEPSPs before (1) and after LTP stimulation (2) are shown (horizontal bar: 0.5 mV, vertical bar: 10 ms). Histograms show the mean amplitude (± SEM) of fEPSPs measured between 50 and 60 min after HFS. A) LTP induced by weak HFS (= arrow) lasts about two hours and is not affected by anisomycin. B) LTP induced by repeated strong HFS (= arrows) lasts up to 7 h and is significantly reduced and finally completely abolished by anisomycin.

In this setting, fEPSPs were found to be not affected by APV (30 µM; Huang and Malenka, 1993), a selective NMDA receptor antagonist, but to be completely abolished by CNQX (10 µM; Takeda et al., 2007), a competitive AMPA glutamate receptor antagonist

(data not shown). Thus, AMPA receptors are exclusively responsible for the basal fEPSPs generated in this setting.

LTP induced by weak HFS lasts about one and a half to two hours (Fig. 22A), whereas LTP induced by repeated strong HFS lasts longer than three hours (Fig. 22B). The classification of LTP into different phases is not sufficiently clear based on the duration only, but also on protein-synthesis dependency. Therefore, the effect of anisomycin, a reversible protein translation inhibitor, was analyzed in both settings. Anisomycin was used at a concentration of 30 µM, a concentration reported to be sufficient to significantly impair protein-synthesis (Lu et al., 1999). It had no effect on LTP induced by weak HFS (= early LTP; Fig. 22A) (one hour after weak HFS; control: 141 ± 3%, n = 10; anisomycin: 145 ± 3%, n = 5, not significant (ns)). In contrast, LTP induced by repeated strong HFS was significantly decreased up to a complete abolishment (= late LTP; Fig. 22B) (one hour after repeated strong HFS; control: 306 ± 10%, n = 10; anisomycin: 236 ± 11%, n = 5, $p < 0.01$).

3.3.2 Involvement of NMDA receptors and L-VDCCs in the induction of early and late LTP

To assess the role of NMDA receptors in early LTP generated by weak HFS, the effect of MK-801, a potent antagonist of NMDA receptors, was analyzed (Fig. 23A). Early LTP was decreased by MK-801 (0.1 – 100 µM) in a concentration dependent manner with a complete blockade at a concentration of 10 µM (one hour after weak HFS; control: 138 ± 6%, n = 10; 0.1 µM MK-801: 136 ± 7%, n = 5, ns; 1 µM MK-801: 113 ± 5%, n = 5, $p < 0.01$; 10 µM MK-801: 100 ± 2%, n = 5, $p < 0.001$; 100 µM MK-801: 100 ± 1%, n = 5, $p < 0.001$). To assess the role of L-VDCCs in LTP, the L-type calcium channel blocker nifedipine (Grover and Teyler, 1990) was applied at different concentrations (0.1 - 500 µM). As shown in Fig. 23B, nifedipine had no effect on early LTP (one hour after weak HFS; control: 137 ± 5%, n = 10; 0.1 µM nifedipine: 137 ± 3%, n = 5, ns; 1 µM nifedipine: 140 ± 5%, n = 5, ns; 10 µM nifedipine: 139 ± 3%, n = 5, ns; 100 µM nifedipine: 138 ± 5%, n = 5, ns; 500 µM nifedipine: 139 ± 4%, n = 5, ns).

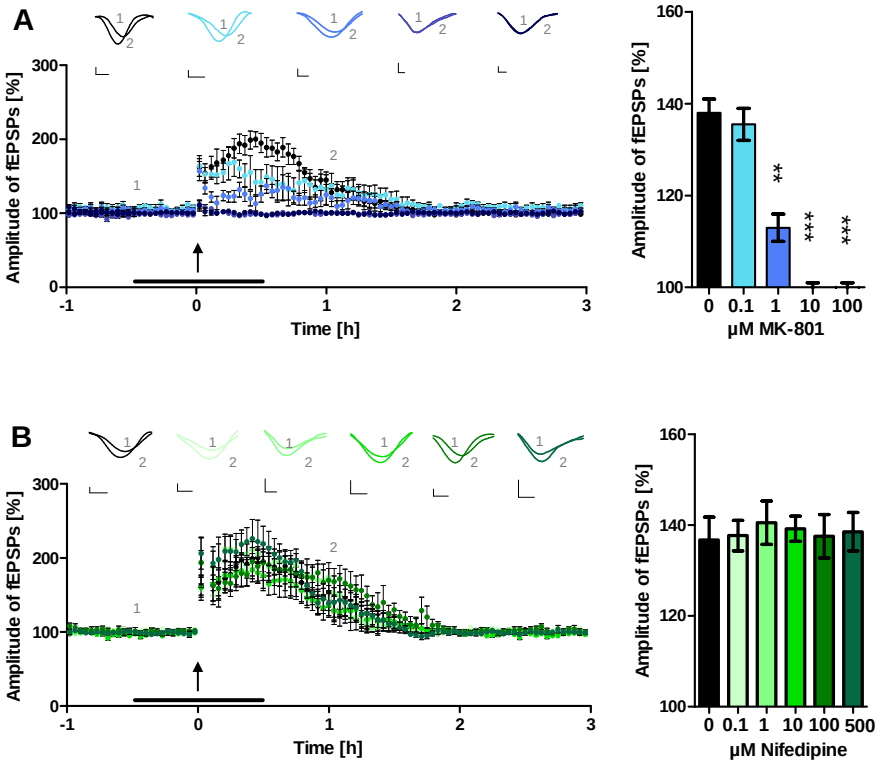


Figure 23: Role of NMDA receptor and L-VDCC in early LTP.
Effects of MK-801, a potent antagonist of NMDA receptors, and nifedipine, an L-type calcium channel blocker, on early LTP induced by weak HFS. A) Early LTP was reduced by MK-801 in a concentration dependent manner with a complete abolishment at 10 µM. MK-801 (0.1 – 100 µM) was applied 6.8 ± 0.4 h before LTP stimulation (= arrow) and was washed out 30 min after LTP stimulation (= bar). Individual fEPSPs before (1) and after LTP stimulation (2) are shown (horizontal bar: 0.5 mV, vertical bar: 10 ms). Histogram shows the (mean ± SEM) amplitude of fEPSPs measured 50–60 min after delivery of LTP stimulation. B) Nifedipine had no effect on early LTP. Nifedipine (0.1 – 500 µM) was applied 30 min before LTP stimulation (= arrow) and remained until 30 min after LTP stimulation (= bar). Histogram shows the mean amplitude (± SEM) of EPSPs measured between 50 and 60 min after HFS.

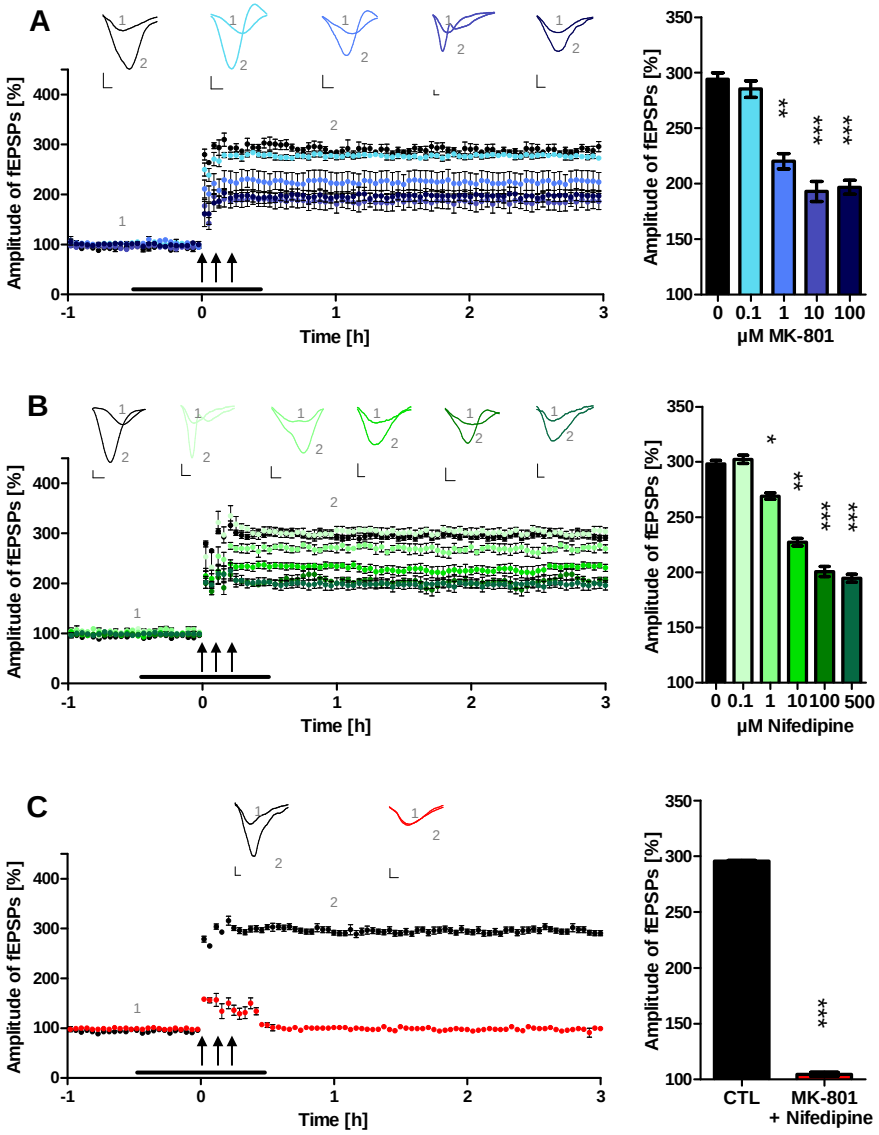


Figure 24: Role of NMDA receptor and L-VDCC in late LTP.
Effects of MK-801, a potent antagonist of NMDA receptors, and nifedipine, an L-type calcium channel blocker, on late LTP induced by repeated strong HFS. A) Late LTP was reduced by MK-801 in a concentration dependent manner with maximum effect at 10 µM. At this concentration late LTP was reduced by approximately 50%. MK-801 (0.1 – 100 µM) was applied 6.8 ± 0.4 h before LTP stimulation (= arrows) and was washed out 30 min after LTP stimulation (= bar). Histogram shows the mean amplitude (± SEM) of fEPSPs measured between 50 and 60 min after HFS. B) Nifedipine reduced late LTP in a concentration dependet manner. The maximal effect of nifedipine on late LTP was reached at 100 µM causing a reduction by approximately 50%. Nifedipine (0.1 – 500 µM) was applied 30 min before LTP stimulation (= arrows) and was washed out 30 min after LTP stimulation (= bar). Individual fEPSPs before (1) and after LTP stimulation (2) are shown (horizontal bar: 0.5 mV, vertical bar: 10 ms). Histogram shows the mean amplitude (± SEM) of fEPSPs measured between 50 and 60 min after HFS. C) A mixture of MK-801 (10 µM) and nifedipine (100 µM) completely abolished late LTP. MK-801 (0.1 – 100 µM) was applied 6.8 ± 0.4 h before LTP stimulation (= arrows) and nifedipine (0.1 – 500 µM) was applied 30 min before LTP stimulation (= arrows). Both substances remained until 30 min after LTP stimulation (= bars). Histogram shows the mean amplitude (± SEM) of fEPSPs measured between 50 and 60 min after HFS.

To assess the role of NMDA receptors in late LTP generated by repeated strong HFS, the effects of different concentrations of MK-801 were analyzed (0.1 – 100 µM; Fig. 24A). Late LTP was decreased by MK-801 in a concentration dependent manner with a maximum effect at a concentration of 10 µM (one hour after repeated strong HFS; control: 297 ± 5%, n = 10; 0.1 µM MK-801: 289 ± 6%, n = 5, ns; 1 µM MK-801: 220 ± 7%, n = 5, $p < 0.01$; 10 µM MK-801: 193 ± 9%, n = 5, $p < 0.001$; 100 µM MK-801: 196 ± 7%, n = 5, $p < 0.001$). At this concentration late LTP was reduced by approximately 50%. The L-type calcium channel blocker nifedipine (0.1 - 500 µM; Fig. 24B) reduced late LTP in a concentration dependent manner (one hour after repeated strong HFS; control: 298 ± 6%, n = 10; 0.1 µM nifedipine: 302 ± 7%, n = 5, ns; 1 µM nifedipine: 269 ± 10%, n = 5, $p < 0.05$; 10 µM nifedipine: 227 ± 7%, n = 5, $p < 0.01$; 100 µM nifedipine: 201 ± 8%, n = 5, $p < 0.001$; 500 µM nifedipine: 195 ± 7%, n = 5, $p < 0.001$). The maximal effect of nifedipine on late LTP was reached at 100 µM resulting in approximately 50% reduction. A combination of maximally effective concentrations of MK-801 (10 µM) and nifedipine (100 µM) completely abolished late LTP (Fig. 24C) (one hour after repeated strong HFS; control: 297 ± 6%, n = 5; MK-801 + nifedipine: 100 ± 1%, n = 5, $p < 0.001$). Neither MK-801 nor nifedipine nor the combination of both affected basal fEPSPs.

3.3.3. The effect of the mGlu5 receptor positive allosteric modulator ADX-47273 on early and late LTP.

The effect of ADX-47273 was analyzed on early and late LTP at different concentrations (0.03, 0.3 and 3 µM). None of the tested concentrations of ADX-47273 showed a significant effect on early LTP (Fig. 25A) (one hour weak HFS; control: 144 ± 4%, n = 10; 0.03 µM ADX-47273: 142 ± 5%, n = 5, ns; 0.3 µM ADX-47273: 143 ± 5%, n = 5, ns; 0.3 µM ADX-47273: 145 ± 5%, n = 5, ns). In contrast, the compound showed a bell-shaped concentration–response relation with a significant enhancement of late LTP at 0.3 µM (Fig. 25B) (one hour after repeated strong HFS; control: 297 ± 6%, n = 10; 0.03 µM ADX-47273: 300 ± 8%, n = 5, ns; 0.3 µM ADX-47273: 337 ± 9%, n = 5, $p < 0.05$; 3 µM ADX-47273: 294 ± 9%, n = 5, ns). Basal fEPSPs were not affected by any concentration of ADX-47273.

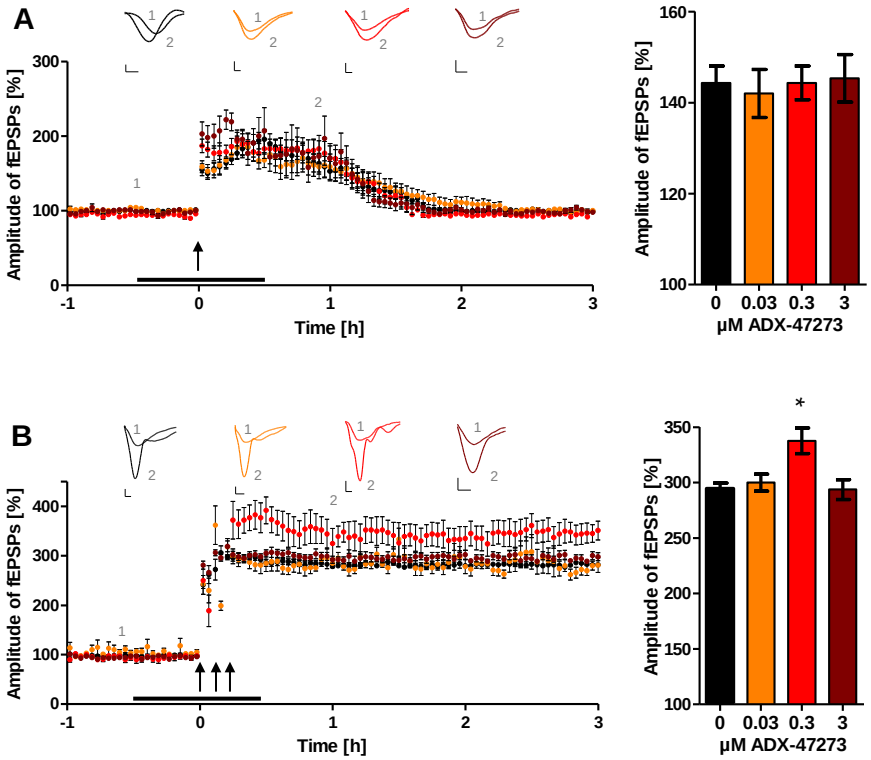


Figure 25: Effect of ADX-47273 on early and late LTP.

Effect of ADX-47273, an mGlu5 receptor positive allosteric modulator, on early LTP induced by weak HFS and late LTP induced by repeated strong HFS. ADX-47273 (0.03 – 3 µM) was applied 30 min before LTP stimulation (= arrow/s) and was washed out 30 min after LTP stimulation (= ar). Individual fEPSPs before (1) and after LTP stimulation (2) are shown (horizontal bar: 0.5 mV, vertical bar: 10 ms). Histograms show the mean amplitude (± SEM) of fEPSPs measured between 50 and 60 min after HFS. A) None of the tested concentrations of ADX-47273 showed a significant effect on early LTP. B) Late LTP was enhanced in a bell-shaped concentration dependent manner by ADX-47273.

3.4. Discussion

In vitro LTP measurements represent a fundamental experimental model of mammalian learning and memory (Bliss and Collingridge, 1993) and have been widely used for decades, however, certain limitations apply (Eichenbaum, 1996). Traditionally, different labs employ slightly different conditions, implying limited comparability. However, qualitative mechanistic statements usually have general validity. As it is known that the details of the experimental conditions in LTP measurements are important (Larkman and Jack, 1995), we made a point of setting up a standardized LTP setup (Kroker et al., 2011) with the stimulation protocol being the only variable parameter. Thus, the results obtained in this study are perfectly comparable, whereas alignment with other studies is warranted only after careful comparison of experimental conditions.

3.4.1. Duration and protein-synthesis dependency of early and late LTP

In the present work, the capabilities of two different stimulation protocols to induce early and late CA1 LTP were analyzed and validated by investigating the duration and protein-synthesis dependency of both types of LTP. To analyze protein-synthesis dependency, anisomycin was used, because it is known to successfully block late LTP (Ahmed and Frey, 2005; Frey et al., 1988; Krug et al., 1984; Lu et al., 1999; Mullany and Lynch, 1997; Reymann and Frey, 2007). LTP induced by weak HFS lasted about two hours and was demonstrated not to be affected by anisomycin. In contrast, LTP induced by repeated strong HFS lasted longer than three hours and was completely abolished within three hours by anisomycin. Thus, LTP induced by weak HFS can be considered to be early LTP, whereas LTP induced by repeated strong HFS is late LTP by definition. The discrimination between early and late LTP by protein-synthesis dependency, as it is made here, is commonly used and is based on the need for *de novo* protein-synthesis to establish LTP that lasts longer than a few hours (Frey et al., 1988; Huang, 1998; Huang and Kandel, 1994; Huang et al., 1996; Nguyen et al., 1994; Nguyen and Kandel, 1997; Reymann and Frey, 2007; Stanton and Sarvey, 1984). It is postulated that early LTP, which lasts for up to maximum three hours, relies only on post-translational modifications of pre-existing proteins, whereas late LTP is believed to depend on protein-synthesis triggered by the LTP-inducing stimulation. Besides the commonly used differentiation between early and

late LTP, a further categorization can be found in literature describing LTP1, LTP2 and LTP3 (Raymond, 2007). Thereby, LTP1 is considered to be equivalent to early LTP, in that it is rapidly decaying, protein-synthesis independent and probably involves post-translational modifications of various synaptic proteins. LTP2 is considered to be an intermediate phase of late LTP that requires protein-synthesis but is independent of gene transcription. Finally, LTP3 represents the durable, translation- and transcription-dependent component of late LTP, for which L-VDCCs play an important role besides the NMDA receptors. Using this definition, the LTP induced by weak HFS can also be classified as LTP1, whereas LTP induced by repeated strong HFS is considered to be LTP3.

3.4.2 NMDA receptors and L-VDCCs involved in the induction of early and late LTP

For decades in LTP research, there was a strong emphasis on the role of NMDA receptors in LTP. In this study, we show that different protocols induce not only different types of LTP concerning their duration and protein-synthesis dependency, but also regarding involvement of NMDA receptors and L-VDCCs. Early LTP induced by weak HFS is shown to be dependent on NMDA receptors only. These results are in agreement with many studies demonstrating that LTP is dependent on activation of NMDA receptors (Collingridge et al., 1983; Harris et al., 1984; Collingridge, 2003). Late LTP induced by repeated strong HFS is demonstrated to be equally dependent on NMDA receptors and L-VDCCs. This result confirms the very few studies on calcium channels showing that, depending on the stimulation protocols, not only NMDA receptors, but also L-VDCCs can play an important role in LTP (Cavus and Teyler, 1996; Freir and Harron, 2003; Grover and Teyler, 1990; Morgan and Teyler, 1999, 2001). Since hippocampal pyramidal neurons express Cav1.2 as well as Cav1.3 channels (Davare et al., 2001; Hell et al., 1993), but only a knock-out mouse model lacking the Cav1.2 channel showed deficits in hippocampal LTP and hippocampus-dependent learning tasks (Clark et al., 2003; Moosmang et al., 2005), it is assumed that Cav1.2 channels are the L-VDCCs involved in LTP. Interestingly, Shankar et al. (1998) found that LTP induced in young rats was largely NMDA receptor dependent while LTP in slices of aged animals was more dependent on L-VDCC activation.

Furthermore, it seems that NMDA receptor and L-VDCC driven LTP are two different processes that involve distinct molecular downstream mechanisms (Cavus and Teyler 1996; Bayazitov et al., 2007; Moosmang et al., 2005; Zakharenko et al., 2003). The presence of two forms of LTP in hippocampal slices should not be considered to be a marginal phenomenon; rather it has important behavioral consequences: blockade of either NMDA receptors or L-VDCC driven LTP impairs spatial learning in animals (Borroni et al., 2000; Moosmang et al., 2005; Morris et al., 1986; Tsien et al., 1996). Thus, both forms of LTP are important for learning and memory.

3.4.3 The effect of the mGlu5 receptor positive allosteric modulator ADX-47273 on early and late LTP

To elucidate the potential for mGlu5 receptor modulation in LTP (Bortolotto et al., 2005; Fitzjohn et al., 1998; Francesconi et al., 2004; Jia et al., 1998; Lu et al., 1997; Manahan-Vaughan and Braunewell, 2005; Raymond et al., 2000; Rush et al., 2002; Shalin et al., 2006), we decided to reinvestigate this issue by examining the effect of the mGlu5 receptor positive allosteric modulator ADX-47273 on our early and late CA1 LTP settings. The use of selective allosteric modulators may help defining the physiological role and therapeutic potential of mGlu receptors in neurological disorders (Kew, 2004; Kew and Kemp, 2005; Marino and Conn, 2006; Nicoletti et al., 2010; Ritzen et al., 2005). mGlu5 receptor positive allosteric modulators do not activate mGlu5 receptors directly but act at an allosteric site to potentiate their (endogenous) activation by glutamate (Chen and Conn, 2008; Conn et al., 2009a). ADX-47273 is described as a potent mGlu5 receptor positive allosteric modulator *in vivo* and *in vitro* (Ayala et al., 2009; Le Poul et al., 2005; Liu et al., 2008; Rosenbrock et al., 2010). The findings in this work demonstrate that late LTP induced by repeated strong HFS is increased in a bell-shaped manner by ADX-47273 which is in good agreement with Rosenbrock et al., 2010. The lack of effectiveness at high concentrations might result from activation of compensatory mechanisms leading to alleviation of LTP enhancement. Here, we analyzed the effect of ADX-47273 on early LTP. Surprisingly, ADX-47273 has no effect on LTP induced by weak HFS.

In general, there exist multiple factors controlling the reliable induction of early and late LTP, e.g. the age, strain and developmental stage of the animals used, pre-incubation time

and temperature (Raymond, 2007). However, as shown recently, in our setting the decisive parameter can only be the induction protocol as this is the only variable factor (Kroker et al., 2011). However, it cannot be assumed, for example, that late LTP induced with the same protocol as ours in an older animal shares all traits with the late LTP discussed here. Taking a closer look at the conflicting reports mentioned above, all studies demonstrating a significant role of the mGlu5 receptor in CA1 LTP used strong or repeated stimulation protocols (Francesconi et al., 2004; Jia et al., 1998; Lu et al., 1997; Raymond et al., 2000; Shalin et al., 2006), which are believed to induce late LTP (Albensi et al., 2007; Huang and Kandel, 1994). Therefore, the findings of this study and the examination of other studies dealing with mGlu5 receptors, suggest that mGlu5 receptors have a differential role in early and late LTP.

Why positive modulation of the mGlu5 receptor is only efficacious in late LTP remains unknown. One possible explanation is that active NMDA receptors only potentiate mGlu5 receptor responses - via serine/threonine protein phosphatase and dephosphorylation (Alagarsamy et al., 1999) - if a specific activation threshold is reached, which is only the case with strong or repeated stimulation. Another possibility is that only intense stimulation leads to spillover of synaptically released glutamate to the perisynaptic location of mGlu5 receptors (Lujan et al., 1996) and hence to their activation. Last but not least, another explanation might be that only the enormous increase of calcium via NMDA receptors and L-VDCCs is sufficient to induce a relevant number of mGlu5 receptors at the surface, as CaM stabilizes the surface expression of mGlu5 receptors via the PKC-dependent regulation of trafficking (Lee et al., 2008). The first and the last alternatives seem to be more likely, since despite presence of ADX-47273 (0.3 µM) late LTP is completely abolished by a mixture of MK-801 (10 µM) and nifedipine (100 µM) (data not shown), even though all compounds were used in concentrations having the maximal effect on LTP (see Fig. 24, 25 and 26). This indicates that mGlu5 receptor per se is not able to cause synaptic potentiation, even at high concentrations, and rather requires NMDA receptor and L-VDCC function to extend its modulary action.

3.4.4. Conclusion

We used distinct protocols to induce early and late CA1 LTP which were validated by addressing the hallmarks accepted for these forms of LTP: protein-synthesis independence and NMDA receptor dependence without contribution of L-VDCCs for early LTP, as opposed to protein-synthesis dependence and NMDA / L-VDCCs dependence for late LTP. For these forms of LTP the modulatory role of mGlu5 receptors was probed using the positive allosteric modulator ADX-47273. ADX-47273 had only an effect on late CA1 LTP (i.e. enhancement) and none on early CA1 LTP, which corroborates previous findings on the facilitatory role of the mGlu5 receptor for late LTP in particular (Balschun and Wetzel, 2002; Bikbaev et al., 2008; Rosenbrock et al., 2010; Wu et al., 2008). We conclude that the stimulus intensity sufficient for induction of early LTP does not reach a threshold for functionally relevant stimulation of mGlu5 receptors, whereas a strong stimulus suitable for late LTP is sufficient to do so.

CHAPTER 4

Differential effects of subtype-specific nAChR agonists on early and late LTP

Abstract

Brain nicotinic acetylcholine receptors (nAChRs) are involved in several neuropsychiatric disorders, e.g. Alzheimer's and Parkinson's diseases, Tourette's syndrome, schizophrenia, depression, autism, attention deficit hyperactivity disorder, and anxiety. Currently, approaches selectively targeting the activation of specific nAChRs are in clinical development for treatment of memory impairment of Alzheimer's disease patients. These are α4β2 and α7 nAChR agonists which are believed to enhance cholinergic and glutamatergic neurotransmission, respectively. In order to gain a better insight into the mechanistic role of these two nAChRs in learning and memory, we investigated the effects of the α4β2 nAChR agonist TC-1827 and the α7 nAChR receptor partial agonist SSR180711 on hippocampal long-term potentiation (LTP), a widely accepted cellular experimental model of memory formation. Generally, LTP is distinguished in an early and a late form, the former being protein-synthesis independent and the latter being protein-synthesis dependent. TC-1827 was found to increase early LTP in a bell-shaped dose-dependent manner, but did not affect late LTP. In contrast, the α7 nAChR partial agonist SSR180711 showed enhancing effects on both early and late LTP in a bell-shaped manner. Furthermore, SSR180711 not only increased early LTP, but transformed it into late LTP, which was not observed with the α4β2 nAChR agonist. Therefore, based on these findings α7 nAChR (partial) agonists appear to exhibit stronger efficacy on memory improvement than α4β2 nAChR agonists.

4.1. Introduction

Brain nicotinic acetylcholine receptors (nAChRs) participate in complex cognitive functions such as attention as well as learning and memory. Clinical data suggest their involvement in several neuropsychiatric disorders, e.g. Alzheimer's and Parkinson's diseases, Tourette's syndrome, schizophrenia, depression, autism, attention deficit hyperactivity disorder, and anxiety (Lindstrom et al., 1997; Philip et al., 2010; Taly et al., 2009; Woodruff-Pak and Gould, 2003; Quik et al., 2009). For the majority of these disorders, the use of nAChR agonists may represent a symptomatic treatment. In this study, we focus on nAChRs as potential targets for improvement of cognitive function (Haydar and Dunlop, 2010; Holladay et al., 1997; Lloyd and Williams, 2000; Mudo et al., 2007; Sarter et al., 2009). In fact, nicotine was shown to exhibit pro-cognitive effects in animals and humans (Herman and Sofuoglu, 2010; Newhouse et al., 1988; Poorthuis et al., 2009; Rezvani and Levin, 2001; Sahakian et al., 1989; Wilson et al., 1995) and PET studies indicated that the number of radiolabeled nicotine binding sites decrease in proportion with the cognitive alteration in Alzheimer's disease (AD) patients (Paterson and Nordberg, 2000). Different nAChR subtypes are expressed in the brain, of which homopentameric α7 and heteropentameric α4β2 nAChRs are the predominant neuronal subtypes (Flores et al., 1992; Paterson and Nordberg, 2000; Tribollet et al., 2004). For both, α4β2 and α7 nAChR agonists, memory enhancing efficacy was demonstrated in several animal cognition tests (Bohme et al., 2004; Chan et al., 2007; Pichat et al., 2007; Wallace et al., 2011). Thus, specific α4β2 and α7 nAChR agonists are gaining more and more interest as clinical targets and some are already being evaluated in clinical trials for treatment of AD and/or cognitive impairment in schizophrenia (Freedman et al., 2001; Grassi et al., 2003; Hajós and Rogers, 2010; Leiser et al., 2009; Nordberg, 2001; Radek et al., 2010; Sarter et al., 2009; Thomsen et al., 2010).

In order to gain more insight into the mechanistic role of these two nAChRs in a cellular model of learning and memory, we analyzed the effects of the selective α4β2 nAChR agonist TC-1827 and the selective α7 nAChR partial agonist SSR180711 on hippocampal long-term potentiation (LTP). LTP is a widely-used cellular experimental model of memory formation (Bliss and Collingridge, 1993), which can be distinguished into early and late forms of LTP being protein-synthesis independent and protein synthesis dependent, respectively (Kroker et al., 2011b; Reymann and Frey, 2007). Therefore, in this study, we compared the effects of the selective α4β2 and α7 nAChR (partial) agonists on

early and late LTP. The results provide a more detailed insight into the potential clinical use of selective α4β2 and α7 nAChR agonists for LTP modulation and hence give a rationale for a ranking of approaches to enhance cognitive function in patients.

4.2. Materials and methods

4.2.1. Preparation of brain slices

Procedures involving animals and their care were conducted in conformity with institutional and European Union guidelines (EEC Council Directive 86/609) and were approved by the Ethical Committee of the responsible regional council (Tübingen). Male Wistar rats (Janvier, Le Genest Saint Isle, France) aged six to seven weeks were shortly anaesthetized with isoflurane and sacrificed by decapitation. The brains were quickly removed and immersed in ice-cold ACSF (containing in mM NaCl 124, KCl 4.9, $MgSO_4$ 8, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25.6, D-glucose 10, pH 7.4, saturated with 95% O_2 and 5% CO_2). Transverse hippocampal brain slices (400 µm thickness) were cut using a Vibratome. Slices were allowed to recover in a holding chamber containing ACSF bubbled with 95% O_2 and 5% CO_2 at room temperature for at least one hour. Slices were then transferred to integrated brain slice chambers and continuously superfused (at a flow rate of 2.5 mL min^{-1}, 25 ± 0.2 °C) with the same ACSF composition used before except for 0.5 mM $MgSO_4$. Prior to doing any electrophysiological recordings, the slices were allowed to equilibrate for at least 30 minutes.

4.2.2. Multi-slice recording

To record field excitatory postsynaptic potentials (fEPSPs) the SliceMaster multi-slice recording system (Scientifica Limited, East Sussex, UK) was used. The system is semi-automated allowing one operator to record simultaneously from an assembly of up to eight brain slices. For the present study, an optimized system described by Kroker et al. (2011a) was used. fEPSPs were elicited in the CA1 region by stimulation of the Schaffer collateral–commissural fibres in the stratum radiatum using glass electrodes with broken tips (filled with ACSF). For recording, glass electrodes (2 – 6 MΩ, filled with ACSF) were placed in the apical dendritic layer. The amplitudes of fEPSPs were used as the parameter of interest (Collingridge et al., 1983). To generate fEPSPs at a constant sub-threshold stimulus, as in previous studies (Seabrook et al., 1997), the stimulus strength of the pulses was adjusted to 20 - 30% of the fEPSP maximum and this voltage was used for the experiment. During baseline recording each slice was stimulated every 30 seconds for at

least one hour. Early LTP was induced by weak high frequency stimulation (HFS) made up of 20 pulses at the frequency of 100 Hz (Bashir et al., 1991; Kroker et al., 2011a,b), whereas late LTP was induced by repeated strong HFS consisting of 100 pulses at the frequency of 100 Hz, repeated two times in five minute intervals (Kroker et al., 2011a,b; Lu et al., 1999).

4.2.3. Data acquisition, software and analysis

A modular electrophysiology system, supplied by npi electronic GmbH (Tamm, Germany), conducts the low noise recordings of extracellular signals. AC coupled signals are amplified 1000-fold and internally filtered with a five kHz low-pass filter as well as a three Hz high-pass filter. In our study, a customized system (programmable pattern generator) was used allowing simultaneous stimulation of all slices either with a single stimulus or a specific stimulus pattern (Kroker et al., 2011a). For data acquisition and analysis the software Notocord® was used.

Data are shown as mean percent (± S.E.M.) of the baseline fEPSP amplitude. Data were analyzed using either *t* tests to compare two conditions or one-way ANOVA (post-hoc test: Dunnett-Test) to compare multiple conditions. In both cases a p-value of ≤ 0.05 was considered significant. Histograms show the mean amplitude (± S.E.M.) of fEPSPs measured between 50 and 60 minutes after weak or repeated strong HFS according to Jia et al. (2010) and Kroker et al. (2011b).

4.2.4. Drug solutions and application

The following drugs were used: (5S,10R)-(–)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5,10-imine hydrogen maleate (= MK-801), (2R,3S,4S)-2-(4-Methoxybenzyl)-3,4-pyrrolidinediol-3-acetate, 2-[(4-Methoxyphenyl)methyl]-3,4-pyrrolidinediol 3-acetate (= anisomycin) and 1,4-Dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester (= nifedipine) were purchased from Sigma-Aldrich Corporation (St. Louis, USA). The compounds (S)-N-methyl-5-(5-pyrimidinyl)-4-penten-2-amine hemigalactarate (= TC-1827) and 1,4-Diazabicyclo[3.2.2]nonane-4-carboxylic acid, 4-

bromophenyl ester (= SSR180711) were synthesized in the Department of Medicinical Chemistry of Boehringer Ingelheim Pharma GmbH & Co KG.

The drugs were prepared as stock solutions and diluted in ACSF immediately before application. Nifedipine and SSR180711 were prepared in DMSO (the final concentration of DMSO was 0.1%). All drugs, except MK-801, were applied 30 minutes before LTP stimulation and remained until 30 minutes after LTP stimulation. MK-801 was used in such a way that, corresponding to Frankiewicz et al. (1996), true equilibrium blockade of N-methyl D-aspartate excitotoxic amino acid (NMDA) receptors can be expected: MK-801 was present immediately after preparing slices, thereafter for an average of 6.8 ± 0.4 hours prior to LTP stimulation and remained until 30 minutes after LTP induction.

4.3. Results

4.3.1. Targeting the α4β2 nAChR: the effect of TC-1827 on early and late LTP

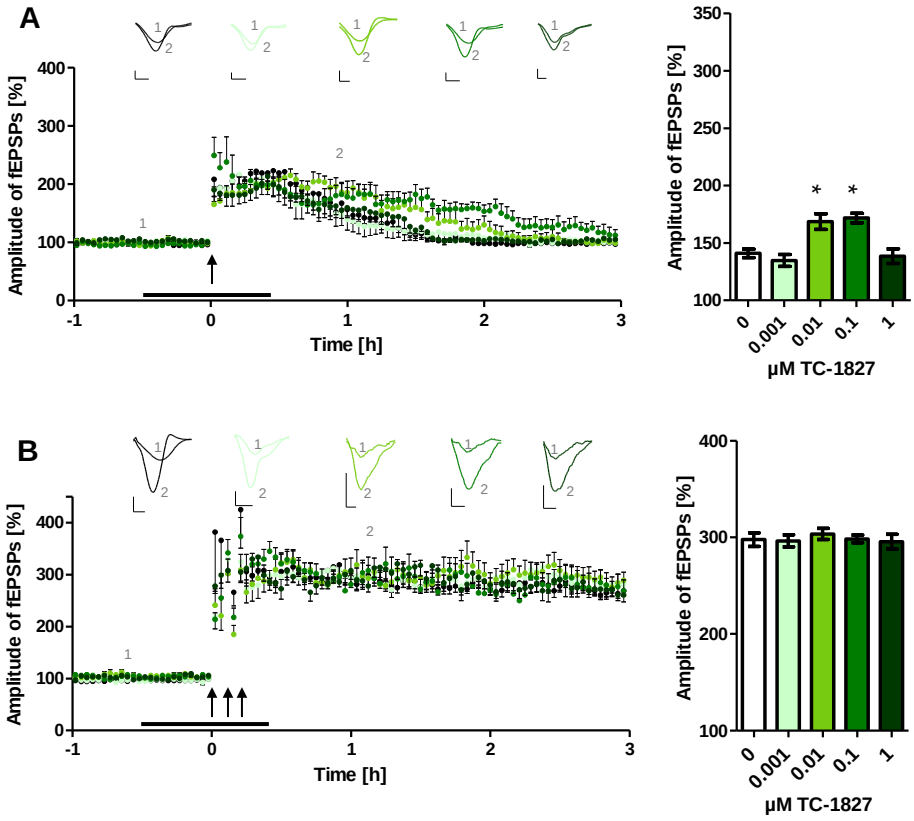


Figure 26: Effect of TC-1827 on early and late LTP.
Effect of the selective α4β2 nAChRs agonist TC-1827 on early LTP induced by weak HFS and late LTP induced by repeated strong HFS. TC-1827 (0.001 – 1 µM) was applied 30 min before LTP induction (arrow/s) and washed out 30 min after LTP induction (horizontal bar). None of the concentrations of TC-1827 had an effect on basal fEPSPs. Individual fEPSPs before (1) and after LTP stimulation (2) are shown (horizontal bar: 0.5 mV, vertical bar: 10 ms). Histograms show the amplitude (mean ± S.E.M.; *n* = 5) of fEPSPs measured between 50 and 60 min after HFS. A) Early LTP was enhanced in a bell-shaped concentration dependent manner by TC-1827. B) None of the tested concentrations of TC-1827 showed a significant effect on late LTP.

Fig. 26 shows the action of the α4β2 nAChR agonist TC-1827 on early and late LTP. This compound increased early LTP with a bell-shaped concentration-response relationship (0.001 – 1 µM) demonstrating a significant prolongation at 0.01 and 0.1 µM, which results in an increase of amplitude at one hour after LTP stimulation (Fig. 26A) (one hour after

weak HFS; control: 141 ± 4%, *n* = 10; 0.001 µM TC-1827: 135 ± 5%, *n* = 5, not significant (= ns); 0.01 µM TC-1827: 169 ±7%, *n* = 5, *p* < 0.05; 0.1 µM TC-1827: 172 ± 4%, *n* = 5, *p* < 0.05; 1 µM TC-1827: 139 ± 6%, *n* = 5, ns). This enhancement was transient and lasted up to maximal three hours after weak HFS. In contrast, none of the tested concentrations (0.001 – 1 µM) of TC-1827 showed a significant effect on late LTP (Fig. 26B) (one hour after repeated HFS; control: 298 ± 7%, *n* = 10; 0.001 µM TC-1827: 297 ± 6%, *n* = 5, ns; 0.01 µM TC-1827: 304 ± 6%, *n* = 5, ns; 0.1 µM TC-1827: 299 ± 6%, *n* = 5, ns; 1 µM TC-1827: 296 ± 8%, *n* = 5, ns). Basal fEPSPs were not affected by any concentration of TC-1827.

4.3.2. Targeting the α7 nAChR

4.3.2.1. The effect of SSR180711 on early and late LTP

In Fig. 27, the effects of the α7 nAChR partial agonist SSR180711 on early and late LTP are shown. This compound increased both early LTP (Fig. 27A) (one hour after weak HFS; control: 142 ± 6%, *n* = 10; 0.003 µM SSR180711: 139 ± 10%, *n* = 5, ns; 0.03 µM SSR180711: 297 ± 35%, *n* = 5, *p* < 0.001; 0.3 µM SSR180711: 213 ± 18%, *n* = 5, *p* < 0.01; 3 µM SSR180711: 140 ± 11%, *n* = 5, ns) and late LTP (Fig. 27B) (one hour after repeated HFS control: 301 ± 8%, *n* = 10; 0.003 µM SSR180711: 310 ± 6%, *n* = 5, ns; 0.03 µM SSR180711: 356 ± 6%, *n* = 5, *p* < 0.05; 0.3 µM SSR180711: 361 ± 7%, *n* = 5, *p* < 0.05; 3 µM SSR180711: 312 ± 5%, *n* = 5, ns), with a bell-shaped concentration-response relation (0.003 – 3 µM) showing a significant enhancement at 0.03 and 0.3 µM for both forms of LTP. These enhancements lasted for more than three hours after LTP stimulation. Basal fEPSPs were not affected by any concentration of SSR180711.

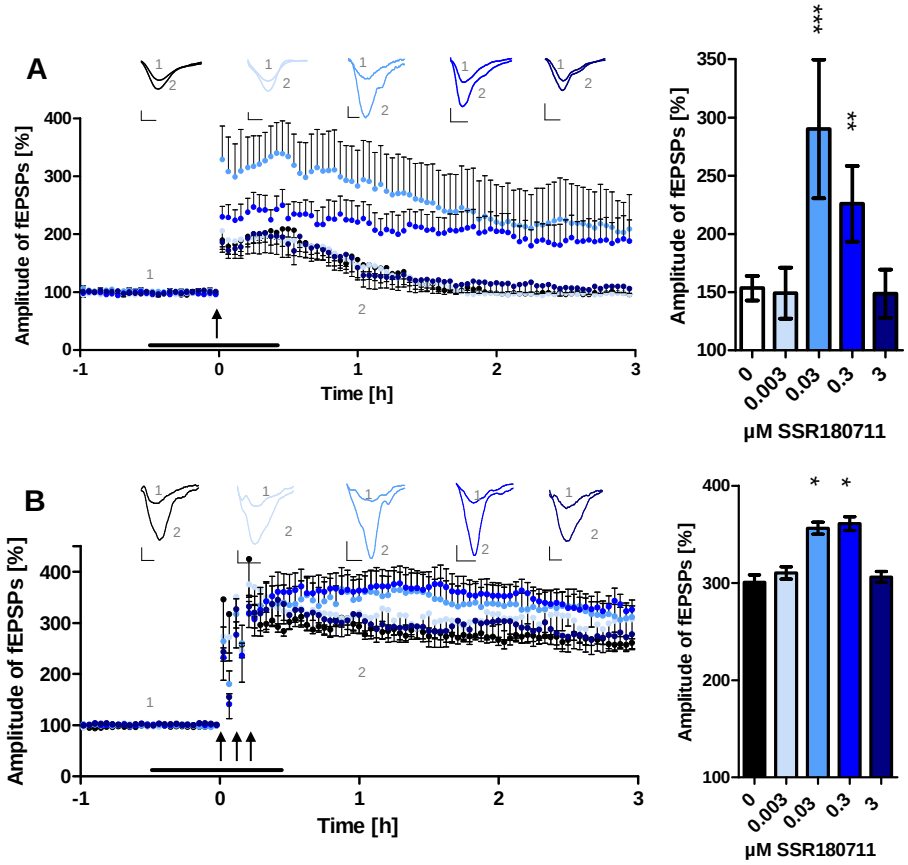


Figure 27: Effect of SSR180711 on early and late LTP.
Effect of the selective α7 nAChR partial agonist SSR180711 on early LTP induced by weak HFS and late LTP induced by repeated strong HFS. SSR180711 (0.003 – 3 µM) was applied 30 min before LTP induction (arrow/s) and washed out 30 min after LTP induction (horizontal bar). None of the concentrations of SSR180711 had an effect on basal fEPSPs. Individual fEPSPs before (1) and after LTP stimulation (2) are shown (horizontal bar: 0.5 mV, vertical bar: 10 ms). Histograms show the amplitude (mean ± S.E.M.; *n* = 5) of fEPSPs measured between 50 and 60 min after HFS. A) Early LTP was enhanced in a bell-shaped concentration dependent manner by SSR180711. B) SSR180711 increased late LTP in a bell-shaped concentration dependent manner.

4.3.2.1. Transformation of early into late LTP

To analyze whether the long-lasting increase of early LTP by SSR180711 could be attributed to the transformation of protein-synthesis independent early LTP into protein-synthesis dependent late LTP, the reversible protein translation inhibitor anisomycin was used at a concentration of 30 µM, which is known to be sufficient to significantly impair protein-synthesis dependent late LTP (Lu et al., 1999; Kroker et al., 2011b). The addition of 30 µM anisomycin to 0.3 µM SSR180711, a concentration shown to increase early LTP (see Fig. 27A), caused a significant decrease of SSR180711 enhanced LTP up to a

complete abolishment within two to three hours after LTP induction (Fig. 28). This indicates that protein-synthesis independent early LTP was transformed into protein-synthesis dependent late LTP by SSR180711. Basal fEPSPs were not affected by the mixture of SSR180711 and anisomycin.

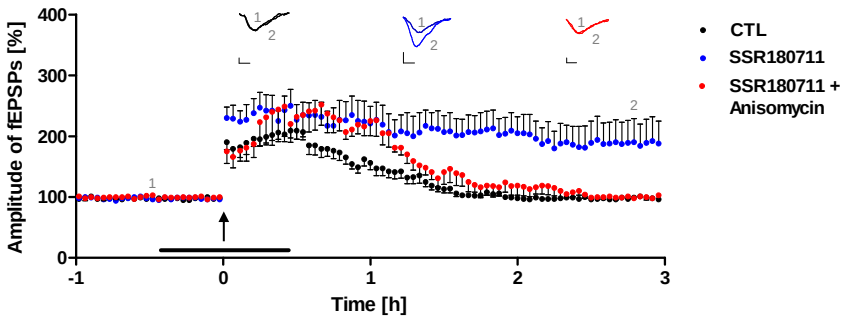


Figure 28: Transformation of early LTP into late LTP by a selective α7 nAChR partial agonist

To analyze whether SSR180711 transforms protein-synthesis independent LTP (= control shown in black, *n* = 5) into protein-synthesis dependent LTP, a mixture of SSR180711 (0.3 µM) and the protein-synthesis inhibitor anisomycin (at 30 µM) was applied 30 min before weak HFS stimulation (arrow) and remained until 30 min after LTP stimulation (horizontal bar). Individual fEPSPs before (1) and after LTP stimulation (2) are shown (horizontal bar: 0.5 mV, vertical bar: 10 ms). SSR180711-enhanced LTP (shown in blue, *n* = 5) lasts longer than 3 h, but is significantly reduced up to a complete abolishment by addition of anisomycin (shown in red, *n* = 5). Neither SSR180711 alone, nor the mixture of SSR180711 and anisomycin, had an effect on basal fEPSPs.

Next, the receptors putatively responsible for the SSR180711 mediated increase in early LTP were investigated (Fig. 29). To analyze the role of NMDA receptors, 10 µM of MK-801 was applied, a concentration reported to be sufficient to block NMDA receptor dependent early and late LTP (Kroker et al., 2011b). As shown in Fig. 29A, MK-801 completely blocked the SSR180711 mediated enhanced early LTP (one hour after weak HFS; 0.03 µM SSR180711: 213 ± 18%, *n* = 5; 0.03 µM SSR180711 + 10 µM MK-801: 103 ± 2%, *n* = 5, $p < 0.001$). Basal fEPSPs were not affected by the mixture of SSR180711 and MK-801. To analyze the role of L-type voltage dependent calcium channels (L-VDCC), 100 µM of the L-type calcium channel blocker nifedipine was applied, a concentration reported to be sufficient to block L-VDCCs dependent late LTP (Kroker et

al., 2011b). As shown in Fig. 29B, nifedipine had a significant, but small effect on the SSR180711 mediated enhanced early LTP (one hour after weak HFS; 0.03 µM SSR180711: 213 ± 18%, n = 5; 0.03 µM SSR180711 + 100 µM nifedipine: 180 ± 7%, n = 5, $p < 0.05$). Basal fEPSPs were not affected by the mixture of SSR180711 and nifedipine.

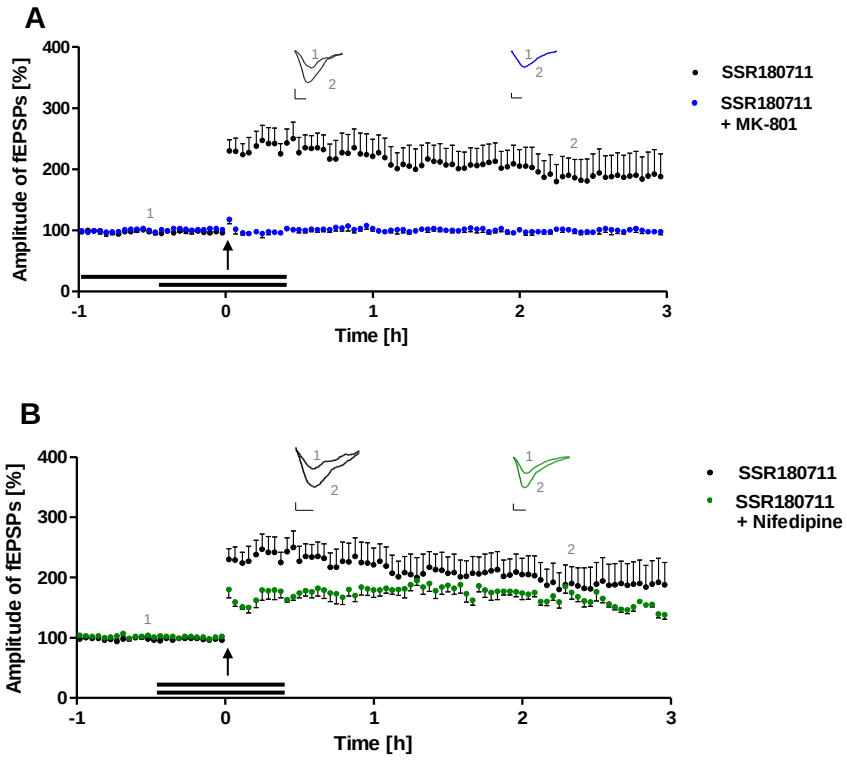


Figure 29: Role of NMDA receptors and L-VDCCs in SSR180711 mediated transformation of early into late LTP.

Effects of MK-801, a potent antagonist of NMDA receptors, and nifedipine, an L-type calcium channel blocker, on SSR180711-enhanced LTP induced by weak HFS. A) Late LTP transformed from early LTP by SSR180711 was completely abolishment at 100 µM MK-801. MK-801 was applied 6.8 ± 0.4 h before weak HFS (arrow) and was washed out 30 min after weak HFS (horizontal bar). Individual fEPSPs before (1) and after LTP stimulation (2) are shown (horizontal bar: 0.5 mV, vertical bar: 10 ms). B) Late LTP transformed from early LTP by SSR180711 was reduced by 100 µM nifedipine. Nifedipine was applied 30 min before weak HFS (arrow) and remained until 30 min after weak HFS (horizontal bar).

4.4. Discussion

Acetylcholinesterase inhibitors, like donepezil, rivastigmine or galantamine, are used for symptomatic treatment of mild-to-moderate AD disease patients. However, experience from clinical practice with acetylcholinesterase inhibitors has raised concerns about the clinical relevance of the only moderate treatment effect, the appearance of tolerance after long-term use, interaction of cholinergic and non-cholinergic effects and possible effects of amyloid precursor protein release (Courtney et al., 2004; Diniz et al., 2009; Helou and Rhalimi, 2010; Hernandez et al., 2009; Kaduszkiewicz et al., 2005; Rosenbloom et al., 2010; Rozzini et al., 2004). Currently, an alternative approach targeting cholinergic neurotransmission through direct activation of nAChRs has come into the focus of interest for the treatment of AD and other cognitive diseases (Arneric et al., 2007; Buccafusco et al., 2004; D'hoedt and Bertrand, 2009; Melnikova, 2007; Nordberg, 2001; Perry et al., 2001; Woodruff-Pak and Gould, 2002). Non-selective nAChR agonists like nicotine and epibatidine, but also sub-type selective nAChR agonists lacking the side effects caused by stimulation of peripheral nAChR such as TC-1827, ispronicline, TC-5619, ABT-089 and SSR180711 have demonstrated memory enhancing effects in various animal cognition tasks (Barak et al., 2009; Bohme et al., 2004; Decker et al., 1994; Dukat and Glennon, 2003; Lippiello et al., 2006; Pichat et al., 2007; Rezvani and Levin, 2001). Furthermore, the α4β2 nAChR agonists ispronicline and AZD-1446 as well as the α7 nAChR agonists TC-5619 and RO5313534 (formerly R3487/MEM3454) were progressed into phase II clinical development (Geerts, 2006; Hauser et al., 2009; Haydar and Dunlop, 2010; Toyohara and Hashimoto, 2010). Thus, the α4β2 and α7 nAChR represent attractive potential drug targets for treatment of diseases linked to cognitive dysfunction. However, their exact mode-of-action on the molecular and cellular processes underlying memory formation, i.e. synaptic plasticity and LTP, are not fully understood.

The potent α4β2 nAChR full agonist TC-1827 (Bohme et al., 2004; Grinevich et al., 2009) and the selective α7 nAChR partial agonist SSR180711 (Barak et al., 2009; Biton et al., 2007; Hashimoto et al., 2008; Kristensen et al., 2007; Pichat et al., 2007) were previously described to enhance hippocampal LTP (Biton et al., 2007; Bohme et al., 2004). However, in these studies, only a single drug concentration was tested and no distinction was made between effects on early and late LTP. This distinction is of importance for the evaluation of downstream effects associated with these targets. Early LTP is independent of protein-synthesis and involves modifications of pre-existing synapses as a result of rapid calcium

influx through NMDA receptors and subsequent protein phosphorylation events (Bliss and Collingridge, 1993; Malenka and Nicoll, 1999). Late LTP is clearly separated from early LTP by its dependence on *de novo* protein-synthesis (Frey et al., 1988; Huang, 1998; Huang and Kandel, 1994; Huang et al., 1996; Kroker et al., 2011b; Nguyen et al., 1994; Nguyen and Kandel, 1997; Reymann and Frey, 2007). Protein-synthesis may contribute both to the modification of existing synapses and to the establishment of new synaptic contacts (Engert and Bonhoeffer, 1999; Lisman, 2003). Moreover, long-lasting forms of memory are generally believed to be protein-synthesis dependent (Pang and Lu, 2004).

The findings in the present study demonstrate that early LTP can be enhanced in a bell-shaped manner by the α4β2 nAChR full agonist TC-1827 with efficacious concentrations of 0.01 and 0.1 µM confirming published results (Bohme et al., 2004) which showed that 0.1 µM TC-1827 increased LTP induced by weak stimulation supposed to induce early LTP (Albensi et al., 2007; Huang and Kandel, 1994). Contrary to the effects on early LTP, TC-1827 did not show any effects on late LTP. Hence, in our setting the activation of the α4β2 nAChR seems to be able to enhance early LTP, but not late LTP. For the α7 nAChR partial agonist SSR180711, we show that both early and late LTP are increased in a bell-shaped manner with efficacious concentrations of 0.03 and 0.3 µM. The result on late LTP confirms published data (Biton et al., 2007) showing that 0.3 µM SSR180711 increased LTP induced by repeated stimulation thought to induce late LTP (Albensi et al., 2007; Huang and Kandel, 1994). Furthermore, for the first time, it was demonstrated that SSR180711 not only increases protein-synthesis independent LTP (early LTP) in a bell-shaped manner, but transforms it into protein-synthesis dependent LTP (late LTP). This transformation of early into late LTP might result from a lowered induction threshold of late LTP. Further investigations revealed that the SSR180711 mediated increase and transformation was dependent mainly on NMDA receptors and in part on L-VDCCs. Thus, as late LTP induced by repeated HFS is dependent on NMDA receptors and L-VDCCs to equal parts (Kroker et al., 2011b), a different type of late LTP seems to be induced by administration of SSR180711 together with weak HFS.

Both the α4β2 and the α7 nAChR (partial) agonists showed a bell-shaped concentration dependence with respect to their enhancement of LTP. An explanation might be provided by the finding that full activation of nAChRs using high agonist concentrations can lead to receptor endocytosis (St. John and Gordon, 2001). Another explanation concerning the lack of effect at high concentrations might be an overstimulation of the nAChRs, not strong enough to cause long-term depression, but sufficient to alleviate the enhancement of

LTP. In fact, it has been shown that nicotine is able to induce long-term depression at high concentrations (Maggi et al., 2004).

Regarding receptor specificity, both TC-1827 and SSR180711 were proven to be specific (partial) agonists, since their enhancing effects on LTP were reversed by addition of the respective specific nAChR antagonists dihydro-beta-erythroidine (for the α4β2 nAChR) and methyllycaconitine (for the α7 nAChR; data not shown).

What might be the explanation for α4β2 and α7 nAChR agonists having differential effects on early and late LTP? Both α4β2 and α7 nAChRs are localized on GABAergic interneurons (Alkondon et al., 1999, 2004; Fabian-Fine et al., 2001; Jones and Yakel, 1997; Khiroug et al., 2003). Disinhibition can take place due to activation of α4β2 and/or α7 nAChRs on GABAergic interneurons having inhibitory connections to other inhibitory neurons producing a net increase in glutamatergic principal cell activity (Alkondon et al., 2004; Ji and Dani, 2000). Furthermore, both receptors are reported to be located on the presynapse of acetylcholinergic neurons (McGehee et al., 1995; Wonnacott, 1997) and, due to the significant calcium permeability of these receptors (Role and Berg, 1996; Séguéla et al., 1993; Vernino et al., 1994), can provide positive feedback by enhancing the release probability of acetylcholine (Bohme et al., 2004; McGehee et al., 1995; Role and Berg, 1996; Wonnacott, 1997). Therefore, activation of α4β2 and/or α7 nAChRs may cause dishinhibition and enhancement of acetylcholine release - mechanisms which presumably are able to enhance early, but not late LTP. The decisive difference between α4β2 and α7 nAChR function is the additional location of α7 nAChRs on Schaffer collateral terminals (Fabian-Fine et al., 2001; Buccafusco et al., 2005) causing strengthening of the glutamatergic system through the facilitation of glutamate release (Aramakis and Metherate, 1998; Biton et al., 2007; Gray et al., 1996; Jones et al., 1999; Mansvelder and McGehee, 2000; McGehee et al., 1995; Pidoplichko et al., 2004; Radcliffe and Dani, 1998). Due to their high calcium permeability α7 nAChRs can enhance the synaptic release of glutamate, increase the coincidence between presynaptic release and postsynaptic depolarization and therefore enhance the strength of LTP (Ge and Dani, 2005; Ji et al., 2001). This might explain the enhancement of early as well as late LTP and the transformation of early into late LTP by activation of α7 nAChRs, but not α4β2 nAChRs lacking this feature on glutamatergic transmission. Thus, concerning cognitive functions, activation of α7 nAChRs might be more effective than activation of α4β2 nAChRs as also suggested by Graef et al. (2011).

In conclusion, this study demonstrates that activation of α7 nAChRs is more efficient in enhancing and prolonging LTP than activation of α4β2 nAChRs. We hypothesize that the better performance of the α7 nAChR agonist is not due to disinhibition or cholinergic transmission as these mechanisms are activated by both receptors, but due to the additional strengthening of the glutamatergic system via the α7 nAChR. Therefore, the direct comparison of the effects of selective α4β2 and α7 nAChR (partial) agonists on early and late LTP suggests that α7 nAChR activation might be the more attractive approach for enhancing cognitive function in patients.

CHAPTER 5

Inhibition of AChE and PDE9A has differential effects on early and late LTP

Abstract

Donepezil is the current standard symptomatic treatment of mild-to-moderate Alzheimer's disease (AD) patients. It aims to compensate for the deficit in cholinergic neurotransmission by blocking acetylcholinesterase (AChE) and thus increases the concentration of extracellular acetylcholine. However, experience from clinical practice demonstrated that AChE inhibitors only have moderate treatment effects. As a potential new approach for memory enhancement, inhibition of specific phosphodiesterases (PDEs) has gained attention. Among those are PDE9A inhibitors which increase the levels of the second messenger cyclic guanosine monophosphate (cGMP) intracellularly. In order to gain more insight into the potential impact of extracellularly acting AChEs and intracellularly acting PDE9A inhibitors on synaptic plasticity, we analyzed the effects of the AChE inhibitor donepezil and the PDE9A inhibitor BAY 73-6691 on long-term potentiation (LTP) in rat hippocampal slices, a widely accepted cellular experimental model of memory formation. Generally, LTP can be differentiated into an early and a late form, being protein-synthesis independent and protein-synthesis dependent, respectively. Donepezil was found to increase early LTP, but did not affect late LTP. In contrast, BAY 73-6691 demonstrated enhancing effects on both early and late LTP and even transformed early into late LTP. Furthermore, it was shown that this transformation into late LTP was dependent on the NO-cGMP-PKG pathway. In conclusion, this study demonstrates that BAY 73-6691 exhibits a stronger effect in enhancing and prolonging LTP than donepezil suggesting that PDE9 inhibition might be more efficacious in enhancing learning and memory.

5.1. Introduction

Alzheimer's disease (AD) is a prevalent neurodegenerative disorder characterized by progressive cognitive decline (Castellani, 2010). A prominent feature linked to cognitive dysfunction of AD patients is a deficit in cholinergic neurotransmission (Bartus et al., 1982; Whitehouse et al., 1982), which has led to the development of acetylcholinesterase (AChE) inhibitors (Francis et al., 1999; Tsuno, 2009). The most widely prescribed AChE inhibitor is donepezil, which is used for symptomatic treatment of AD patients (Birks and Harvey, 2003; Whitehead et al., 2004). However, clinical experience with AChE inhibitors has demonstrated only moderate efficacy, thereby questioning their clinical relevance, (Courtney et al., 2004; Diniz et al., 2009; Kaduszkiewicz et al., 2005). Recently, phosphodiesterase (PDE) inhibitors have been proposed as an alternative approach for treatment of cognitive dysfunction (Beavo, 1995; Menniti et al., 2006; Reneerkens et al., 2009; Schmidt et al., 2010), since they were shown to enhance memory performance in animal cognition models (Barad et al., 1998; Boess et al., 2004; Prickaerts et al., 2004; Rutten et al., 2005, 2007, 2008; Zhang and O'Donnell, 2000). One of the eleven PDE isoforms (Beavo, 1995), showing expression in cognition relevant brain regions, is PDE9A (Andreeva et al., 2001; Lakics et al., 2010; Schmidt et al., 2009; van Staveren et al., 2002). As PDE9A is specific for hydrolyzing cGMP, its inhibitors specifically block the breakdown of cGMP and were shown to increase cGMP *in vitro* and *in vivo* (Hutson et al., 2011; Verhoest et al., 2009; Wunder et al., 2005). Furthermore, they were demonstrated to be efficacious in various rodent memory tasks (Hutson et al., 2011; van der Staay et al., 2008).

In order to gain more insight into the mechanistic roles of the extracellularly acting AChE and the intracellularly acting PDE9A in learning and memory, we analyzed the effects of the respective inhibitors donepezil and BAY 73-6691 (Wunder et al., 2005) on long-term potentiation (LTP) in rat hippocampal slices. LTP is a widely-used cellular experimental model of memory formation (Bliss and Collingridge, 1993), which can be differentiated into early and late forms of LTP being protein-synthesis independent and protein-synthesis dependent, respectively (Kroker et al., 2011b; Reymann and Frey, 2007). Interestingly, there have been only few studies exploring the underlying cellular mechanism by which donepezil might mediate its cognitive enhancing effect (Barnes et al., 2000; Spencer et al., 2010). To our knowledge, its effect on synaptic plasticity and LTP in hippocampal slices has not been investigated so far. Regarding BAY 73-6691, only its effect on early LTP has

been published (van der Staay et al., 2008), but neither a full concentration-response curve nor an assessment of its effect on late LTP were reported. Therefore, in this study, we analyzed and compared the effects of donepezil and BAY 73-6691 on early and late LTP. The results provide a deeper insight into the mechanism of action of AChE inhibitors and the PDE9A inhibitor for LTP modulation and hence into their potential roles in learning and memory.

5.2. Materials and methods

5.2.1. Tissue preparation

5.2.1.1. Animals

Procedures involving animals and their care were conducted in conformity with institutional and European Union guidelines (EEC Council Directive 86/609) and were approved by the Ethical Committee of the respective regional council. For all experiments male Wistar rats (Janvier, Le Genest Saint Isle, France) aged six to seven weeks were used.

5.2.1.2. Preparation of brain slices

Rats were shortly anaesthetized with isoflurane and euthanized by decapitation. The brains were quickly removed and immersed in ice-cold ACSF (containing in mM NaCl 124, KCl 4.9, $MgSO_4$ 8, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25.6, D-glucose 10, pH 7.4, saturated with 95% O_2 and 5% CO_2). Transverse hippocampal brain slices (400 µm thickness) were cut using a Vibratome. Slices were allowed to recover in a holding chamber containing ACSF bubbled with 95% O_2 and 5% CO_2 at room temperature for at least three hours. For estimation of protein concentration, western blot analysis and measurement of AChE activity the slices were homogenated by douncing in PBS (Phosphate Buffered Saline, Bio-Rad Laboratories, Inc.) supplemented with a cocktail of protease inhibitors (Roche Diagnostic GmbH), partitioned into three tubes and stored at -80 °C until use.

For electrophysiological recordings, slices were transferred into the integrated brain slice chambers and continuously superfused (at a flow rate of 2.5 mL min^{-1}, 25 ± 0.2 °C) with the same ACSF composition used before except for 0.5 mM $MgSO_4$. Prior to doing any electrophysiological recordings, the slices were allowed to equilibrate for at least 30 minutes.

5.2.1.3. Perfusion of rats and brain homogenate preperation

Perfusion and homogenate preparation were performed as described by Das et al. (2001) with minor modifications. Briefly, rats were perfused through the heart with saline (0.9% NaCl) under ketamine (70 mg/kg) and xylazine (6 mg/kg) anesthesia. The brain was

removed and a 10% (w/v) homogenate of the hippocampus was prepared by douncing in PBS supplemented with a cocktail of protease inhibitors. 3 x 33 µl of homogenate were collected, diluted in the ratio of 1:10 and stored at -80 °C. The rest of the homogenate was centrifuged at 100,000 x g at 4 °C for 60 minutes. The supernatant as well as the pellet were each collected in separate tubes, diluted in the ratio of 1:10, stored at -80 °C and used as a source of estimation of protein concentration and measurement of acetylcholinesterase activity, respectively. Jackisch et al. (2009) verified that choline esterase activity does not decrease under these storage conditions.

5.2.2. Electrophysiological measurements

5.2.2.1. Extracellular recording and stimulation

An optimized SliceMaster multi-slice recording system (Scientifica Limited, East Sussex, UK; Kroker et al., 2011a) was used to record field excitatory postsynaptic potentials (fEPSPs). fEPSPs were elicited in the CA1 region by stimulation of the Schaffer collateral–commissural fibres in the stratum radiatum using glass electrodes with broken tips (filled with ACSF). For recording, glass electrodes (2 – 6 MΩ, filled with ACSF) were placed in the apical dendritic layer. The amplitudes of fEPSPs were used as the parameter of interest (Collingridge et al., 1983; Kroker et al., 2011a). To generate fEPSPs at a constant sub-threshold stimulus, as in previous studies (Kroker et al., 2011a; Seabrook et al., 1997), the stimulus strength of the pulses was adjusted to 20 - 30% of the fEPSP maximum and this voltage was used for the experiment. During baseline recording, each slice was stimulated every 30 seconds for at least one hour. Early LTP was induced by weak high frequency stimulation (HFS) made up of 20 pulses at the frequency of 100 Hz (Bashir et al., 1991; Kroker et al., 2011b), whereas late LTP was induced by repeated strong HFS consisting of 100 pulses at the frequency of 100 Hz, repeated two times in five minute intervals (Kroker et al., 2011b; Lu et al., 1999).

5.2.2.2. Recording, data acquisition and analysis

A modular electrophysiology system, supplied by npi electronic GmbH (Tamm, Germany), conducts the low noise recordings of extracellular signals. AC coupled signals are

amplified 1000 x and internally filtered with a five kHz low-pass filter as well as a three Hz high-pass filter. In our study, a customized system (programmable pattern generator) was used allowing simultaneous stimulation of all slices either with a single stimulus or a specific stimulus pattern (Kroker et al., 2011a). For data acquisition and analysis the software Notocord® was used. Data are shown as mean (± SEM) percent of the baseline fEPSP amplitude. Data were analyzed using either *t* tests to compare two conditions or one-way ANOVA (post-hoc test: Dunnett-Test) to compare multiple conditions. In both cases a *p*-value of 0.05 was considered significant. Histograms show the (mean ± SEM) amplitude of EPSPs measured 50 – 60 min after delivery of LTP stimulation according to Jia et al. (2010) and Kroker et al. (2011b).

5.2.2.3. Drug application

Drugs were prepared as stock solutions and diluted in ACSF immediately before application. ODQ and BAY 73-6691 were prepared in DMSO (the final concentration of DMSO in ACSF was 0.1%). All drugs were applied 30 minutes before LTP stimulation and remained until 30 minutes after LTP stimulation

5.2.3. Determination of protein concentration

Samples of the slice homogenate, the hippocampal homogenate, supernatant and pellet were used for protein measurement. Protein concentration was determined by using a Pierce BCA protein assay kit according to the manufacture's procedure (Thermo Scientific Pierce BCA Protein Assay Kit).

5.2.4. Western blot analysis

A total of 20 µg of protein (from samples of hippocampal homogenate, slice homogenate, supernant and pellet) per lane was run on 4 – 12% Bis-Tris gels (NuPAGE® Novex Bis-Tris Gels) and then transferred onto PVDF membranes for western blotting (Trans-Blot Transfer Medium; Bio-Rad). The membranes were blocked for one hour in Odyssey

blocking buffer (Licor). The blots were then probed with affinity purified goat polyclonal anti-AChE antibodies over night (E-19, dilution 1:200, Santa Cruz Bioctechnology Inc.; Fischer et al., 2002; Jevsek et al., 2004; Santos et al., 2007; Sternfeld et al., 2000). Following three washes, the blots were incubated for one hour with secondary anti-goat antibodies made in donkey purchased from Licor (926-32223, dilution 1:2000, Odyssey®, Licor). After additional washing, immunoreactive bands were detected using a Licor Odyssey imaging system according to the manufacture's procedure.

5.2.5. Measurement of achetylcholinesterase activity

Choline esterase activity was determined according to Ellman et al. (1961), as modified by Ashour et al. (1987) and Jackisch et al. (2009). In brief, 250 µl of the reagent buffer (0.01% DTNB in 0.05 M sodium diphosphate buffer, pH 7.4) and 6 µl tissue homogenate (corresponding to about 24 – 42 µg protein) were added to the wells of a 96-well microtiter plate cooled on ice. Each concentration was determined in triplicate. The enzymatic reaction was started at room temperature by the addition of 50 µl acetylthiocholine iodide solutions at various concentrations (final concentrations of acetylthiocholine in the wells (µM): 31.25, 62.5, 125, 250, 500, 1000 and 2000). Absorbance change at 405 nm was measured at room temperature using a Flex Station 3 (Molecular Devices). Values were recorded every 30 seconds for one hour. Negative controls (in triplicate, without tissue homogenate) were always run in parallel. For determination of the inhibitory potency of donepezil, the assay was performed as described above, except that only one concentration of acetylthiocholine iodide (500 µM) was used. The inhibitory potency of donepezil was then determined in the presence of its increasing concentrations (1 nM to 100 µM). Each concentration was determined in triplicate.

Enzyme kinetic parameters were determined according to Jackisch et al. (2009). Data were analyzed using either *t* tests to compare two conditions or one-way ANOVA (post-hoc test: Dunnett-Test) to compare multiple conditions. In both cases a *p*-value of 0.05 was considered significant.

5.2.6. Drugs

The following drugs were used: (2R,3S,4S)-2-(4-Methoxybenzyl)-3,4-pyrrolidinediol-3-acetate, 2-[(4-Methoxyphenyl)methyl]-3,4-pyrrolidinediol 3-acetate (= anisomycin), 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (= ODQ), KT 5823, donepezil hydrochloride monohydrate (= donepezil), 5,5′-Dithiobis(2-nitrobenzoic acid) (= DTNB), 5,11-Dihydro-11-[(4-methyl-1-piperazinyl)acetyl]-6H-pyrido[2,3-b][1,4]benzodiazepin-6-one dihydrochloride (= pirenzepine) and acetylthiocholine iodide were purchased from Sigma-Aldrich Corporation (St. Louis, USA). The compound BAY 73-6691 (1-(2-Chlorophenyl)-6-[(2R)-3,3,3-trifluoro-2-methylpropyl]-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidine-4-one) was synthesized as a racemate in the Department of Medicinical Chemistry of Boehringer Ingelheim Pharma GmbH & Co KG.

5.3. Results

5.3.1. Targeting AChE

5.3.1.1. The role of ACh in early and late LTP

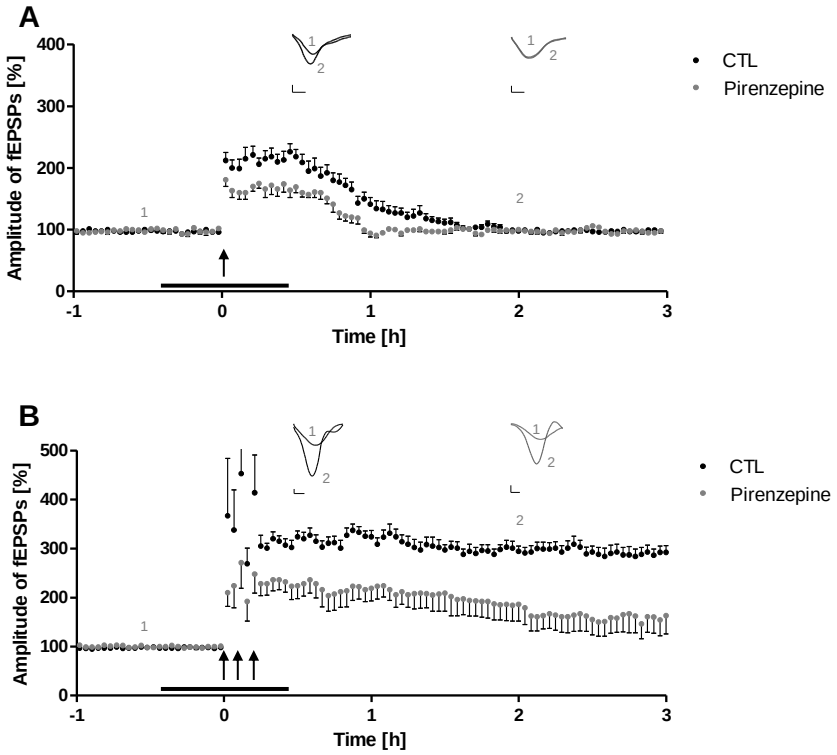


Figure 30: Role of ACh in early and late LTP.
Effect of the selective M1 muscarinic acetylcholine receptor antagonist pirenzepine on early LTP induced by weak HFS and late LTP induced by repeated strong HFS. Pirenzepine (0.1 µM) was applied 30 min before LTP induction (arrow/s) and washed out 30 min after LTP induction (horizontal bar). Individual fEPSPs before (1) and after LTP stimulation (2) are shown (horizontal bar: 0.5 mV, vertical bar: 10 ms). 0.1 µM Pirenzepine had no effect on basal fEPSPs. A) Pirenzepine decreased early LTP (control: n = 10; pirenzepine: n = 5). B) Late LTP was significantly reduced by addition of pirenzepine (control: n = 10; pirenzepine: n = 5).

To analyze whether the neurotransmitter ACh is involved in early and late LTP, the effect of pirenzepine, a selective M1 muscarinic acetylcholine receptor antagonist (Eberlein et al., 1988), was examined (Fig. 30). As pirenzepine is known to affect basal fEPSPs at some concentrations (Buchanan et al., 2010; Tabata et al., 2000; Wang et al., 2006), escalating concentrations were tested to determine a concentration having no effect on basal synaptic

transmission (data not shown). In fact, 0.1 µM was found to be the highest concentration of pirenzepine not compromising basal fEPSPs which is supported by two other studies (Calabresi et al., 1999; Bonsi et al., 2008). Therefore, 0.1 µM pirenzepine was used for LTP experiments, and this concentration caused a significant impairment of early LTP (Fig. 30A) (one hour after weak HFS; control: 141 ± 10%, n = 10; 0.1 µM pirenzepine: 1 ± 3, n = 5, $p < 0.05$) and late LTP (Fig. 30B) (one hour after repeated HFS control: 308 ± 15%, n = 10; 0.1 µM pirenzepine: 214 ± 27%, n = 5, $p < 0.01$) indicating that the neurotransmitter ACh is involved in both forms of LTP.

5.3.1.2. Expression and activity of AChE in hippocampal slices and inhibitory potency of donepezil

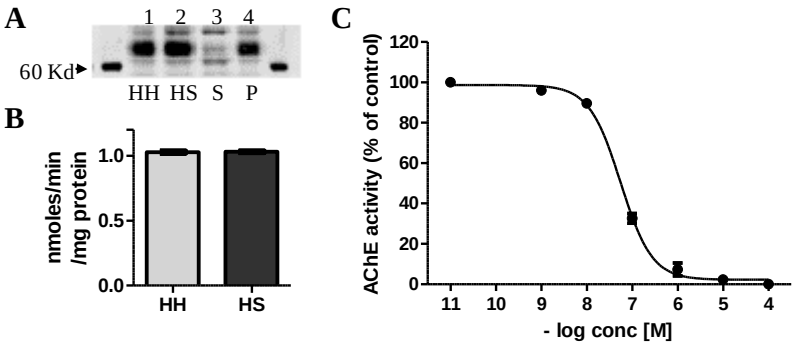


Figure 31: AChE in hippocampal slices.
Analysis of the expression and activity of AChE and the inhibitory potency of donepezil in hippocampal slices. A) Western blot analysis of the AChE expression of different samples. Lane 1 shows AChE expression in freshly prepared hippocampal homogenate (HH), lane 2 shows AChE expression in hippocampal slices homogenated 5 h after cutting (HS), lane 3 shows AChE expression in the supernatant of the hippocampal homogenate representing the cytosolic fraction (S), and lane 4 shows AChE expression in the pellet of the hippocampal homogenate representing the membrane fraction (P). B) Comparison of the AChE activity of the freshly prepared hippocampal homogenate (HH) and the hippocampal slices homogenated 5 h after cutting (HS). C) Inhibitory potency of donepezil on the AChE activity of hippocampal slice homogenate indicating that AChE activity can be completely abolished.

As shown by western blotting (Fig. 31A), AChE expression can be detected in freshly prepared hippocampal homogenate (HH, lane 1) and in hippocampal slices homogenated five hours after cutting (HS, lane 2). The supernatant (S) of the hippocampal homogenate after its centrifugation (i.e. the cytosolic fraction), shown in lane three, exhibits only a weak AChE immunoreactive band compared to the pellet (P) (i.e. the membrane fraction), shown in lane four, indicating that AChE is mainly attached to the membrane of cells. In Fig. 31B the AChE activity of HH and HS is compared demonstrating that both probes have a specific activity of about 1 nmol/min/mg protein (HH: 1.029 ± 0.01288; HS: 1.032 ± 0.01100). Fig. 31C shows the inhibitory potency of donepezil on the AChE activity in HS indicating that AChE activity can be completely abolished. The IC50 for donepezil was determined to be 55 nM.

5.3.1.3. The effect of donepezil on early and late LTP

Fig. 32 shows the effects of donepezil on early and late LTP. This compound affects early LTP in a concentration dependent manner (0.01 – 10 µM) demonstrating a significant prolongation at 0.1 – 10 µM. This prolongation results in an increased early LTP at one hours after LTP stimulation (Fig. 32A) (one hour after weak HFS control: 142 ± 11%, $n = 10$; 0.01 µM donepezil: 146 ± 14%, $n = 5$, not significant (= ns); 0.1 µM donepezil: 192 ± 17%, $n = 5$, $p < 0.05$; 1 µM donepezil: 189 ± 16%, $n = 5$, $p < 0.05$; 10 µM donepezil: 185 ± 23%, $n = 5$, $p < 0.05$). This enhancement was transient and lasted up to maximally three hours after weak HFS. In contrast, none of the tested concentrations (0.01 – 10 µM) of donepezil showed a significant effect on late LTP (Fig. 32B) (one hour after repeated HFS control: 312 ± 17%, $n = 10$; 0.01 µM donepezil: 296 ± 21%, $n = 5$, ns; 0.1 µM donepezil: 312 ± 18%, $n = 5$, ns; 1 µM donepezil: 313 ± 30%, $n = 5$, ns; 10 µM donepezil: 296 ± 39%, $n = 5$, ns). Basal fEPSPs were not affected by any concentration of donepezil.

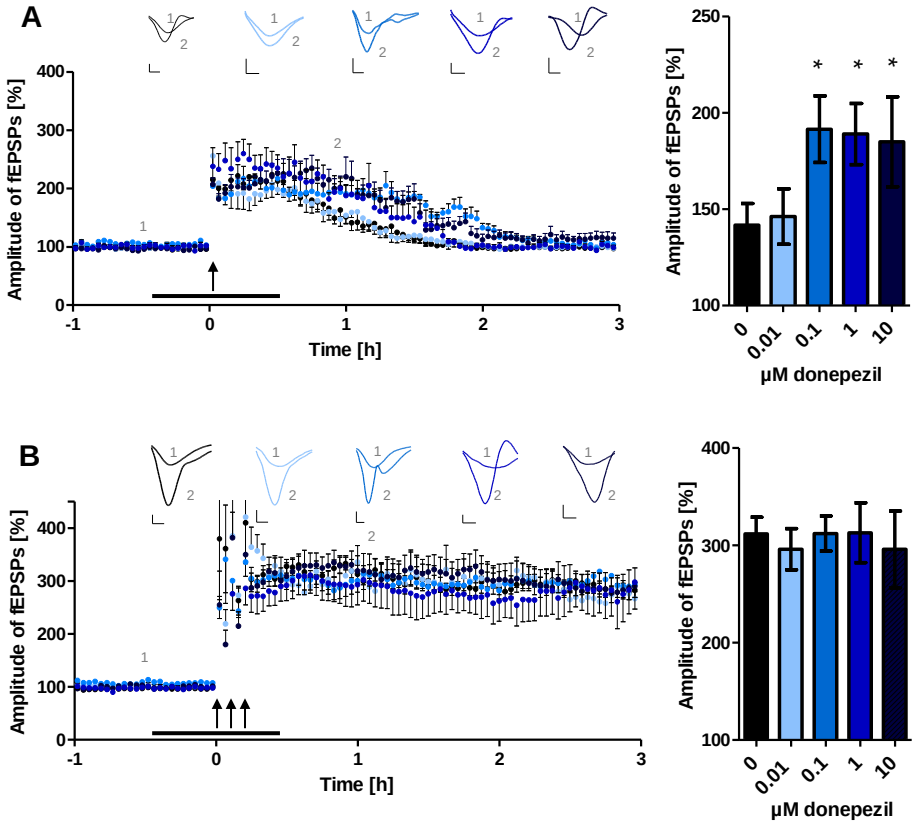


Figure 32: Action of donepezil on early and late LTP.
Effect of the AChE inhibitor donepezil on early LTP induced by weak HFS and late LTP induced by repeated strong HFS. Donepezil (0.01 – 10 µM) was applied 30 min before LTP induction (arrow/s) and washed out 30 min after LTP induction (horizontal bar). Individual fEPSPs before (1) and after LTP stimulation (2) are shown (horizontal bar: 0.5 mV, vertical bar: 10 ms). None of the concentrations of donepezil had an effect on basal fEPSPs. Histograms show the amplitude (mean ± S.E.M.; control: *n* = 10; each concentration of donepezil: *n* = 5) of fEPSPs measured between 50 and 60 min after HFS. A) Early LTP was enhanced in a concentration dependent manner by donepezil. B) None of the tested concentrations of donepezil showed a significant effect on late LTP.

5.3.2. Targeting PDE9A

5.3.2.1. The NO pathway in early and late LTP

To check whether the NO pathway is involved in early and late LTP, the effect of ODQ, a specific inhibitor of soluble guanylyl cyclase (Garthwaite et al., 1995), was examined (Fig. 33). 5 µM of ODQ, a concentration known to be sufficient to block soluble guanylyl cyclase (Lu et al., 1999), had no effect on early LTP (Fig. 33A) (one hour after weak HFS;

control: 128 ± 11%, *n* = 10; 5 µM ODQ: 131 ± 17%, *n* = 5, ns), but completely blocked late LTP within three hours (Fig. 33B) (one hour after repeated HFS; control: 321 ± 15%, *n* = 10; 5 µM ODQ: 218 ± 20%, *n* = 5, $p < 0.01$). Basal fEPSPs were not affected by 5 µM of ODQ.

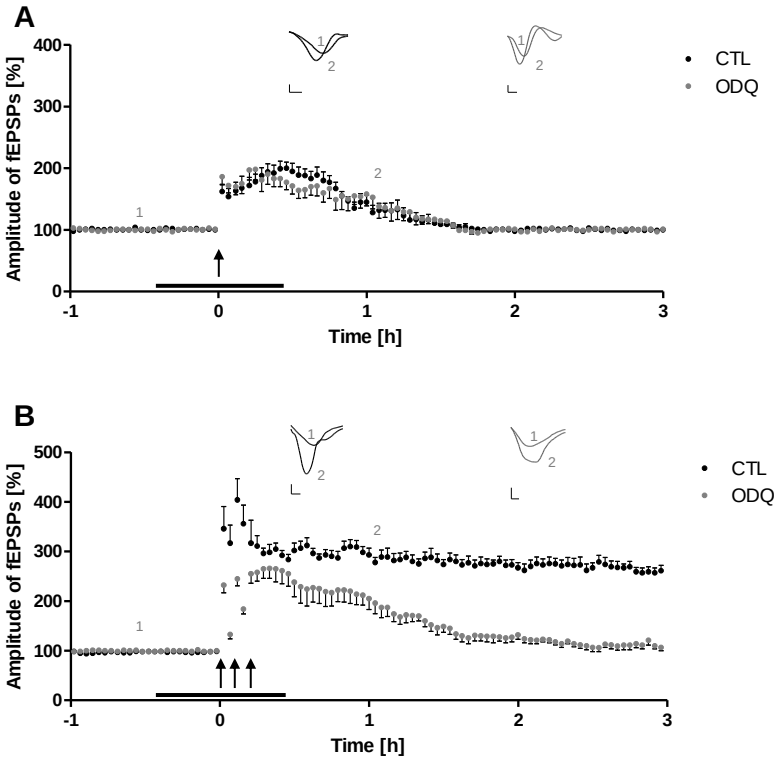


Figure 33: The NO pathway in early and late LTP.
Effect of the specific soluble guanylyl cyclase inhibitor ODQ on early LTP induced by weak HFS and late LTP induced by repeated strong HFS. ODQ (5 µM) was applied 30 min before LTP induction (arrow/s) and washed out 30 min after LTP induction (horizontal bar). Individual fEPSPs before (1) and after LTP stimulation (2) are shown (horizontal bar: 0.5 mV, vertical bar: 10 ms). ODQ had no effect on basal fEPSPs. A) ODQ did not affect early LTP (control: *n* = 10; ODQ: *n* = 5). B) Late LTP was significantly reduced up to a complete abolishment by addition of ODQ (control: *n* = 10; ODQ: *n* = 5).

5.3.2.2. The effect of BAY 73-6691 on early and late LTP

In Fig. 34, the effects of BAY 73-6691 on early and late LTP are shown. This compound increased both early LTP (Fig. 34A) (one hour after weak HFS; control: 135 ± 12%,

n = 10; 0.1 µM BAY 73-6691: 128 ± 18, *n* = 5, ns; 1 µM BAY 73-6691: 220 ± 20, *n* = 5, *p* < 0.01; 10 µM BAY 73-6691: 195 ± 11%, *n* = 5, *p* < 0.01) and late LTP (Fig. 34B) (one hour after repeated HFS; control: 316 ± 16%, *n* = 10; 0.1 µM BAY 73-6691: 308 ± 14%, *n* = 5, ns; 1 µM BAY 73-6691: 357 ± 14%, *n* = 5, *p* < 0.05; 10 µM BAY 73-6691: 345 ± 20%, *n* = 5, *p* < 0.05) in a concentration dependent manner (0.01 – 10 µM) showing a significant enhancement at 1 - 10 µM for both forms of LTP. In both cases, these enhancements lasted for more than three hours after LTP stimulation. Basal fEPSPs were not affected by any concentration of BAY 73-6691.

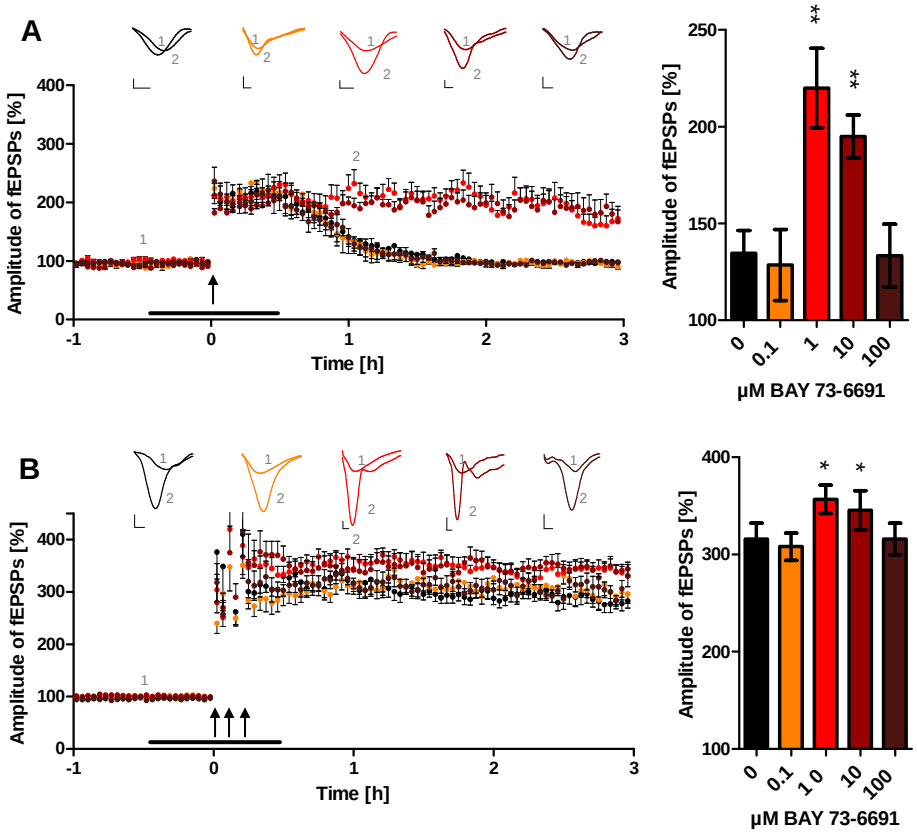


Figure 34: Effect of BAY 73-6691 on early and late LTP.
Effect of the PDE9A inhibitor BAY 73-6691 on early LTP induced by weak HFS and late LTP induced by repeated strong HFS. BAY 73-6691 (0.1 – 10 µM) was applied 30 min before LTP induction (arrow/s) and washed out 30 min after LTP induction (horizontal bar). None of the concentrations of BAY 73-6691 had an effect on basal fEPSPs. Individual fEPSPs before (1) and after LTP stimulation (2) are shown (horizontal bar: 0.5 mV, vertical bar: 10 ms). Histograms show the amplitude (mean ± S.E.M.; control: *n* = 10; each concentration of BAY 73-6691: *n* = 5) of fEPSPs measured between 50 and 60 min after HFS. A) Early LTP was enhanced by BAY 73-6691 in a concentration dependent manner. B) BAY 73-6691 increased late LTP concentration dependently.

5.3.2.3. Transformation of early into late LTP due to BAY 73-6691

To analyze whether the long-lasting increase of early LTP by BAY 73-6691 is attributed to the transformation of protein-synthesis independent early LTP into protein-synthesis dependent late LTP, the reversible protein translation inhibitor anisomycin was used at a concentration of 30 µM, which is known to be sufficient to significantly impair protein-synthesis dependent late LTP (Kroker et al., 2011b; Lu et al., 1999). As shown in Fig. 35A, the addition of 30 µM anisomycin to 1 µM BAY 73-6691, a concentration shown to increase early LTP (see Fig. 34A), caused a significant decrease of BAY 73-6691-mediated enhancement of early LTP up to a complete abolishment within two to three hours after LTP induction (one hour after weak HFS; control: 135 ± 12%, n = 10; 1 µM BAY 73-6691: 220 ± 20%, n = 5, p < 0.01; 1 µM BAY 73-6691 + 30 µM anisomycin: 150 ± 14%, n = 5, ns). This indicates that protein-synthesis independent early LTP was transformed into protein-synthesis dependent late LTP by BAY 73-6691. Basal fEPSPs were not affected by the mixture of BAY 73-6691 and anisomycin.

To test whether the transformation from early into late LTP by BAY 73-6691 is attributed to the NO pathway, the effect of the soluble guanylyl cyclase inhibitor ODQ was analyzed. 5 µM of ODQ, a concentration shown to be sufficient to significantly impair protein-synthesis dependent late LTP (see Fig. 34B), was used. As shown in Fig. 35B, the addition of 5 µM ODQ to 1 µM BAY 73-6691, a concentration shown to increase early LTP (see Fig. 5A), caused a reversal of the BAY 73-6691-mediated enhancement of early LTP (one hour after weak HFS; control: 135 ± 12%, n = 10; 1 µM BAY 73-6691: 220 ± 20%, n = 5, p < 0.01; 1 µM BAY 73-6691 + 5 µM ODQ: 139 ± 16%, n = 5, ns). This indicates that the transformation from early into late LTP by BAY 73-6691 is based on the NO pathway. Basal fEPSPs were not affected by the mixture of BAY 73-6691 and ODQ.

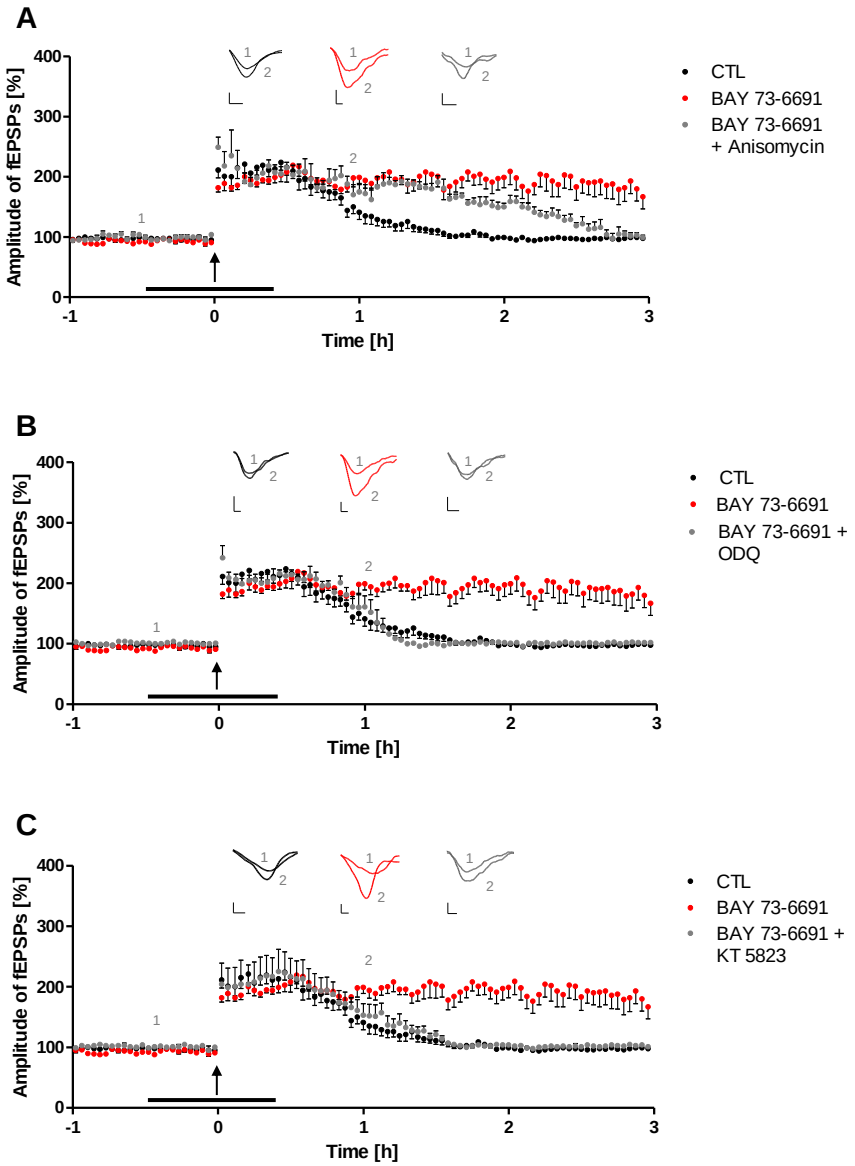


Figure 35: Transformation of early LTP into late LTP by BAY 73-6691.
BAY 73-6691 transforms protein synthesis independent LTP into protein-synthesis dependent LTP via the NO-pathway and activation of PKG. Individual fEPSPs before (1) and after LTP stimulation (2) are shown (horizontal bar: 0.5 mV, vertical bar: 10 ms). A) To analyze whether BAY 73-6691 transforms protein-synthesis independent LTP (= control: n = 10) into protein-synthesis dependent LTP, a mixture of BAY 73-6691 (1 µM) and the protein-synthesis inhibitor anisomycin (at 30 µM) was applied 30 min before weak HFS stimulation (arrow) and remained until 30 min after LTP stimulation (horizontal bar). BAY 73-6691-enhanced LTP (n = 5) lasted longer than 3 h, but was significantly reduced up to a complete abolishment by addition of anisomycin (n = 5). Neither BAY 73-6691 alone, nor the mixture of BAY 73-6691 and anisomycin, had an effect on basal fEPSPs. B) To test whether the transformation from early LTP (= control: n = 10) into late LTP by BAY 73-6691 is based on the NO pathway, a mixture of BAY 73-6691 (1 µM) and the soluble guanylyl cyclase inhibitor ODQ (at 5 µM) was applied 30 min before weak HFS stimulation (arrow) and remained until 30 min after LTP stimulation (horizontal bar).

continued:
BAY 73-6691-enhanced LTP (*n* = 5) was significantly reduced and the effect of BAY 73-6691 was completely reversed (*n* = 5). Neither BAY 73-6691 alone, nor the mixture of BAY 73-6691 and ODQ, had an effect on basal fEPSPs. C) To analyze whether BAY 73-6691 transforms early LTP (= control: *n* = 10) into late LTP through the activation of PKG, a mixture of BAY 73-6691 (1 µM) and the PKG-inhibitor KT 5823 (at 2 µM) was applied 30 min before weak HFS stimulation (arrow) and remained until 30 min after LTP stimulation (horizontal bar). BAY 73-6691-enhanced LTP (*n* = 5) was significantly reduced and the effect of BAY 73-6691 was completely reversed (*n* = 5). Neither BAY 73-6691 alone, nor the mixture of BAY 73-6691 and KT 5823, had an effect on basal fEPSPs.

To analyze whether BAY 73-6691 transforms early into late LTP through the activation of PKG, the PKG-inhibitor KT 5823 was used at a concentration of 2 µM, which is known to be sufficient to significantly impair protein-synthesis dependent late LTP (Lu et al., 1999). As shown in Fig. 35C, the addition of 2 µM KT 5823 to 1 µM BAY 73-6691, a concentration shown to increase early LTP (see Fig. 34A), caused a reversal of the BAY 73-6691-mediated enhancement of early LTP (one hour after weak HFS; control: 135 ± 12%, *n* = 10; 1 µM BAY 73-6691: 220 ± 20, *n* = 5, $p < 0.01$; 1 µM BAY 73-6691 + 2 µM KT 5823: 138 ± 19%, *n* = 5, ns). This indicates that the transformation from early into late LTP by BAY 73-6691 is attributed to the activation of PKG. Basal fEPSPs were not affected by the mixture of BAY 73-6691 and KT 5823.

5.4. Discussion

The AChE inhibitor donepezil is the current gold standard for symptomatic treatment of mild-to-moderate AD patients (Birks and Harvey, 2003; Whitehead et al., 2004). By blocking AChE, it increases and prolongs the concentration of ACh in the synaptic cleft in order to compensate for the cholinergic deficit in AD patients. These increased extracellular levels of ACh may activate metabotropic muscarinic and ionotropic nicotinic acetylcholine receptors, which finally lead to the pro-cognitive effects of donepezil. However, experience from clinical practice with AChE inhibitors has shown only moderate efficacy, emergence of tolerance after long-term use, interaction of cholinergic and non-cholinergic effects and possible effects of amyloid precursor protein release (Courtney et al., 2004; Diniz et al., 2009; Helou and Rhalimi, 2010; Hernandez et al., 2009; Kaduszkiewicz et al., 2005; Rosenbloom et al., 2010; Rozzini et al., 2004). As a potential new approach, inhibition of specific PDEs has come into the focus of interest for the treatment of memory impairment (Menniti et al., 2006; Reneerkens et al., 2009; Schmidt, 2010). One of these PDEs is PDE9A, which contributes to the intracellular NO-sGC-cGMP signalling cascade by its cGMP hydrolytic activity (Arancio et al., 2001; Son et al., 1998; Zhuo et al., 1994). Under physiological conditions, cGMP is formed by NO-sensitive soluble guanylyl cyclases (NO-GCs) (Garthwaite, 2008). These NO-GCs are activated by NO, which is generated by calcium/calmodulin-dependent neuronal NO synthases (Christopherson et al., 1999). NO is believed to act as a retrograde messenger and has been implicated as a neuromodulator in synaptic transmission (Böhme et al., 1991; Boehning and Snyder, 2003; Garthwaite, 2008; O'Dell et al., 1991; Schuman and Madison, 1991). Indeed, presynaptic cGMP facilitates glutamate release (Neitz et al., 2011). Furthermore, cGMP acts postsynaptically as part of the sGC-cGMP-PKG pathway, known to indirectly activate the transcription factor CREB (Ko and Kelly, 1999; Lu et al., 1999). Thus, overall elevation of cGMP results in increased glutamate release presynaptically and increased phosphorylation of CREB postsynaptically, both being important mechanisms for learning and memory (Blokland et al., 2006; Prickaerts et al., 2004; Rutten et al., 2007; Silva, et al., 1998; Son et al., 1998). Indeed, it was shown that PDE9A inhibitors can increase cGMP levels in the brain of animals (Verhoest et al., 2009) and in CSF of animals and humans (Hutson et al., 2011; Nicholas et al., 2009), and furthermore, memory enhancing efficacy of the PDE9A inhibitor BAY 73-6691 was demonstrated in various

animal cognition tasks (Hutson et al., 2011; van der Stay et al., 2008). In order to gain more insight into the mechanistic roles of AChE inhibitors and PDE9A inhibitors in learning and memory, we analyzed the effects of donepezil and the potent, selective PDE9A inhibitor BAY 73-6691 (Wunder et al., 2005) on LTP measured in rat hippocampal slices.

We report here that donepezil is capable of prolonging and hence enhancing early LTP in rat hippocampal slices in a concentration dependent manner with a minimal efficacious concentration of 0.1 µM (see Fig. 32A). This supports previous findings showing that donepezil is able to delay the decay of LTP *in vivo* (Barnes et al., 2000) - the only LTP study published so far using donepezil - and to enhance cognitive function in animals (McCarthy et al., 2011; Prickaerts et al., 2005; van der Staay and Bouger, 2005) and humans (Jacobson and Sabbagh, 2008; Rogers et al., 1998; Seltzer et al., 2004; Tsao and Heilman, 2005). In our study, donepezil had no effect on late LTP (see Fig. 32B) which is in agreement with a previous report on the failure of the AChE inhibitor galantamine to increase LTP induced by two tetani (100 Hz for 1 s, 5 s time interval; Lagostena et al., 2008), a stimulation protocol which is believed to induce late LTP (Albensi et al., 2007; Huang and Kandel, 1994). Donepezil having no effect on late LTP cannot be attributed to an insufficient increase of ACh by LTP stimulation, since the muscarinic acetylcholine receptor antagonist pirenzepine caused an impairment of late LTP (see Fig. 30) suggesting that the cholinergic system is indeed involved in late LTP and functional in the slice preparation. Moreover, AChE expression, AChE activity and blockade of AChE activity by donepezil were confirmed in hippocampal slices (see Fig. 31A-C). It also cannot be attributed to changes of AChE expression in hippocampal slices (see Fig. 31A), nor to a decrease of AChE activity in hippocampal slices versus freshly prepared homogenate (Fig. 31B), nor a failure of donepezil to block AChE activity in hippocampal slices (Fig. 31C: the IC50 of donepezil is roughly in the range of published data (Geerts et al., 2005; Jackisch et al., 2009; Naik et al., 2009)). Thus, even though donepezil has the potential to augment early LTP, we show that it has only limited impact on synaptic plasticity, since it cannot enhance late LTP.

The differentiation between early and late LTP is of importance for the evaluation of downstream effects. Early LTP is independent of protein-synthesis (Bliss and Collingridge, 1993; Kroker et al., 2011b; Malenka and Nicoll, 1999; Reyman and Frey, 2007) and involves modifications of pre-existing synapses as a result of rapid calcium influx through NMDA receptors and subsequent phosphorylation events (Derkach et al., 1999; Malenka

and Bear, 2004) as well as insertion of additional AMPA receptors into the postsynaptic membrane (Davies et al., 1989; Liao et al., 2001; Lu et al., 2001; Malenka and Bear, 2004; Malinow, 2003; Shi et al., 1999, 2001). The time-window of early LTP corresponds with the duration of short-term memory as defined by some researchers, i.e. short-term memory is a form of memory which does not require gene expression and protein-synthesis (Grecksch and Matthies, 1980; Izquierdo et al., 2002). Late LTP is clearly separated from early LTP by its dependence on *de novo* protein-synthesis (Frey et al., 1988; Huang, 1998, 1996; Huang and Kandel, 1994; Kroker et al., 2011b; Nguyen et al., 1991; Nguyen and Kandel, 1997; Reymann and Frey, 2007). Protein-synthesis may contribute to both the modification of existing synapses and to the establishment of new synaptic contacts (Buchs and Muller, 1996; Chang and Greenough, 1984; Desmond and Levy, 1988; Lee et al., 1980; Lisman, 2003). Moreover, long-lasting forms of memory are generally believed to be protein-synthesis dependent (Costa-Mattioli et al., 2009; Grecksch and Matthies, 1980; Pang and Lu, 2004).

In this study, the role of the NO pathway in LTP was assessed using the sGC inhibitor ODQ and the results demonstrate that this pathway is important for late, but not for early LTP (see Fig. 33). These results confirm other studies showing that the involvement of the NO pathway is strongly dependent on the LTP stimulation protocol (Chetkovich et al., 1993; Gribkoff and Lum-Ragan, 1992; Haley et al., 1996; Lu et al., 1999; Malen and Chapman, 1997; O'Dell et al., 1994; Williams et al., 1993; Zhuo et al., 1999). For the PDE9A inhibitor BAY 73-6691, we show that both early and late LTP are increased in a bell-shaped manner with efficacious concentrations of 1 and 10 µM. The lack of efficacy of BAY 73-6691 at the highest concentration might result from an excessive increase in cGMP relative to the augmentation in the cAMP level as suggested by van der Staay et al. (2008). This might cause activation of compensatory mechanisms leading to alleviation of LTP enhancement. Our early LTP data confirm previous findings (van der Staay et al., 2008) showing an increase of early LTP in a bell-shaped manner. Compared to this study, we observed a slight left shift of effective concentrations, which may be due to the different protocols used to induce early LTP. The present study further demonstrates that BAY 73-6691 not only increases protein-synthesis independent LTP (early LTP) in a concentration dependent manner, but transforms it into protein-synthesis dependent LTP (late LTP) due to the activation of the NO pathway and particularly PKG (see Fig. 35). This transformation into late LTP is consistent with findings regarding the transformation of early into late LTP in mice hippocampal slices by Br-cGMP (Lu et al., 1999) and with

the basic idea that information in the short-term memory can be transferred into long term memory (Baddeley 2003; Pang et al., 2004). Hence, this study demonstrates that donepezil showed no enhancement of late LTP and only a moderate increase in early LTP, whereas inhibition of PDE9A led to a strong enhancement of early LTP with its transformation into late LTP. Furthermore, PDE9A inhibition was even able to increase protein-synthesis dependent late LTP.

In conclusion, this study demonstrates that BAY 73-6691 is more efficacious than donepezil in enhancing and prolonging LTP in rat hippocampal slices. This direct comparison of the effects the AChE inhibitor and the PDE9A inhibitor on early and late LTP indicates that PDE9A inhibition might also be more efficacious in enhancing cognitive function *in vivo*. These results may also provide a mechanistic rationale for the apparently modest efficacy of donepezil for treating memory impairment in AD patients, and suggest that there is a potential for alternative or adjunctive therapies with greater impact on synaptic plasticity, which may be superior to AChE inhibition alone.

CHAPTER 6

Effects of cognitive enhancing drugs on amyloid-beta oligomer induced impairment of LTP

Abstract

Brains of Alzheimer's disease (AD) patients are histopathologically characterized by extracellular senile plaques consisting of fibrillary amyloid-β (Aβ) deposits and by intracellular neurofibrillary tangles composed of hyperphosphorylated tau protein. Soluble Aβ oligomers, also referred to as Aβ derived diffusible ligands (ADDLs), are thought to be neuro- or synaptotoxic Aβ-species causative for AD. In order to gain more insight into the mechanistic role of ADDLs in a cellular model of learning and memory, we analyzed their effect on hippocampal long-term potentiation (LTP) in the CA1 region of rat hippocampal slices. This study demonstrates that early LTP, being protein-synthesis independent, and late LTP, being protein-synthesis dependent, are affected to different extents by ADDLs. Furthermore, ADDLs were shown to exclusively target NMDA receptors and/or their signaling cascades. These results corroborate the hypothesis that soluble Aβ oligomers cause synaptic dysfunction which might lead to cognitive decline seen in AD patients. Furthermore, cognitive enhancing (pre-)clinical drugs acting on different targets tested at their efficacious concentration regarding LTP enhancement were evaluated for their ability to ameliorate ADDLs induced LTP deficits with the aim to determine the potential therapeutic value of these targets as alternative strategies for the treatment of AD. The current gold standard for treatment of AD patients is the acetylcholine esterase inhibitor donepezil, which was demonstrated to slightly restore ADDLs induced impairment of early LTP, but to have no effect on ADDLs induced impairment of late LTP. The same was shown for the α4β2 nicotinic acetylcholine receptor (nAChR) agonist TC-1827. Both, the α7 nAChR partial agonist SSR180711 and the phosphodiesterase (PDE) 9A inhibitor BAY 73-6691 completely rescued early as well as late LTP impaired by ADDLs. The metabotropic glutamate 5 receptor positive allosteric modulator ADX-47273 had no effect on ADDLs impaired early LTP, but partly restored ADDLs impaired late LTP. Thus, activating α7 nAChRs and inhibiting PDE9 were found to be most effective in ameliorating ADDLs induced LTP deficits, which suggests that targeting α7 nAChRs and PDE9 might represent powerful alternative approaches for the treatment of AD.

6.1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive cognitive decline and specific pathological findings in patients. Histopathological hallmarks of AD brains are extracellular senile plaques of amyloid-β (Aβ) peptides and intracellular neurofibrillary tangles of tau protein (Gouras et al., 2005; LaFerla and Oddo, 2005; Querfurth and LaFerla, 2010). The predominant types of Aβ peptides in senile plaques are Aβ42 and Aβ40 (Fodale et al., 2006). As a result of aging and AD, subtle changes in Aβ generation, or its metabolism, may occur causing an increased production especially of the Aβ42 peptide leading to its accumulation and deposition in the brain (Borchelt et al., 1996; Duff et al., 1996; Hardy and Selkoe, 2002). As for the relationship between Aβ and neurofibrillary tangles in AD, it is suggested that the development of tau is a downstream consequence of the Aβ pathology (Hardy and Selkoe, 2002; Kitazawa et al., 2005; Oddo et al., 2008; Tseng et al., 2008). Due to the apparent involvement of Aβ in AD, the "amyloid cascade hypothesis" was postulated, which originally links the pathological process of AD and neuronal cell death to the aggregation and deposition of Aβ (Hardy and Higgins, 1992). Current investigations in animal models and human brain samples have placed a special emphasis on soluble Aβ aggregates (Dahlgren et al., 2002; Klein, 2002; Lue et al., 1999; McLean et al., 1999; Tabaton and Piccini, 2005; Walsh et al., 1999; Youssef et al., 2008), also referred to as amyloid-β derived diffusible ligands (ADDLs). Indeed, these soluble oligomers are currently believed to be the putative cause of AD, rather than insoluble plaques (Hardy and Selkoe, 2002; Small, 1998).

In order to gain more insight into the mechanistic role of ADDLs in a cellular model of learning and memory, we analyzed the effects of synthetic ADDLs on hippocampal long-term potentiation (LTP). LTP is a widely-used cellular experimental model of memory formation (Bliss and Collingridge, 1993), which can be subclassified into early and late forms of LTP having different mechanisms (Kroker et al., 2011b; Reymann and Frey, 2007). Some studies already demonstrated that ADDLs elicit an impairment of LTP in the CA1 region of the hippocampus (Chen et al., 2000; Kim et al., 2001; Klybin et al., 2004; Raymond et al., 2003; Shankar et al., 2008; Walsh et al., 2002), but the effects of ADDLs on the different forms of LTP have not been addressed so far. Therefore, in this study, we analyzed the effects of synthetic ADDLs on validated early and late hippocampal CA1 LTP. These results provide a more detailed insight into the mechanistic role of soluble Aβ oligomers in CA1 LTP and their effect on NMDA receptor signaling. Furthermore, we

evaluated several cognitive enhancing (pre-)clinical drugs acting on different targets - the acetylcholine esterase (AChE) inhibitor donepezil, the α4β2 nicotinic acetylcholine receptor (nAChR) agonist TC-1827, the α7 nAChR partial agonist SSR180711, the phosphodiesterase (PDE) 9A inhibitor BAY 73-6691 and the metabotropic glutamate 5 (mGlu5) receptor positive modulator ADX-47273 - for their ability to ameliorate ADDLs induced LTP deficits in order to determine the potential therapeutic value of these targets for the treatment of AD. All these drugs were tested at their efficacious concentration regarding LTP enhancement (Kroker et al., 2011b,c,d).

6.2. Materials and methods

6.2.1. Preparation of brain slices

Procedures involving animals and their care were conducted in conformity with institutional and European Union guidelines (EEC Council Directive 86/609) and were approved by the Ethical Committee of the responsible regional council (Tübingen). Male Wistar rats (Janvier, Le Genest Saint Isle, France) aged six to seven weeks were shortly anaesthetized with isoflurane and sacrificed by decapitation. The brains were quickly removed and immersed in ice-cold ACSF (containing in mM NaCl 124, KCl 4.9, $MgSO_4$ 8, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25.6, D-glucose 10, pH 7.4, saturated with 95% O_2 and 5% CO_2). Transverse hippocampal brain slices (400 µm thickness) were cut using a Vibratome. Slices were allowed to recover in a holding chamber containing ACSF bubbled with 95% O_2 and 5% CO_2 at room temperature for at least one hour. Slices were then transferred to integrated brain slice chambers and continuously superfused (at a flow rate of 2.5 mL min^{-1}, 25 ± 0.2 °C) with the same ACSF composition used before except for 0.5 mM $MgSO_4$. Prior to doing any electrophysiological recordings, the slices were allowed to equilibrate for at least 30 minutes.

6.2.2. Multi-slice recording

To record field excitatory postsynaptic potentials (fEPSPs) the SliceMaster multi-slice recording system (Scientifica Limited, East Sussex, UK) was used. The system is semi-automated allowing one operator to record simultaneously from an assembly of up to eight brain slices. For the present study, an optimized system described by Kroker et al. (2011a) was used. fEPSPs were elicited in the CA1 region by stimulation of the Schaffer collateral–commissural fibres in the stratum radiatum using glass electrodes with broken tips (filled with ACSF). For recording, glass electrodes (2 – 6 MΩ, filled with ACSF) were placed in the apical dendritic layer. The amplitudes of fEPSPs were used as the parameter of interest (Collingridge et al., 1983). To generate fEPSPs at a constant sub-threshold stimulus, as in previous studies (Seabrook et al., 1997), the stimulus strength of the pulses was adjusted to 20 - 30% of the fEPSP maximum and this voltage was used for the experiment. During baseline recording each slice was stimulated every 30 seconds for at

least one hour. Early LTP was induced by weak high frequency stimulation (HFS) made up of 20 pulses at the frequency of 100 Hz (Bashir et al., 1991; Kroker et al., 2011a,b), whereas late LTP was induced by repeated strong HFS consisting of 100 pulses at the frequency of 100 Hz, repeated two times in five minute intervals (Kroker et al., 2011a,b; Lu et al., 1999).

6.2.3. Data acquisition, software and analysis

A modular electrophysiology system, supplied by npi electronic GmbH (Tamm, Germany), conducts the low noise recordings of extracellular signals. AC coupled signals are amplified 1000-fold and internally filtered with a five kHz low-pass filter as well as a three Hz high-pass filter. In our study, a customized system (programmable pattern generator) was used allowing simultaneous stimulation of all slices either with a single stimulus or a specific stimulus pattern (Kroker et al., 2011a). For data acquisition and analysis the software Notocord® was used.

Data are shown as mean percent (± S.E.M.) of the baseline fEPSP amplitude. Data were analyzed using either *t* tests to compare two conditions or one-way ANOVA (post-hoc test: Dunnett-Test) to compare multiple conditions. For rescue experiments, the effect of ADDLs on LTP was compared with the effect of ADDLs and the compound on LTP. In all cases a p-value of ≤ 0.05 was considered significant. Histograms show the mean amplitude (± S.E.M.) of fEPSPs measured between 25 and 35 minutes after weak or repeated strong HFS according to Jia et al. (2010) and Kroker et al. (2011b).

6.2.4. Aβ (ADDLs) preparation and application

ADDLs were prepared according to Lambert et al. (1998) with slight modifications (Moreth et al., 2011). Briefly, Aβ42 (purchased from Bachem) was solubilized in 1,1,1,3,3,3-hexafluoro-2-propanol by sonication (10 minutes) to a final concentration of 1 mM. Then, it was flash-frozen in liquid nitrogen and lyophilized. The lyophilized Aβ42 was solubilized in 10 mM NaOH and brought up in ice cold Ham's F12 medium (w/o phenol red) to a final concentration of 100 µM (pH 7.4 at 4 °C for at least 14 hours). The

samples were centrifuged at 15.000 x g for ten minutes at 4 °C. The supernatant containing Aβ oligomers (= ADDLs) was then used in all assays as within three hours. Quality tests using atomic force microscopy, electron microscopy and dynamic light scattering DLS revealed that the ADDLs were globular shaped aggregates with a mean size of 6 ± 2 nm in height and that they were stable in ACSF for at least one hour at room temperature (Moreth et al., 2011). ADDLs were applied 30 minutes before LTP stimulation and remained in the superfusion buffer until 30 minutes after LTP stimulation. For rescue experiments, a combination of ADDLs and the respective compound was applied 30 minutes before LTP stimulation and remained in the superfusion buffer until 30 minutes after LTP stimulation.

6.2.5. Drug solutions and application

The following drugs were used: MK-801 ((5S,10R)-(–)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate), nifedipine (1,4-Dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester) and donepezil (donepezil hydrochloride monohydrate) were purchased from Sigma-Aldrich Corporation (St. Louis, USA). The compounds ADX-47273 (S-(4-fluoro-phenyl)-{3-[3-8fluorophenyl)-[1,2,4]-oxadiazol-5-yl]-piperidin-1-yl}-methanone), TC-1827 ((S)-N-methyl-5-(5-pyrimidinyl)-4-penten-2-amine hemigalactarate), SSR180711 (1,4-Diazabicyclo[3.2.2] nonane-4-carboxylic acid, 4-bromophenyl ester) and BAY 73-6691 (1-(2-Chlorophenyl) - 6- [(2R)-3,3,3-trifluoro-2-methylpropyl] - 1,5 - dihydro - 4H-pyrazolo [3,4-d]pyrimidine-4-one; synthesized as a racemate) were synthesized in the Department of Medicinical Chemistry of Boehringer Ingelheim Pharma GmbH & Co KG.

The drugs were prepared as stock solutions and diluted in ACSF immediately before application. Nifedipine, ADX-47273, SSR180711 and BAY 73-6691 were prepared in DMSO (the final concentration of DMSO was 0.1%). All drugs, except MK-801, were applied 30 minutes before LTP stimulation and remained until 30 minutes after LTP stimulation. MK-801 was used in such a way that, corresponding to Frankiewicz et al. (1996), full blockade of NMDA receptors can be expected: MK-801 was present immediately after preparing slices, thereafter for an average of 6.8 ± 0.4 hours prior to LTP stimulation and remained until 30 minutes after LTP stimulation (Kroker et al., 2011b).

6.3. Results

6.3.1. Effects of ADDLs on LTP

6.3.1.1. Effects of ADDLs on early and late LTP

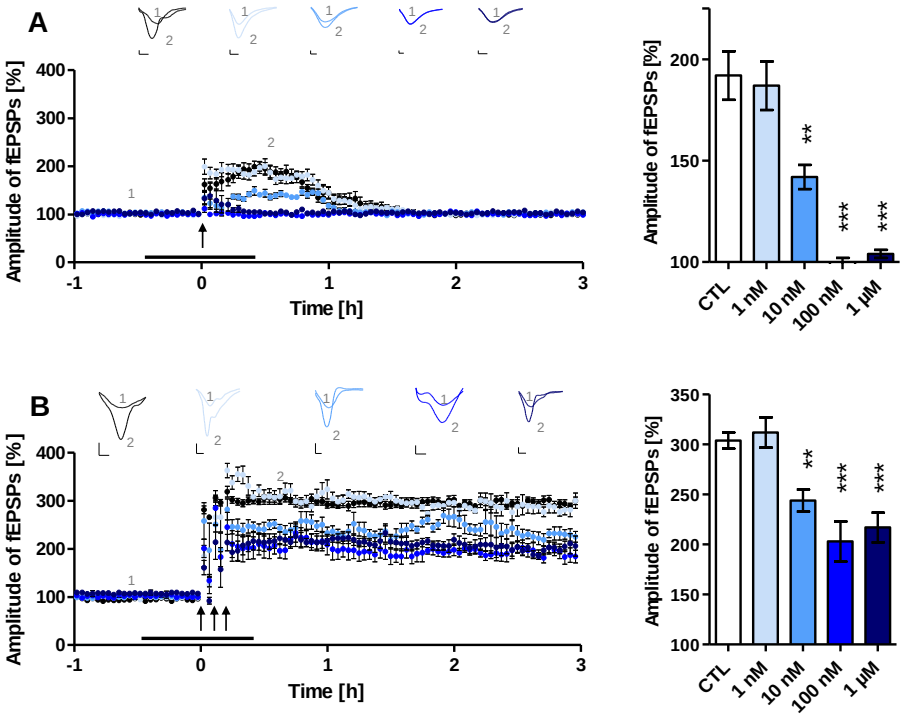


Figure 36: Impairing effects of ADDLs on early and late LTP.
Effect of ADDLs on early LTP induced by weak HFS and late LTP induced by repeated strong HFS. ADDLs (1 – 1000 nM) were applied 30 min before LTP stimulation (arrow/s) and were washed out 30 min after LTP stimulation (horizontal bar). Histograms show the amplitude of fEPSPs measured 25–35 min after delivery of LTP stimulation (mean ± SEM, *n* = 5). Individual fEPSPs before (1) and after LTP stimulation (2) are shown (horizontal bar: 0.5 mV, vertical bar: 10 ms). A) Early LTP was reduced by ADDLs in a concentration dependent manner with a complete abolishment at 100 nM. B) Late LTP was reduced by ADDLs in a concentration dependent manner with maximum effect at 100 nM. At this concentration late LTP was reduced by approximately 50%.

The effects of different concentrations of ADDLs on early and late LTP are shown in Fig. 36. Early LTP was decreased by ADDLs (1 – 1000 nM tested) in a concentration dependent manner with a complete blockade at 100 – 1000 nM (Fig. 36A) (0.5 hours after weak HFS: control: 138 ± 12%, *n* = 10; 1 nM ADDLs: 147 ± 12%, *n* = 5, not significant (ns); 10 nM ADDLs: 127 ± 7%, *n* = 5, *p* < 0.01; 100 nM ADDLs: 100 ± 3%, *n* = 5,

$p < 0.001$; 1000 nM ADDLs: 104 ± 2%, $n = 5$, $p < 0.001$). ADDLs (1 – 1000 nM tested) also decreased late LTP in a concentration dependent manner with a maximum effect at 100 – 1000 nM (Fig., 36B) (0.5 hours after repeated strong HFS; control: 299 ± 8%, $n = 10$; 1 nM ADDLs: 303 ± 10%, $n = 5$, ns; 10 nM ADDLs: 254 ± 11, $n = 5$, $p < 0.01$; 100 nM ADDLs: 217 ± 10%, $n = 5$, $p < 0.001$; 1000 nM ADDLs: 214 ± 11%, $n = 5$, $p < 0.001$). At 100 – 1000 nM ADDLs concentration, late LTP was not completely abolished, but reduced by approximately 50%. None of the concentrations of ADDLs affected basal fEPSPs.

6.3.1.2. ADDLs impair late LTP by targeting NMDA receptor signaling

The signaling pathways putatively being impaired by ADDLs in late LTP were investigated (Fig. 37). To analyze the potential effects of ADDLs on NMDA receptor signaling, 10 µM of the NMDA receptor antagonist MK-801 was applied in addition to 100 nM ADDLs. This concentration of MK-801 was reported to be sufficient to block the NMDA receptor dependent part of late LTP (Kroker et al., 2011b). As shown in Fig. 37A, MK-801 had no further effect on ADDLs mediated impairment of late LTP (0.5 hours after weak HFS; control: 299 ± 8%, $n = 10$; 100 nM ADDLs: 217 ± 10%, $n = 5$, $p < 0.001$; 100 nM ADDLs + 10 µM MK-801: 212 ± 11%, $n = 5$, $p < 0.001$). Basal fEPSPs were not affected by the mixture of ADDLs and MK-801.

To analyze the role of L-type voltage dependent calcium channel (L-VDCC) signaling, 100 µM of the L-VDCC blocker nifedipine was applied in addition to 100 nM ADDLs. This nifedipine concentration was reported to be sufficient to block the L-VDCCs dependent part of late LTP (Kroker et al., 2011b). As shown in Fig. 37B, nifedipine completely blocked the ADDLs impaired late LTP (0.5 hours after weak HFS; control: 299 ± 8%, $n = 10$; 100 nM ADDLs: 217 ± 10%, $n = 5$, $p < 0.001$; 100 nM ADDLs + 100 µM nifedipine: 99 ± 2%, $n = 5$, $p < 0.001$). Basal fEPSPs were not affected by the mixture of ADDLs and nifedipine. Thus, NMDA receptors and/or NMDA receptor downstream signaling, but not L-VDCCs and/or their downstream signaling, were apparently targeted by ADDLs.

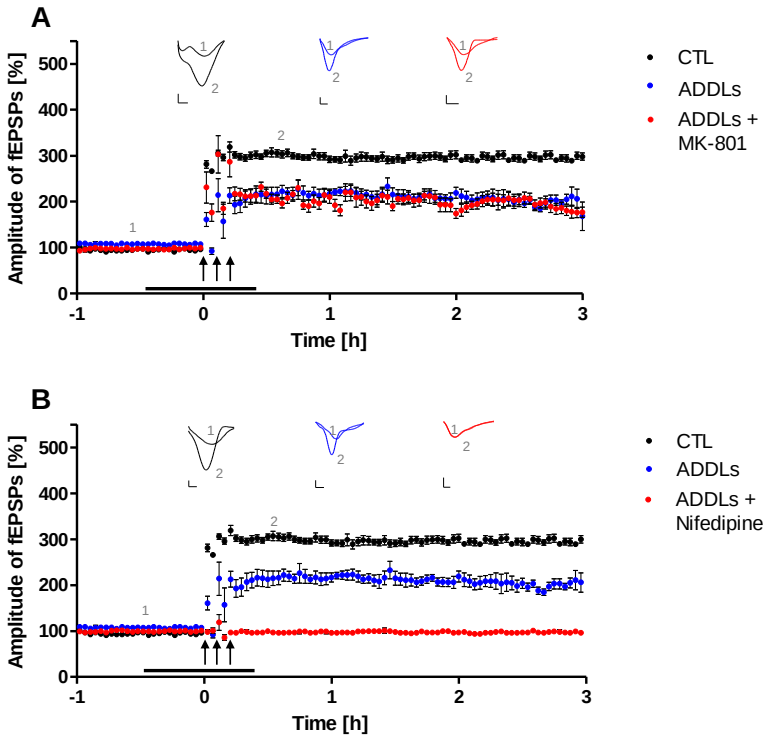


Figure 37: ADDLs impair late LTP by exclusively targeting NMDA receptors and/or their downstream signaling.

Effects of MK-801, a potent antagonist of NMDA receptors, and nifedipine, a L-VDCC blocker, on ADDLs impaired late LTP. The histograms in A and B show the amplitude (mean ± S.E.M; *n* = 5) of fEPSPs measured 25–35 min after LTP stimulation. Individual fEPSPs before (1) and after LTP stimulation (2) are shown (horizontal bar: 0.5 mV, vertical bar: 10 ms). A) 10 µM MK-801 had no effect on ADDLs mediated impairment of late LTP (100 nM). MK-801 was applied 6.8 ± 0.4 h before LTP stimulation (arrows) and was washed out 30 min after LTP stimulation (horizontal bar). ADDLs were applied 30 min before LTP stimulation (arrows) and remained until 30 min after weak HFS (horizontal bar). B) ADDLs mediated impairment of late LTP (100 nM) was completely abolished by 100 µM nifedipine. The mixture of ADDLs and nifedipine was applied 30 min before LTP stimulation (arrows) and remained until 30 min after weak HFS (horizontal bar).

6.3.2. Evaluation of cognitive enhancing (pre-)clinical drugs acting on different targets for their ability to ameliorate ADDLs induced LTP deficits

6.3.2.1. Effect of inhibiting AChE by donepezil on ADDLs mediated impairment ofv LTP

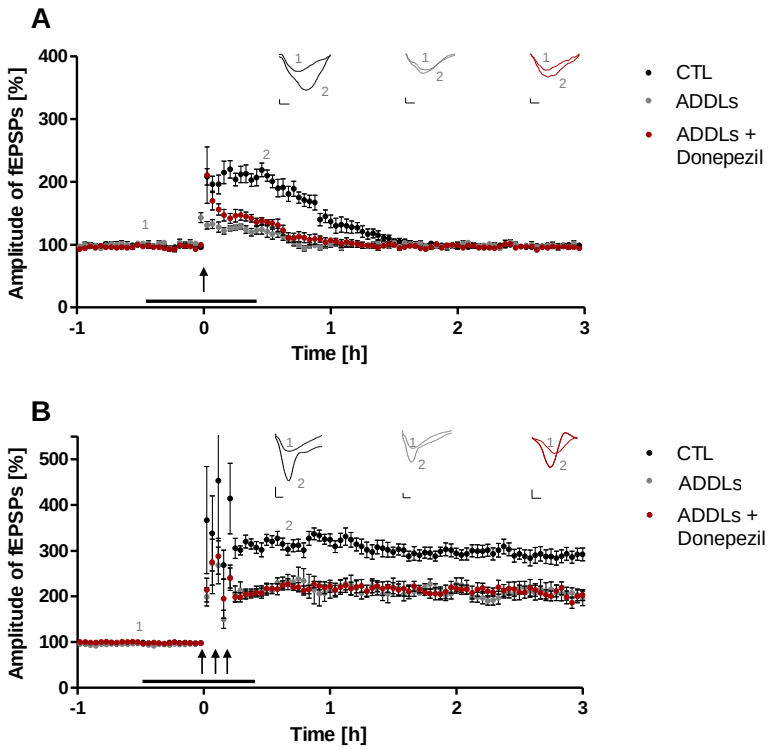


Figure 38: Effect of donepezil on ADDLs mediated impairment of LTP.
Effect of the AChE inhibitor donepezil on ADDLs mediated impairment of early and late LTP. A combination of ADDLs (20 nM) and donepezil (1 µM) was applied 30 min before LTP induction (arrow/s) and washed out 30 min after LTP induction (horizontal bar). Individual fEPSPs before (1) and after LTP stimulation (2) are shown (horizontal bar: 0.5 mV, vertical bar: 10 ms). A) Donepezil slightly restored ADDLs mediated impairment of early LTP. B) Donepezil had no effect on ADDLs mediated impairment of late LTP.

Fig. 38 shows the effect of 1 µM donepezil - a concentration known to be most efficacious in enhancing LTP (Kroker et al., 2011d) - on early and late LTP impaired by ADDLs (20 nM). Donepezil slightly restored ADDLs mediated impairment of early LTP (Fig. 38A) (0.5 hours after weak HFS; control: 198 ± 11%, *n* = 10; 20 nM ADDLs: 116 ± 7%, *n* = 5; 20 nM ADDLs + 1 µM donepezil: 129 ± 7%, *n* = 5, ns), but had no effect

on ADDLs mediated impairment of late LTP (Fig. 38B) (0.5 hours after repeated strong HFS; control: 315 ± 14%, *n* = 10; 20 nM ADDLs: 223 ± 11%, *n* = 5, 20 nM ADDLs + 1 µM donepezil: 218 ± 11%, *n* = 5, ns). Neither 20 nM ADDLs, nor 1 µM donepezil or a combination of both affected basal fEPSPs.

6.3.2.2. Effect of the a4β2 nAChR agonist TC-1827 on ADDLs mediated impairment of LTP

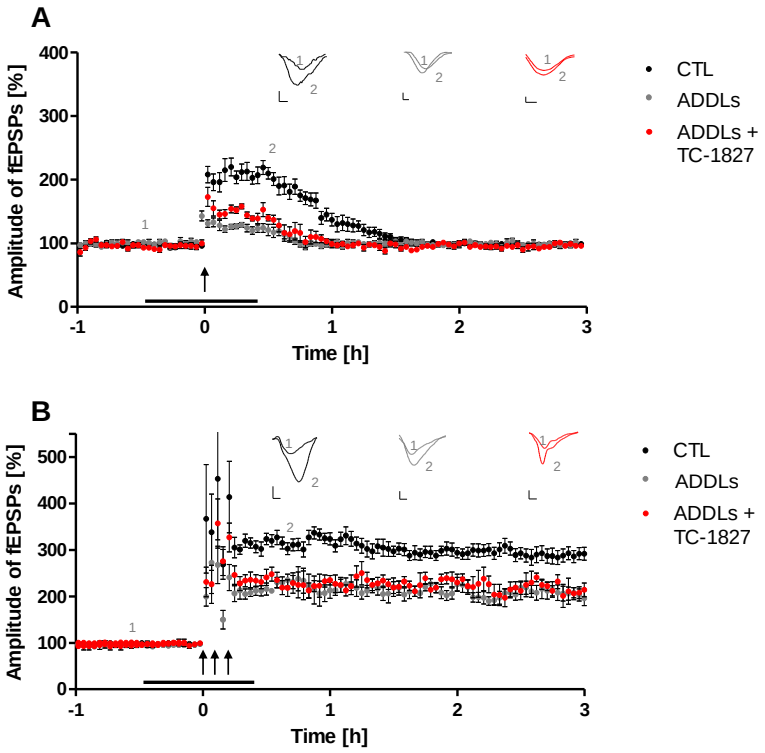


Figure 39: Effect of TC-1827 on ADDLs mediated impairment of LTP.
Effect of the α4β2 nAChR agonist TC-1827 on ADDLs mediated impairment of early and late LTP. A combination of ADDLs (20 nM) and TC-1827 (0.1 µM) was applied 30 min before LTP induction (arrow/s) and washed out 30 min after LTP induction (horizontal bar). Individual fEPSPs before (1) and after LTP stimulation (2) are shown (horizontal bar: 0.5 mV, vertical bar: 10 ms). A) TC-1827 slightly restored ADDLs mediated impairment of early LTP. B) TC-1827 had no effect on ADDLs mediated impairment of late LTP.

Fig. 39 shows the effect of 0.1 µM TC-1827 - a concentration known to be most efficacious in enhancing LTP (Kroker et al., 2011c) - on early and late LTP impaired by ADDLs (20 nM). TC-1827 slightly restored ADDLs induced impairment of early LTP (Fig. 39A) (0.5 hours after weak HFS; control: 197 ± 12%, *n* = 10; 20 nM ADDLs: 117 ± 8%, *n* = 5; 20 nM ADDLs + 0.1 µM TC-1827: 131 ± 9%, *n* = 5, ns), but had no effect on ADDLs induced impairment of late LTP (Fig. 39B) (0.5 hours after repeated strong HFS; control: 313 ± 15%, *n* = 10; 20 nM ADDLs: 222 ± 10%, *n* = 5; 20 nM ADDLs + 0.1 µM TC-1827: 232 ± 16%, *n* = 5, ns). Neither 20 nM ADDLs, nor 0.1 µM TC-1827 or a combination of both affected basal fEPSPs.

6.3.2.3. Effect of the α7 nAChR partial agonist SSR180711 on ADDLs mediated impairment of LTP

Fig. 40 shows the effect of 0.3 µM SSR180711 - a concentration known to be most efficacious in enhancing LTP (Kroker et al., 2011c) - on early and late LTP impaired by ADDLs (20 nM). SSR180711 completely rescued ADDLs mediated impairment of early LTP (Fig. 40A) (0.5 hours after weak HFS; control: 200 ±13%, *n* = 10; 20 nM ADDLs: 115 ± 6%, *n* = 5; 20 nM ADDLs + 0.3 µM SSR180711: 198 ± 26%, *n* = 5, $p < 0.05$). Also, ADDLs induced impairment of late LTP was fully restored by SSR180711 (Fig. 40B) (0.5 hours after repeated strong HFS; control: 310 ± 14%, *n* = 10; 20 nM ADDLs: 219 ± 9%, *n* = 5; 20 nM ADDLs + 0.3 µM SSR180711: 293 ± 18%, *n* = 5, $p < 0.01$). Neither 20 nM ADDLs, nor 0.3 µM SSR180711 or a combination of both affected basal fEPSPs.

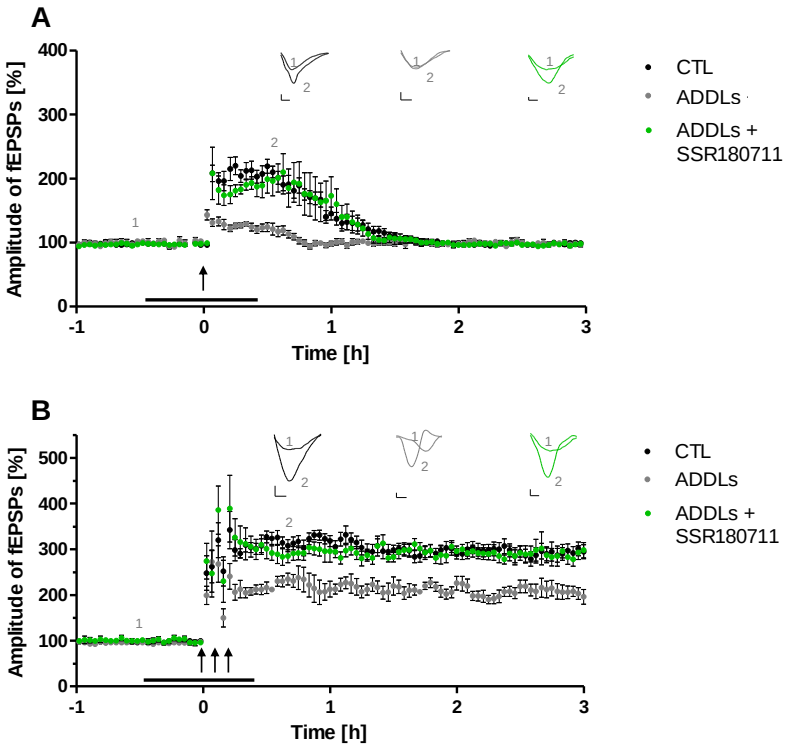


Figure 40: Effect of SSR180711on ADDLs mediated impairment of LTP.
Effect of the α7nAChR partial agonist SSR180711 on ADDLs mediated impairment of early and late LTP. A combination of ADDLs (20 nM) and SSR180711 (0.3 µM) was applied 30 min before LTP induction (arrow/s) and washed out 30 min after LTP induction (horizontal bar). Individual fEPSPs before (1) and after LTP stimulation (2) are shown (horizontal bar: 0.5 mV, vertical bar: 10 ms). A) SSR180711 completely restored ADDLs mediated impairment of early LTP. B) SSR180711 rescued ADDLs mediated impairment of late LTP.

6.3.2.4. Effect of inhibiting PDE9A by BAY 73-6691 on ADDLs mediated impairment of LTP

Fig. 41 shows the effect of 1 µM BAY 73-6691 - a concentration known to be most efficacious in enhancing LTP (Kroker et al., 2011d) - on early and late LTP impaired by ADDLs (20 nM). BAY 73-6691 completely rescued ADDLs induced impairment of early LTP (Fig. 41A) (0.5 hours after weak HFS; control: 199 ± 12%, n = 10; 20 nM ADDLs: 117 ± 7%, n = 5; 20 nM ADDLs + 1 µM BAY 73-6691: 193 ± 18%, n = 5, $p < 0.01$). Also, ADDLs induced impairment of late LTP was fully restored by BAY 73-6691 (Fig. 41B)

(0.5 hours after repeated strong HFS; control: 315 ± 15%, n = 10; 20 nM ADDLs: 225 ± 12%, n = 5; 20 nM ADDLs + 1 µM BAY 73-6691: 303 ± 17%, n = 5, p < 0.01). Neither 20 nM ADDLs, nor 1 µM BAY 73-6691 or a combination of both affected basal fEPSPs.

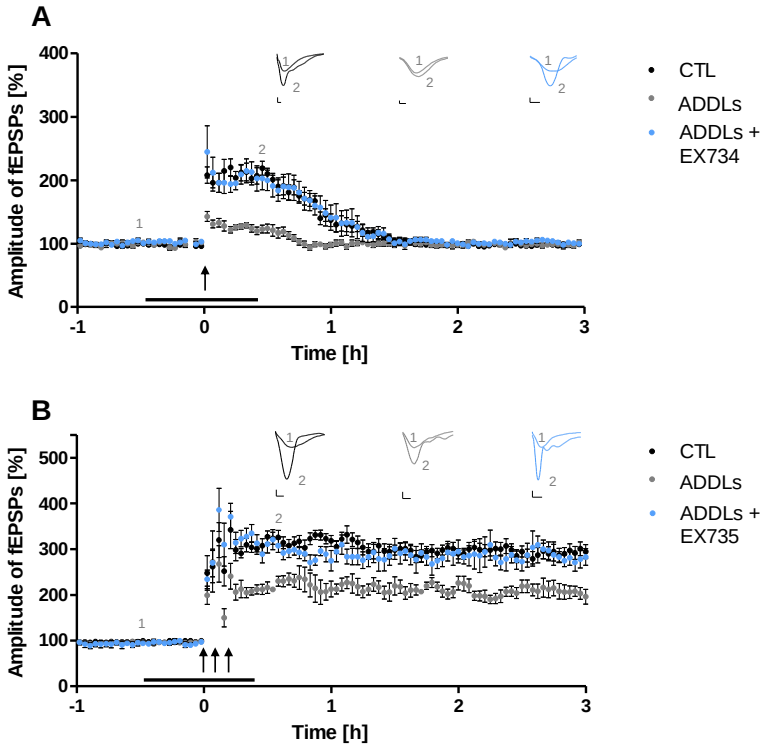


Figure 41: Effect of BAY 73-6691 on ADDLs mediated impairment of LTP.
Effect of inhibiting PDE9A by BAY 73-6691 on ADDLs mediated impairment of early and late LTP. A combination of ADDLs (20 nM) and BAY 73-6691 (1 µM) was applied 30 min before LTP induction (arrow/s) and washed out 30 min after LTP induction (horizontal bar). Individual fEPSPs before (1) and after LTP stimulation (2) are shown (horizontal bar: 0.5 mV, vertical bar: 10 ms). A) BAY 73-6691 completely restored ADDLs mediated impairment of early LTP. B) BAY 73-6691 rescued ADDLs mediated impairment of late LTP.

6.3.2.5. Effect of the mGlu5 receptor positive modulator ADX-47273 on ADDLs mediated impairment of LTP

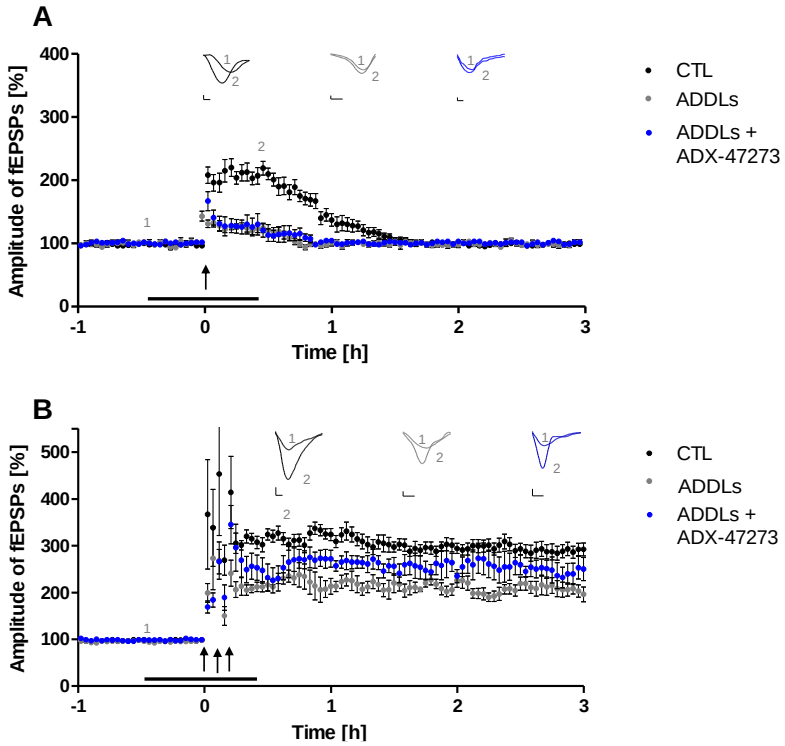


Figure 42: Effect of ADX-47273 on ADDLs mediated impairment of LTP.
Effect of the mGlu5 receptor positive modulator ADX-47273 on ADDLs mediated impairment of early and late LTP. A combination of ADDLs (20 nM) and ADX-47273 (0.3 µM) was applied 30 min before LTP induction (arrow/s) and washed out 30 min after LTP induction (horizontal bar). Individual fEPSPs before (1) and after LTP stimulation (2) are shown (horizontal bar: 0.5 mV, vertical bar: 10 ms). A) ADX-47273 had no effect on ADDLs mediated impairment of early LTP. B) ADX-47273 showed an attenuation of late LTP impaired by ADDLs.

Fig. 42 shows the effect of 0.3 µM ADX-47273 - a concentration known to be most efficacious in enhancing LTP (Kroker et al., 2011b) - on early and late LTP impaired by ADDLs (20 nM). ADX-47273 had no effect on ADDLs mediated impairment of early LTP (Fig. 42A) (0.5 hours after weak HFS; control: 198 ± 10%, n = 10; 20 nM ADDLs: 118 ± 6%, n = 5; 20 nM ADDLs + 0.3 µM ADX-47273: 115 ± 11%, n = 5, ns). However, this concentration partially restored late LTP impaired by ADDLs (Fig.42B) (0.5 hours after repeated strong HFS; control: 310 ± 16%, n = 10; 20 nM ADDLs: 221 ± 11%, n = 5; 20 nM ADDLs + 0.3 µM ADX-47273: 242 ± 29%, n = 5, $p < 0.05$). Neither 20 nM ADDLs, nor 0.3 µM ADX-47273 or a combination of both affected basal fEPSPs.

6.4. Discussion

AD is the most common form of dementia in elderly people, accounting for around 50 – 60% of all cases of mental deterioration among persons over 65 years of age (Blennow et al., 2006). Estimates foresee that more than 80 million individuals will be affected by the disease in 2040 due to population aging worldwide (Brookmeyer et al., 2007; Forlenza et al., 2010). Thus, there is a growing need of efficacious and safe medication for AD patients. Since soluble Aβ oligomers, also called ADDLs, are currently discussed as the putative cause of AD, rather than insoluble plaques (Hardy and Selkoe, 2002; Small, 1998), several research groups have reported on artificially generated Aβ oligomers and their effects *in vitro* and *in vivo* (Catalano et al., 2006; Dahlgren et al., 2002; Klein, 2002; Lue et al., 1999; Tabaton and Piccini, 2005; Youssef et al., 2008). For example, correlation of elevated levels of ADDLs in the AD brain and cognitive decline has been demonstrated (Kar et al., 2004; Näslund et al., 2000). Moreover, ADDLs are known to be responsible for abnormal dendritic spine morphology, changed synaptic receptor composition and spine loss (Lacor et al., 2007). It was reported that acute injection of ADDLs into the hippocampus of rats and mice induces impairments of learning and memory (Li et al., 2005; McDonald et al., 1994). Some studies already demonstrated that ADDLs impair LTP in the CA1 region of the hippocampus in general (Chen et al., 2000; Kim et al., 2001; Klybin et al., 2004; Raymond et al., 2003; Shankar et al., 2008; Walsh et al., 2002), but the effects of ADDLs on early and late LTP have not been addressed so far. Therefore, in this study, we analyzed the effects of synthetic ADDLs on validated early and late hippocampal CA1 LTP. Furthermore, we evaluated several cognitive enhancing drugs acting on different targets for their ability to ameliorate ADDLs induced LTP deficits in order to determine the potential therapeutic value of these targets as new approaches in the treatment of AD.

6.4.1. Effect of ADDLs on early and late LTP

Here we demonstrate for the first time that early and late LTP are affected by ADDLs to a different extent. Early LTP is decreased by ADDLs in a concentration dependent manner to a complete abolishment (see Fig. 36A). Late LTP is also decreased in a concentration dependent manner, but at maximum concentrations it is only reduced by about 50% (see

Fig. 36B). As already shown in a previous study, in this setting early LTP is dependent on NMDA receptors only, whereas late LTP is equally dependent on NMDA receptors and L-type voltage depended calcium channels (L-VDCCs) (Kroker et al., 2011b). Hence, ADDLs can completely abolish NMDA receptor dependent early LTP. Furthermore, by the use of MK-801 and nifedipine, it was demonstrated that the portion of late LTP being impaired by ADDLs was dependent on NMDA receptors and/or NMDA receptor downstream signaling (see Fig. 37). The results confirm published data showing that ADDLs reduce postsynaptic surface NMDA receptors (Goto et al., 2006; Johansson et al., 2006; Snyder et al., 2005) and impair NMDA receptor function (Snyder et al., 2005; Tyszkiewicz and Yan, 2005). Additionally, studies have implicated ADDLs to interfere with several major signaling pathways downstream of the NMDA receptor (Snyder et al., 2005) including the calcium-dependent protein phosphatase calcineurin (Chen et al., 2002), CaMKII (Zhao et al., 2004), protein phosphatase 1 (Knobloch et al., 2007) and CREB (Chen et al., 2002). Indeed, post-mortem tissue analysis of the brains of AD patients indicated a reduction of NMDA receptors (Hynd et al., 2001; Penney et al., 1990; Ulas and Cotman, 1997), especially of the subunits NR1, NR2A and NR2B in the hippocampus (Hynd et al., 2004). Similarly, a trend towards a reduction in NR1 mRNA was found in the hippocampus in AD by in situ hybridization (Panegyres et al., 2002).

The differentiation between early and late LTP, as it is made in this study, is of importance for the evaluation of downstream effects. Early LTP is independent of protein-synthesis (Bliss and Collingridge, 1993; Kroker et al., 2011b; Malenka and Nicoll, 1999; Reyman and Frey, 2007) and involves modifications of pre-existing synapses as a result of rapid calcium influx through NMDA receptors and subsequent phosphorylation events (Derkach et al., 1999; Malenka and Bear, 2004) as well as insertion of additional AMPA receptors into the postsynaptic membrane (Davies et al., 1989; Liao et al., 2001; Lu et al., 2001; Malenka and Bear, 2004; Malinow, 2003; Shi et al., 1999, 2001). Late LTP is clearly separated from early LTP by its dependence on *de novo* protein-synthesis (Frey et al., 1988; Huang, 1996, 1998; Huang and Kandel, 1994; Kroker et al., 2011b; Nguyen et al., 1991; Nguyen and Kandel, 1997; Reymann and Frey, 2007). Protein-synthesis may contribute both to the modification of existing synapses and to the establishment of new synaptic contacts (Buchs and Muller, 1996; Lee et al., 1980; Lisman, 2003). Thus, like memory has different phases, namely short-term and long-term memory, LTP consists of different phases as well. The time-window of early LTP corresponds with the duration of short-term memory as defined by some researchers, i.e. short-term memory is a form of

memory which does not require gene expression and protein-synthesis (Grecksch and Matthies, 1980; Izquierdo et al., 2002). Moreover, long-lasting forms of memory are generally believed to be protein-synthesis dependent (Costa-Mattioli et al., 2009; Grecksch and Matthies, 1980; Pang and Lu, 2004), which is also the case for late LTP.

6.4.2. Cognitive enhancing (pre-)clinical drugs and their ability to ameliorate ADDLs induced LTP deficits

The potential therapeutic value of the different approaches currently pursued in clinical development for the symptomatic treatment of AD was determined by testing (tool) compounds addressing these targets for their ability to ameliorate ADDLs induced LTP deficits.

The AChE inhibitor donepezil is the current gold standard for symptomatic treatment of mild-to-moderate AD patients (Birks and Harvey, 2003; Whitehead et al., 2004). In this study we show that donepezil only slightly restores ADDLs induced LTP impairment in early LTP and has no effect in late LTP (see Fig. 38). The small effect in early LTP might contribute to explain the only moderate therapeutic efficacy of donepezil in AD patients (Courtney et al., 2004; Diniz et al., 2009; Kaduszkiewicz et al., 2005).

Further promising targets for improvement of cognitive function and hence candidate targets for AD treatment are nAChRs (Haydar and Dunlop, 2010; Holladay et al., 1997; Lloyd and Williams, 2000; Mudo et al., 2007; Sarter et al., 2009). For both, α4β2 and α7 nAChR agonists, memory enhancing efficacy was demonstrated in several animal cognition tests (Bohme et al., 2004; Chan et al., 2007; Pichat et al., 2007; Wallace et al., 2011). Hence, these targets are gaining increasing interest and some specific modulators are already being evaluated in clinical trials for treatment of AD and/or cognitive impairment in schizophrenia (Freedman et al., 2001; Grassi et al., 2003; Hajós and Rogers, 2010; Leiser et al., 2009; Nordberg, 2001; Radek et al., 2010; Sarter et al., 2009; Thomsen et al., 2010). In this study, we demonstrate for the first time that the α4β2 nAChR agonist TC-1827 is capable to slightly restore early LTP impaired by ADDLs, but has no effect on late LTP (see Fig. 39). Furthermore, it was shown that complete rescues of ADDLs mediated impairment of early and late LTP are possible using the α7 nAChR partial agonist SSR180711 (see Fig. 40). These results confirm previous data, showing that α7

nAChR agonists can rescue Aβ oligomer induced impairment of LTP (Chen et al., 2006, 2010; Welsby et al., 2007). Therefore, α7 nAChR agonism seems to be more efficacious in ameliorating ADDLs induced LTP deficits than α4β2 nAChR agonism.

Another potential new approach, inhibition of specific PDEs, has come into the focus of interest for the treatment of memory impairment (Menniti et al., 2006; Reneerkens et al., 2009; Schmidt, 2010). It was shown that PDE9A inhibitors can increase cGMP levels in the brain of animals (Verhoest et al., 2009) and in CSF of animals and humans (Nicholas et al., 2009). Furthermore, memory enhancing efficacy of the PDE9A inhibitors PF-04447943 and BAY 73-6691 was demonstrated in various animal cognition tasks (Hutson et al., 2011; van der Stay et al., 2008). Here, using BAY 73-6691, we show for the first time that ADDLs induced impairment of early as well as late LTP can be completely rescued by PDE9-inhibition (see Fig. 41).

As evidence accumulates that amplification of mGlu5 receptor function could be beneficial in enhancing cognitive function (Cleva and Olive, 2011; Simonyi et al., 2010), a series of selective mGlu5-receptor positive allosteric modulators (Conn et al., 2009b) were generated. Among these, CDPPB and ADX-47273 were shown to be active in preclinical models that might predict efficacy for the treatment of positive symptoms and cognitive dysfunction associated with schizophrenia (Liu et al., 2008; Rosenbrock et al., 2010; Schlumberger et al., 2009, 2010; Vardigan et al., 2010). As mGlu5 receptor allosteric modulators might also be candidates for AD treatment, in this study, the ability of ADX-47273 to ameliorate ADDLs induced LTP deficits was evaluated. Here, we show for the first time that the mGlu5 receptor positive modulator ADX-47273 has no effect on ADDLs impaired early LTP, but can partially restore ADDLs induced impairment of late LTP (see Fig. 42).

6.4.3. Conclusion

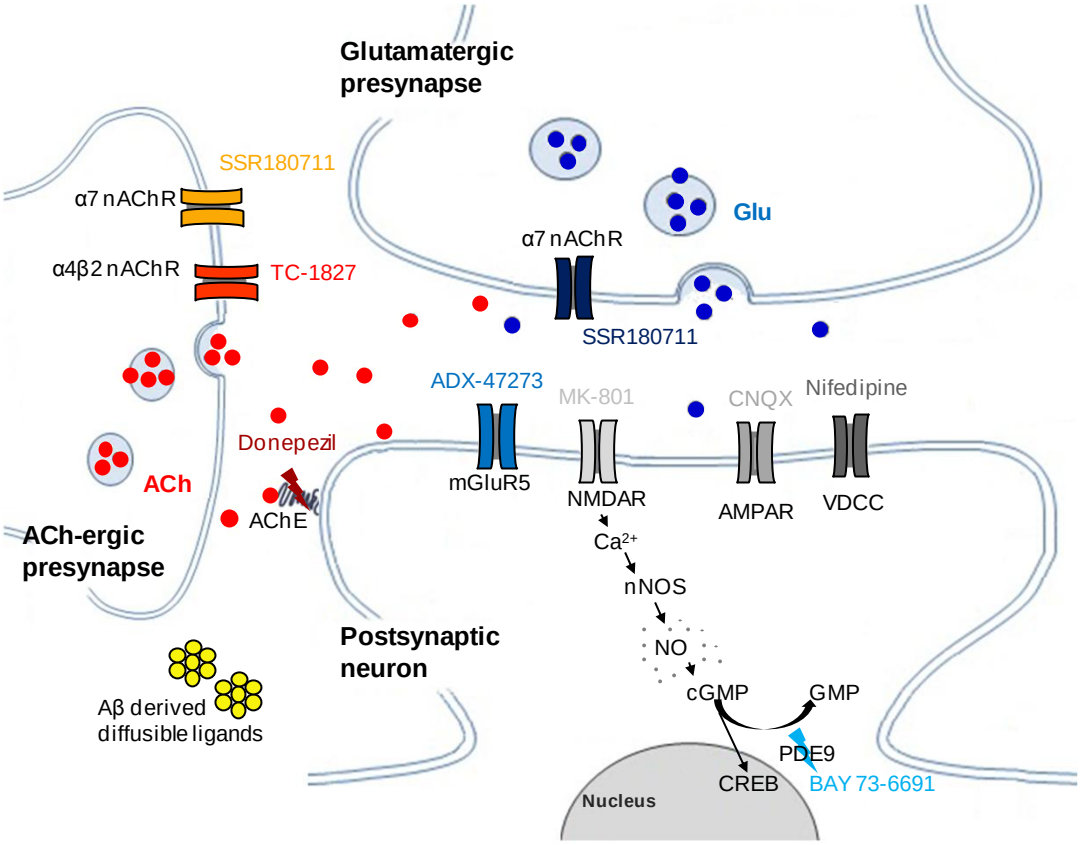
This study demonstrates that early and late LTP are affected to different extents by ADDLs. Due to the differentiation between early and late LTP and the pharmacological dissection of the relevant receptors, ADDLs were shown to exclusively target NMDA receptors and/or their signaling cascade. These results further contribute to the hypothesis that soluble Aβ oligomers cause NMDA receptor related synaptic dysfunction which might lead to cognitive decline seen in AD patients. Furthermore, several cognitive enhancing

(pre-)clinical drugs addressing different targets were evaluated for their ability to ameliorate ADDLs induced LTP deficits in order to assess their potential therapeutic value for symptomatic treatment of AD. The current gold standard for treatment of AD patients is the AChE inhibitor donepezil, which was demonstrated to slightly restore ADDLs induced impairment of early LTP, but to have no effect on ADDLs induced impairment of late LTP. The same was shown for the α4β2 nAChR agonist TC-1827. Both, the α7nAChR partial agonist SSR180711 and the PDE9 inhibitor BAY 73-6691 completely rescued early as well as late LTP impaired by ADDLs. The mGlu5 receptor positive allosteric modulator ADX-47273 had no effect on ADDLs impaired early LTP, but slightly restored ADDLs impaired late LTP. Thus, α7 nAChRs agonism and PDE9 inhibition were found to be most effective in ameliorating ADDLs induced LTP deficits. Therefore, targeting α7 nAChRs and PDE9 might represent powerful approaches for symptomatic treatment of AD. In general, this study shows that targets/drugs acting on glutamatergic NMDAR-related pathways seem to be very promising to translate into clinical benefit.

CHAPTER 7

General discussion and outlook

The present thesis focuses on the establishment and validation of LTP in rat hippocampal slices to characterize memory enhancing drugs as potential treatment of AD. First, a multi-slice recording system was set up enabling stable measurements of LTP for up to seven hours from several slices simultaneously. Then, distinct protocols to induce early and late CA1 LTP, resembling short-term and long-term memory, were established. They were then validated addressing the hallmarks accepted for these forms of LTP: protein-synthesis independence and NMDA receptor dependence without contribution of L-VDCCs for early LTP, as opposed to protein-synthesis dependence and additive NMDA/L-VDCCs dependence for late LTP. As a loss of mainly cholinergic and glutamatergic synapses has been described in AD patients, the validated forms of LTP were used to study drugs potentially enhancing cholinergic and glutamatergic neuronal function. Thus, the potential therapeutic value of approaches aiming at enhancement of cholinergic and glutamatergic neuronal function was determined by analyzing their potential to improve early and late LTP and to restore LTP impaired by soluble Aβ oligomers (Fig. 43). Soluble Aβ oligomers, also referred to as amyloid-β derived diffusible ligands (ADDLs), are thought to be one cause of AD. In this thesis, they were demonstrated to impair early and late LTP to different extents by exclusively targeting NMDA receptors and/or their signaling. These results further contribute to the hypothesis that soluble Aβ oligomers cause synaptic dysfunction which might lead to cognitive decline seen in AD patients. The AChE inhibitor donepezil (Fig. 43 shown in dark red) is the current gold standard for symptomatic treatment of patients suffering from mild-to-moderate AD. It facilitates exclusively cholinergic function by increasing and prolonging the concentration of ACh in the synaptic cleft. The increased extracellular levels of the transmitter ACh can activate cholinergic receptors such as nAChRs, which are thought to cause the cognition enhancing effects of donepezil. In this study, the effect of donepezil on LTP was demonstrated to be only moderate: donepezil was found to increase early LTP, but to not affect late LTP. Similarly, it slightly restored ADDLs induced deficits in early LTP, but had no effect on late LTP impaired by ADDLs. Interestingly, donepezil showed quantitatively the same effects on LTP and ADDLs induced LTP deficits as the α4β2 nAChR agonist TC-1827 (Fig. 43 shown in red). This similarity may indicate that the levels of ACh increased by donepezil activate the α4β2 nAChR, but not sufficiently the α7 nAChR. The reason for the α4β2 nAChR being indirectly targeted by donepezil might be based on the much higher binding affinity of ACh to the α4β2 nAChR as compared to the α7 nAChR (K_i for α4β2:



Drug	(target)	Transmitter system	LTP early	LTP late	LTP - ADDLs early	LTP - ADDLs late
TC-1827	(α4β2 nAChR)	ACh	↑	—	↑	—
Donepezil	(AChE)	ACh	↑	—	↑	—
SSR180711	(α7 nAChR)	ACh / Glu	↑ (late)	↑	↑	↑
BAY 73-6691	(PDE9A)	Glu	↑ (late)	↑	↑	↑
ADX-47273	(mGluR5)	Glu	—	↑	—	↑

Figure 43: Facilitating glutamatergic and cholinergic neuronal function – effect on LTP and ADDLs induced impairment of LTP

Shown in red: The neurotransmitter acetylcholine, the drugs used to facilitate cholinergic function and their targets; i.e. the inhibitor donepezil and its target AChE; the (partial) agonists TC-1827 and SSR180711 as well as their respective targets the α4β2 nAChR and the α7 nAChR.

Shown in blue: The neurotransmitter glutamate, the drugs used to facilitate glutamatergic function and their targets; i.e. the inhibitor BAY73-6691 and its target PDE9A, the positive allosteric modulator ADX-47273 and its target the mGlu5 receptor, the partial agonists SSR180711 and its target the α7 nAChR.

Shown in yellow: Amyloid-beta derived diffusible ligands.

Legend of the table: —; no effect; ↑; enhancing effect; ⇡, slight / partial effect

6.8-57 nM; K_i for α7: 4000-10830 nM; Sharples and Wonnacott, 2001). Activation of the α4β2 nAChR is able to enhance and slightly rescue early LTP impaired by ADDLs, but it has no effect on late LTP. The mechanism responsible for this effect seems likely to be disinhibition, which may take place by activation of α4β2 and/or α7 nAChRs located on GABAergic interneurons having inhibitory connections to other inhibitory neurons, thus producing a net increase in glutamatergic principal neuron activity. The decisive difference between α4β2 and α7 nAChR function is the additional location of α7 nAChRs on Schaffer collateral terminals causing strengthening of the glutamatergic system by facilitation of glutamate release. Hence, the α7 nAChR partial agonist SSR180711 facilitates cholinergic and glutamatergic function (Fig. 43 shown in light red and dark blue depending on the location and hence function) and most likely thereby enhances both LTP types and even transforms early into late LTP. Furthermore, a complete rescue of ADDLs induced impairment of early and late LTP was observed by SSR180711. As the PDE9A inhibitor BAY 73-6691 (Fig. 43 shown in light blue), exclusively targeting glutamatergic function, shows the same effect as SSR180711 on LTP and LTP impaired by ADDLs, the crucial factor for an effective LTP enhancement and rescue seems to be the activation of glutamatergic function. PDE9 is part of the intracellular signaling pathway NO-sGC-cGMP influencing the levels of the second messenger cGMP by its cGMP-specific hydrolytic activity. Elevation of cGMP caused by PDE9 inhibition most likely results in strengthening of the NMDA receptor related postsynaptical pathway NO-sGC-cGMP-PKG leading to CREB phosphorylation being a crucial cellular mechanism for learning and memory. Another drug exclusively interfering with glutamatergic function is the mGlu5 receptor positive allosteric modulator ADX-47273 (Fig. 43 shown in blue), which was found to increase late LTP and to partly rescue late LTP impaired by ADDLs. It had no effect on early LTP, no matter whether it was intact or impaired by ADDLs. The reason for this is likely, that the stimulus intensity sufficient for induction of early LTP does not reach a threshold for functionally relevant stimulation of mGlu5 receptors.

Hence, drugs facilitating glutamatergic function not only seem to be more efficacious in enhancing LTP than drugs facilitating solely cholinergic function, but they are also superior in ameliorating soluble Aβ oligomer induced LTP deficits. Consequently, the findings of this thesis might provide an explanation for the rather moderate therapeutic effects of donepezil in AD patients. Moreover, based on the results of the present thesis, it can be concluded that drugs interfering with glutamatergic function seem to have a high therapeutic value as alternative, and probably more efficacious, approaches for the

symptomatic treatment of AD. Further investigations have to show whether this finding also holds true for animal models addressing learning and memory, and - ultimately - for AD patients in clinical trials.

Even though this thesis comes to an end, "this is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning." (Winston Churchill)

For example, one might not only analyze a single concentration for the rescuing experiments of the ADDLs induced impairment of LTP, but perform a concentration series for each drug. In this thesis, only one concentration of each drug was tested, namely the concentration which showed the best effect in enhancing early and late LTP. To detect the unlikely event of an effectiveness shift of concentration in the rescuing experiments, a concentration series of all drugs would be desirable.

A question, which arose during the thesis, deals with the site of action of some of the tested drugs. For example, it is not completely known whether BAY 73-6691 acts presynaptically and/or postsynaptically. Paired-pulse facilitation, a short-term form of synaptic plasticity, could shed light on the involvement of BAY 73-6691 in the presynapse. The experimental setting is identical to the one used for LTP measurements, but instead of an LTP inducing stimulation pattern, two stimulations are delivered at a very short interval, i.e. 20 - 50 milliseconds. The responses to both stimulations are analyzed and the second fEPSP can be up to five times the size of the first. Facilitation has been shown to occur solely at the presynaptic side. It is thought to be the result of an increase in probability of vesicle release. If BAY 73-6691 acted presynaptically by an increase in the probability of vesicle release, the facilitating effect of the second pulse should exceed the one of the control conditions. Not only electrophysiological measurements could be used to address the site of action of some of the tested drugs, but also immunohistochemistry having a very high resolution going down to the sub-cellular level would be a suitable method to identify the localization of a target protein.

Another very interesting question deals with the potential effect of some drugs on interneurons. For example, donepezil and TC-1827 are thought to increase early LTP by disinhibition. A patch clamp study to address the effect of doenepezil and TC-1827 on interneurons could shed some light on this issue.

It is known that late LTP requires *de novo* protein-synthesis (Fazeli et al., 1993; Frey et al., 1988; Huang, 1998; Malenka and Nicoll, 1999; Nguyen et al., 1994; Reyman and Frey, 2007). Furthermore, transcription of different immediate early genes like *zif268* and *Arc*

after late LTP induction is described (Bozon et al., 2002; Jones et al., 2001; Guzowski et al., 2000). Nevertheless, the knowledge about the processes and detailed signaling pathways remains to be elucidated. One way to shed light onto these downstream mechanisms could be high-resolution transcriptome profiling of the respective neurons after LTP induction, using e.g. deep sequencing technologies. This approach has the potential to further clarify the mode of late LTP induction and might also provide information of further molecular sites of action for its modulation.

In this thesis, LTP experiments were exclusively used to analyze drugs targeting AD. As not only AD patients are impaired in their learning and memory abilities, but also patients suffering from schizophrenia, LTP experiments are not exclusively a model for AD. LTP could also be used as a model to screen for potential drugs for the treatment of impaired cognitive function in schizophrenia patients.

Finally, all LTP experiments in this thesis were conducted in rat hippocampal slices. Transferring this technique onto mouse hippocampal slices could pave the way to new and more disease-specific model systems: genetically engineered mouse models for AD or schizophrenia-like pathology could be used to address the effectiveness of drugs in a more disease relevant context.

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I can no other answer make, but, thanks, and thanks.

William Shakespeare

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