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Effects of Pridopidine and Abeta Oligomers on Synaptic Plasticity

Da Bin Kim

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Vorsitz: apl. Prof. Dr. Bernhard Haslinger

Prüfer*innen der Dissertation:

1. apl. Prof. Dr. Gerhard Rammes
2. Priv.-Doz. Dr. Jennifer Altomonte

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1. Introduction

1.1 Pathophysiology of Alzheimer's disease (AD)

Alzheimer's disease (AD) belongs to a group of neurodegenerative disorders characterized by progressive memory decline. This decline is followed by other cognitive dysfunctions such as attention derangement, visuospatial disorientation (Winblad et al., 2016; Zhu et al., 2020), neuropsychiatric disorders (Harwood et al., 2000) and even vegetative dysfunctions (Zahola et al., 2019). AD comprises 50–70% of all dementia (Winblad et al., 2016) and has multifactorial etiologies. Up to 6% of AD cases are attributed to genetic causes, such as the ε4 allele of the Apolipoprotein E (ApoE) (Zhu et al., 2015). The primary two histopathologic lesions of AD are the intracellular tau neurofibrillary tangles and extracellular amyloid plaque. The amyloid plaques consist of insoluble amyloid-beta (Aβ) peptides produced through the sequential cleavage of the transmembrane amyloid precursor protein (APP) by two enzymes, β- and γ-secretase (Kummer & Heneka, 2014). Aβ oligomers are 36–43 amino acid residues in length, and these soluble oligomeric forms of Aβ are likely responsible for pathogenesis of both sporadic and familiar AD (fAD; (Lesne et al., 2013; Selkoe & Hardy, 2016).

1.1.1 Genetic predispositions to AD

1.1.1.1 Apolipoprotein E (ApoE) polymorphisms

Genetic predisposition is a major factor in hereditary early-onset AD and is associated with mutations in genes associated with the APP cleavage process. Specifically, ApoE polymorphisms are the most common known genetic risk factors of early onset (< 65 years) and late onset (> 65 years) AD (Zhu et al., 2015). ApoE proteins are glycoproteins mostly synthesized in the liver and central nervous system (CNS). ApoE proteins are contained in lipoproteins as ligands and facilitate a receptor-mediated endocytosis of lipoproteins. These ApoE proteins are essential for the transportation and absorption of lipids, such

as the homeostasis of cholesterol (Huang & Mahley, 2014). In 1998, Simons et al. discovered that cholesterol depletion with cholesterol inhibitors such as statin inhibited Aβ formation in rats' hippocampal cell cultures (Simons et al., 1998). Humans have three ApoE alleles: ApoE2, E3 and E4. In the CNS, ApoE is part of the lipoprotein and is referred to as a high-density lipoprotein (HDL)-like particle. Lipoproteins containing ApoE4 are usually poorly lipidated. An antibody experiment targeting ApoE revealed that immunotherapy targeting poorly lipidated ApoE might be effective against AD (Chernick et al., 2018; Kim et al., 2012; Liao et al., 2018); therefore, ApoE4 is considered a risk factor (including, for example, a homozygous allele of ApoE4/E4 and ApoE3/E4). An allele with ApoE2 is considered protective against AD (Cacace et al., 2016; Chernick et al., 2018; Dulewicz et al., 2021; Safieh et al., 2019).

1.1.1.2 Mutations in presenilin genes

Mutations in presenilin genes 1 (PSEN1, in chromosome 14) and 2 (PSEN2, in chromosome 1) are also responsible for pathogenesis of early-onset, familiar forms of AD. Presenilin 1 (PS1) or presenilin 2 (PS2) are parts of the γ-secretase-complex in the ER membrane and function as catalytic compounds in reactions involving aspartyl-proteases. Mutation of PSEN1 or PSEN2 enhances Aβ production (Cacace et al., 2016; Rosenberg et al., 2016).

1.1.1.3 Mutations in amyloid precursor protein (APP) genes

An autosomal-dominant mutation of APP genes causes an early-onset hereditary form of AD. Because APP genes are found on chromosome 21, patients with Down syndrome have high incidence of AD—30% of people over age 50 with Down syndrome suffer from AD. The London Mutation (V717I) was the first APP mutation discovered and is the most common APP mutation of early-onset fAD. This mutation causes the substitution of valine for isoleucine in codon 717 of the APP gene. This mutation is often used in preclinical animal experiments to test novel AD therapeutics (Cruts et al., 2012; Grigor'eva et al., 2019).

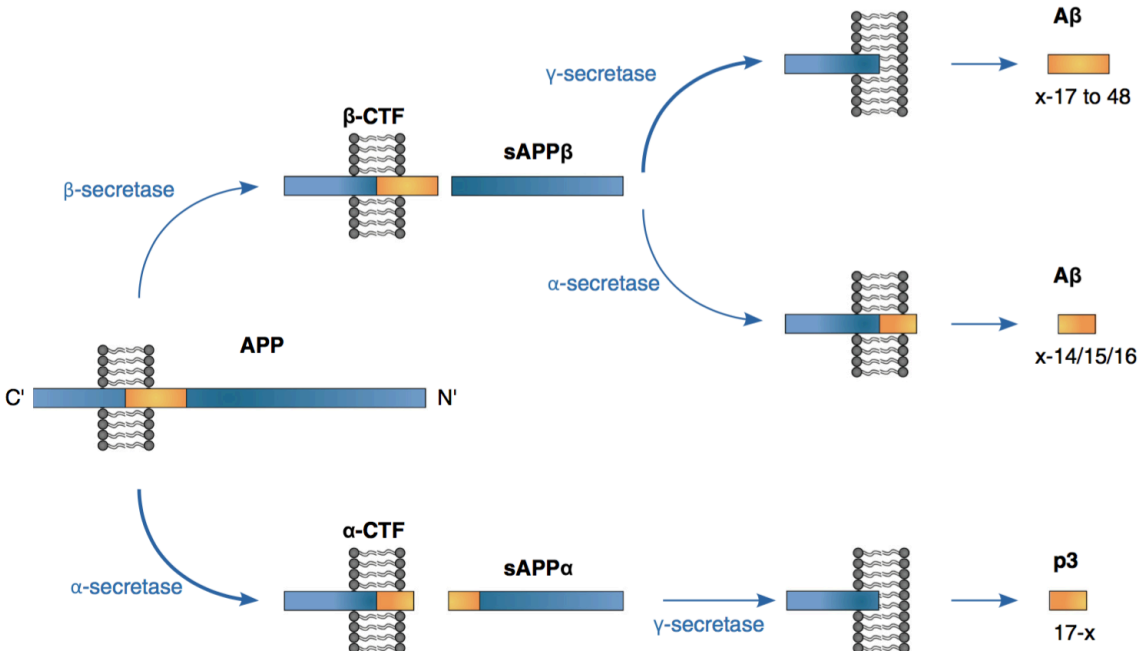

Figure 1. Amyloidogenic (upper) and nonamyloidogenic (lower) APP process pathways (Kummer & Heneka, 2014)

APP is a precursor molecule of Aβ. An amyloidogenic pathway of APP processing requires a cleavage of APP by β-secretase before position 1 of the Aβ domain in its N-terminal. This process yields a C-terminal stub called β-C-terminal fragment (β-CTF), which can be further cleaved by γ-secretase in multiple sites or transmembrane domain from position 17. This process generates Aβ peptides of various lengths or amino acids at position 40, 38, or 42. Shorter isoforms of Aβ are sometimes generated by sequential cleavage of α-secretase before position 17. In the nonamyloidogenic pathway, APP is first cleaved by α-secretase in position 16 or 17, releasing sAPPα and leaving an α-CTF, which can be further cleaved by γ-secretase, liberating P3 peptides.

1.1.2 Physiological functions and pathological effects of Aβ peptides

1.1.2.1 Non-amyloidogenic manners of APP

Aβ peptides are produced through cleavage of APP. APP is a single-transmembrane protein with a cytoplasmic C-terminus and an extracellular glycosylated-N-terminus that has 695–770 amino acids. APP isoforms of 695 amino acids are mainly expressed in neuronal synapses. Soluble amyloid precursor proteins (sAPPα) are released through the non-amyloidogenic pathway by α-secretase **(Figure 1).** The rate of sAPPα release is increased by activation of the muscarinergic acetycholin receptor. Results from preclinical trials suggest that the nonamyloidogenic pathway could be a therapeutic target of AD (Anderson et al., 1999; Borroni et al., 2002; Sennvik et al., 2000). In APP-transgenic mouse models of AD, the expression rate of sAPPα has been negatively correlated with the APP amyloidogenic process (Obregon et al., 2012). In 2013, Hasebe et al. incubated cortical neurons of mice with sAPPα, which promoted neurite outgrowth. However, none of the clinical trials resulted in cognitive improvement for patients (Habib et al., 2017).

1.1.2.2 Physiological functions of Aβ

There is also emerging evidence that the monomer state of Aβ has neuroprotective characteristics as a synaptic modulator that is mediated through activation of the insulin/insulin growth factor (IGF) -1 receptors, a family of tyrosine-kinase receptors (Zimbone et al., 2018). Insulin/IGF-1 receptors are transmembrane proteins that are activated by two IGF-factors (IGF-1/2) and by insulin (Xu et al., 2018). They are widely distributed in mammalian cell surfaces and regulate proliferation, survival and differentiation of normal and neoplastic cells (Brahmkhatri et al., 2015). In the CNS, insulin/IGF-1 receptors are widely expressed in the hypothalamus, hippocampus, olfactory nerve, cerebellum and neocortex (Baltazar-Lara et al., 2020).

Several metabolic diseases could be associated with AD. Existing work has demonstrated that the insulin signaling pathway affects AD pathology. Aβ-

oligomers inhibit autophosphorylation of the tyrosine-kinase receptor and downregulate the insulin/IGF-1 receptor (Giuffrida et al., 2012). Decreased insulin sensitivity is also related to tau hyperphosphorylation via glycogen synthase kinase-3α (GSK-3α), reduced Aβ clearance, and neuroinflammation (Kellar & Craft, 2020). Indeed, insulin infusion has been found to reduce the expression of APP and presenilin in experiments with mononuclear cells (Dandona et al., 2011). Vandal et al. also demonstrated that an insulin injection decreased cortical-soluble Aβ and promoted Aβ efflux through the blood-brain barrier in a diabetic mouse model (Vandal & Calon, 2015). Aβ peptide is also associated with cholesterol metabolism. As mentioned above, increased expression of ApoE4, which also indicates poor lipidation of lipoproteins, is correlated with impaired Aβ clearance in the CNS.

Recent research has also indicated a possible antimicrobial function of Aβ, which has been associated with prolonged cell survival of mice brains infected with herpes encephalitis (Eimer et al., 2018). Increased viral DNA and RNAs were found in postmortem tissues of AD patients (Readhead et al., 2018). Valacyclovir is currently in phase 2 clinical trials (VALAD trial; NCT03282916) as an antiviral therapeutic (Devanand et al., 2020). In experiments with cell-free oxidative systems with metal ions, $A\beta_{1-40}$ also functioned as a radical scavenger at nanomolar concentrations (Baruch-Suchodolsky & Fischer, 2009).

1.1.2.3 Pathophysiology of Aβ through receptor interactions

An extracellular aggregation and accumulation of Aβ peptide through interaction with cellular prion protein (PrP^c) has historically been considered the main form of pathophysiology in AD (Lauren et al., 2009). However, bindings of Aβ to various receptors and their subcellular modulations also cause neuronal cell toxicity and cell death. PrPc interacts not only with Aβ oligomers but also with metabotropic glutamate receptor 5 (mGluR5) and induces Ca^{2+} release from

intracellular ER storage (Beraldo et al., 2016). Soluble oligomeric forms of Aβ also interact with numerous other ionic receptors, such as the N-methyl-D-aspartic acid receptor (NMDAr) and the alpha-7 nicotinic acetylcholine receptor (α7nAChr) (Beraldo et al., 2016; Nagele et al., 2002). Through these receptor interactions, Aβ oligomers disrupt the Ca^{2+} homeostasis that induces neuronal toxicity, synaptic disruption, and cell death (Chen et al., 2017). Low-density lipoprotein receptor-related proteins (LRPs) also react with Aβ and APP, regulating ligand- (i.e., ApoE-) mediated endocytosis of cholesterols and Aβ clearance, suggesting that LRP and Aβ have close functional relationship (Marzolo & Bu, 2009). Aβ oligomers are also related to mitochondrial dysfunction, which induces oxidative stress by producing reactive oxygen species (ROS; (Chen et al., 2017; Swerdlow, 2018).

1.1.3 Diagnosis of AD

In addition to medical history such as behavioral observation or neurological examinations, several neuropsychological tests, such as the mini-mental state test (MMST) or the clock-drawing test, can be used to assess the cognitive decline of patients. The Consortium to Establish a Registry for Alzheimer's Disease (CERAD) was also developed in 1986 and was sponsored by the National Institute of Aging (NIA) in the USA **(Table 1)**. The result of these efforts was a neuropsychological battery that is more accurate than the MMST for assessing AD and even other non-AD forms of dementia, such as Lewy body dementia or Parkinson's disease (PD; (Breton et al., 2019; Camargo et al., 2018; Hellwig et al., 2013; Muller et al., 2019).

Clinical battery
Neuropsychology battery
Neuropathology battery
Neuroimaging battery
Behavior rating scales for dementia
Family history assessment
Service assessment (in home or community based)
Autopsy resources
Educational brochures

Table 1. The main batteries of CERAD (Fillenbaum et al., 2008)

Clinical and neuropsychological batteries were first developed by CERAD investigators. Several standardized assessments were then added, including neuropathological assessment, neuroimaging evaluation, behavioral rating scales, and even autopsy resources from deceased AD patients.

Several cerebrospinal fluid (CSF) abnormalities observed in AD patients are significant biomarkers; these include enhancement of the tau protein, phosphorylated-tau protein and $A\beta_{1\text{-}42}$ (Olsson et al., 2016). $A\beta_{1\text{-}40}$ is the most common form of $A\beta$, followed by $A\beta_{1\text{-}38}$ and $A\beta_{1\text{-}42}$. Among these, $A\beta_{1\text{-}42}$ is 42 amino acids in length and is most likely to aggregate and disrupt synaptic plasticity through NMDA receptors (Kummer & Heneka, 2014; Rammes et al., 2015; Rammes et al., 2011). Since $A\beta_{1\text{-}42}$ predominates in senile plaques, the $A\beta_{1\text{-}42}/A\beta_{1\text{-}40}$ ratio of the CSF in AD patients should be under 0.1. However, according to a meta-analysis conducted by Cochrane in 2014, CSF-$A\beta$ levels and ratios cannot be recommended for diagnosing early-stage AD with mild

cognitive impairment because of poor data quality and huge variations in data sensitivity and specificity (Ritchie et al., 2014).

Computed tomography (CT) and magnetic resonance imaging (MRI) can be used to detect brain atrophy, such as atrophy of the hippocampus in the temporal lobe (Wolinsky et al., 2018). Moreover, 18-fluorine radionuclide tracers have been developed for amyloid or tau-positron emission tomography (amyloid or tau-PET) to detect depositions of neurodegenerative biomarkers in brain networks (Whitwell, 2018). Regional cerebral blood flow imaging that uses single-photon emission computed tomography (rCBF-SPECT) might also be helpful in AD diagnosis (Valotassiou et al., 2018). However, results from a Japanese retrospective multicenter study in 2019 suggested that negative rCBF-SPECT without a typical AD pattern in imaging cannot definitively eliminate the possibility of AD (Takahashi et al., 2019).

Through various neuroimaging methods, it is also crucial to exclude other structural CNS-pathologies, such as normal pressure hydrocephalus (Picascia et al., 2015) or vascular forms of dementia (Emrani et al., 2020; Escobar et al., 2019). Neuropsychological tests can also be used to identify alternative diagnoses such as deliriums or secondary forms of dementia caused by depression (Wang et al., 2021).

1.2 Synaptic plasticity and memory formation

Encoding and retrieval of memories at conscious or unconscious levels requires complex neurochemical changes between hippocampal circuits and cortex. To discover treatments for cognitive decline and memory impairment in AD, the hippocampus and its established roles in synaptic plasticity have received much attention.

Synaptic plasticity, which is crucial for memory and learning, underlies the capacity of the brain to vary its synaptic network and its strength of connectivity in response to external inputs. This occurs through persistent activation of synapses in a bidirectional manner, either as a long-term potentiation (LTP) or long-term depression (LTD). Novel information can be selectively encoded as a long-term memory, and this process is a well-established role of the hippocampus (Bear, 2003; Bliss & Lomo, 1973). Indeed, glutamatergic neurotransmissions in the Schaffer collateral-commissural pathway (SCCP) of the hippocampus are the best-known cellular correlates of LTP (Bliss & Collingridge, 1993; Bubb et al., 2017).

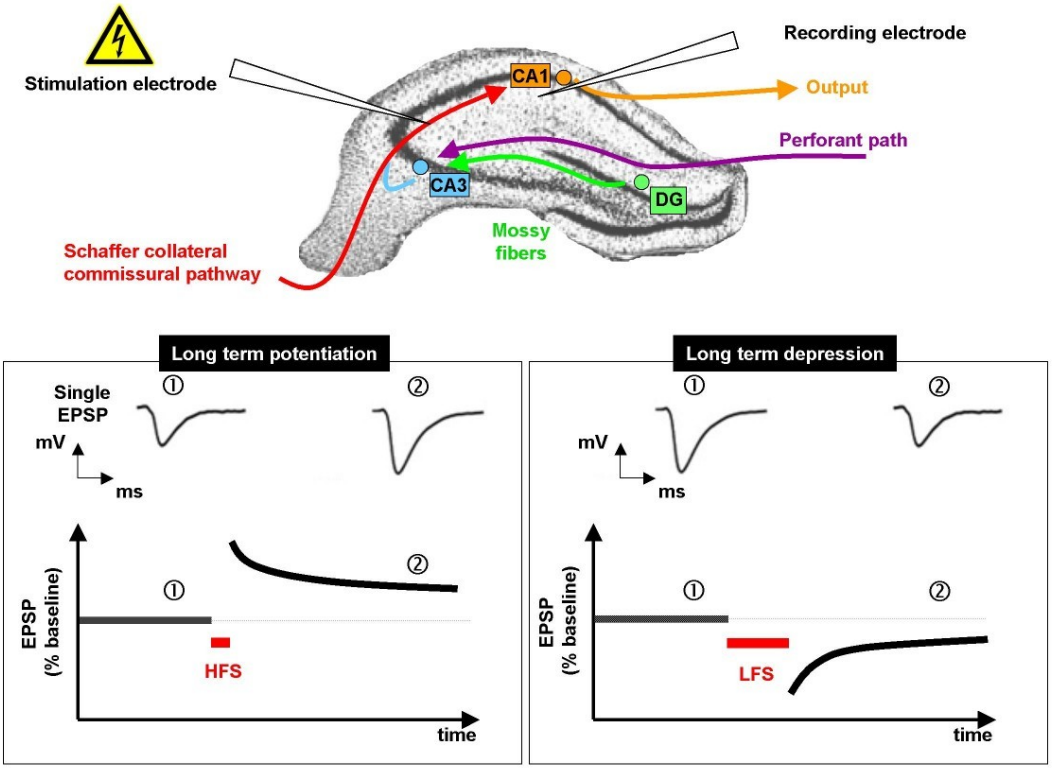

Figure 2. Electrophysiological experiment setup for inducing LTP and LTD in the murine hippocampus (Riedemann et al., 2010)

The upper panel describes the coronal section of a murine hippocampal slice. Hippocampal formation is attributed to three molecular layers of the allocortex. The dentate gyrus (DG) receives inputs from the entorhinal cortex. Inputs are then transferred through CA3 and SCCP to CA1 and out of the hippocampus to the subiculum. A stimulation electrode can be placed in SCCP and a recording electrode in CA1 to induce and record LTP and LTD.

The lower panel visualizes how the amplitude of a single excitatory postsynaptic potential (EPSP) can be quantified by the signal's slope. After a baseline level, high-frequency stimulation (HFS, 100 Hz) can be applied to induce LTP (left). The right panel describes the induction of LTD through application of low-frequency stimulation (LFS, 1–5 Hz) in SCCP.

LTP was conceptualized by Terje Lømo, a Norwegian physiologist. He discovered in his electrophysiological experiments that an HFS yielded prolonged excitatory postsynaptic potential (EPSP) in postsynaptic cells of the dentate gyrus in the rabbit hippocampus (Lomo, 2003). Indeed, LTP can be observed at every excitatory synapse in brain regions such as the anterior cingulate or insular region of the cerebral cortex; these regions offer a synaptic model for pain and emotion (Zhuo, 2018). LTP in Purkinje-cell synapses in the cerebellum plays a key role in motor learning (Gallimore et al., 2018). LTP can also be induced in other limbic systems, such as the amygdala (Kim & Cho, 2017). LTP in the nucleus accumbens and ventral tegmental area is associated with addiction (Thomas & Malenka, 2003), which can also be defined as part of learning and memory processes. Numerous neurotransmitters are involved in physiological processes of LTP, even in signaling pathways between hippocampal subfields. For example, the CA3-CA1 SCCP, which is considered a prototype of LTP, requires NMDA receptors to induce LTP. In contrast, in the mossy fiber pathway, the mGluR plays an important role (Nistico et al., 2011). In addition to the glutamatergic input, dopamine (DA), noradrenaline (NA), acetylcholine (Ach), serotonin (5-HT), and brain-derived neurotrophic factor (BDNF) are involved in these subtle processes in different brain areas (Bubb et al., 2017; Edelmann et al., 2017).

1.2.1 Physiological processes of LTP induction and maintenance

During the early phase of LTP (eLTP), postsynaptic Ca^{2+} concentrations increase through activation of NMDAr, which then activates several protein kinases including protein kinase C (PKC; (Kelly et al., 2007; Patel & Zamani,

2021). The activation of PKC in turn activates an α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAr). Moreover, this process increases the flow of AMPAr into the postsynaptic membrane (Penn et al., 2017). However, these AMPA receptors are not synthesized specifically during eLTP. AMPArs are instead pooled from adjacent intracellular postsynaptic regions, so eLTP occurs without the need for protein synthesis and gene modulation. Moreover, eLTP can be triggered by a single HFS and usually lasts for 2–3 hours (Pang & Lu, 2004; Patel & Zamani, 2021).

If eLTP is extended to the late phase of LTP (lLTP), it requires enlargement of pre- and postsynaptic structures such as postsynaptic densities of dendritic mushroom spines and axonal boutons. Notably, lLTP can last days to months and can be increased through repetitions of HFS (Baltaci et al., 2019; Patel & Zamani, 2021). These processes are induced by activation of a cascade of several protein kinases, such as protein kinase A (PKA), calcium-calmodulin-dependent protein kinase II **(**CAMKII), and mitogen activated protein kinase (MAPK) pathways (Adams et al., 2000; Kelly et al., 2007). This process induces a cascade of phosphorylation of transcription factors such as cAMP-response element binding proteins (CREB) to regulate the transcription of the genes. (Baltaci et al., 2019). A transcription of protein kinase M-zeta (PKMζ) genes is necessary to maintain LTP (Hernandez et al., 2003; Patel & Zamani, 2021; Sacktor, 2008). PKMζ is a constitutively active isoform of PKC, since it lacks the regulatory N-terminal domain (Kelly et al., 2007). PKMζ stabilizes AMPAr at the postsynaptic membrane through interaction with an ATPase called N-ethylmaleimide-sensitive fusion protein (NSF; (Yao et al., 2008). NSF also inhibits endocytosis of AMPAr by blocking the interaction between AMPAr and protein interacting with C kinase-1 (PICK1; (Hanley et al., 2002). Furthermore, PKMζ stabilizes AMPAr at the postsynaptic membrane via its interaction with a scaffolding protein called postsynaptic density protein 95 (PSD-95) and inhibits lateral diffusion of AMPAr, anchoring AMPAr in the synaptic membrane (Shao et al., 2012; Yudowski et al., 2013). PKMζ maintains its activation via a positive feedback mechanism. This mechanism involves phosphorylation and inhibition

of a protein interacting with NIMA 1 (PIN1) that blocks gene translation of PKMζ (Kelly et al., 2007; Patel & Zamani, 2021). Overexpression of PKMζ is also implicated in the maturation of dendritic spines; such overexpression transforms dendritic spines into stubby forms that are stable and can last for months (Holtmaat et al., 2005; Ron et al., 2012).

1.3 Pridopidine

1.3.1 Pridopidine and its pharmacological effects

Pridopidine—formerly ACR16:4-[3-(methylsulfonyl)phenyl]-1-propylpiperidine hydrochloride, Huntexil®—is a phenylpiperidine compound originally developed by Arcid Carlsson Research Laboratories (de Yebenes et al., 2011; Sahlholm et al., 2015). Pridopidine belongs to a pharmacological class of agents called dopidines. It was originally used in preclinical trials to treat motor symptoms of Huntington's disease (HD) because it could correct both hyper- and hypoactive locomotion induced by D-amphetamine in rat models of HD without significant motor side effects (Natesan et al., 2006; Ponten et al., 2010; Ponten et al., 2013). Pridopidine could also reduce L-Dopa-induced unilateral hyperactive locomotion without completely desensitizing L-Dopa effects in rodent PD models (Ponten et al., 2013). This effect was, however, initially believed to be modulated by dopaminergic transmission (Dyhring et al., 2010).

1.3.2 Pridopidine and its dopaminergic neuromodulation

Due to its state-dependent and dual-functioning characteristics, pridopidine has historically been defined as a "dopamine-stabilizer" distinct from other dopamine D2 receptor (D2R) agonists or antagonists **(Figure 3**; (Ponten et al., 2010).

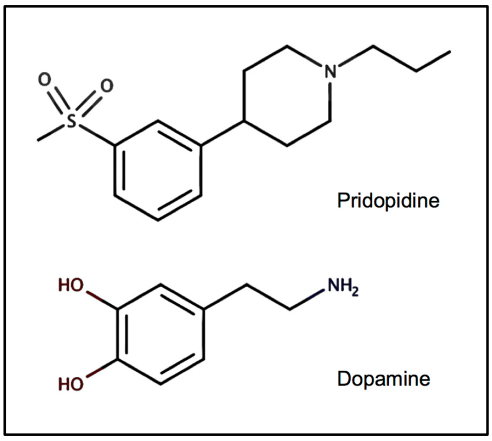

Figure 3. Molecular structure of DA and pridopidine.
Pridopidine was first discovered in 1998 (Ponten et al., 2010) and was initially understood to be a competitive D2R antagonist with 'activity-dependent' D2R occupancy (Dyhring et al., 2010).

Pridopidine was initially thought to modulate D2R. However, pridopidine has a relatively low affinity in the micromolar range (IC50 and Ki for D2R 10uM; (Pettersson et al., 2010; Rung et al., 2008). Pridopidine is a substance with almost no intrinsic activity associated with D2R. Pridopidine interacts preferably to already-activated D2R and has rapid dissociation properties that depend on the preexisting activity state of D2R (Pettersson et al., 2010; Tadori et al., 2007). This quality might also explain why pridopidine produces less antidopaminergic side effects such as anhedonia, dysphoria or extrapyramidal motor symptoms compared to other dopamine antagonists like haloperidol (Nilsson et al., 2004). There are two isoforms of D2R: D2 long and D2 short (D2L and D2S, respectively) that have distinct functions and distributions in the human body (Usiello et al., 2000). Haloperidol is assumed to block D2L, and pridopidine instead interacts with D2S (Nilsson et al., 2004; Usiello et al., 2000).

Nilsson et al., 2004	*Pridopidine improved the behavioral repertoire of mice injected with (+)-MK-801, and this effect might demonstrate this treatment's positive cognitive effects.*
Rung, Carlsson et al., 2005	*Pridopidine reversed (+)-MK-801-induced social withdrawal in rat model of Schizophrenia.*
Natesan, Svensson et al., 2006	*Pridopidine revealed an antipsychotic-like effect in addition to stabilization of amphetamine-induced hyperlocomotor activity (Nilsson & Carlsson, 2013).*
Sahlholm, Valle-Leon et al., 2018	*Pridopidine reversed phencyclidine-induced amnestic deficits in object recognition experiments in rodents. This effect could be blocked by the sigma receptor antagonist NE-100.*

Table 2. Unique functions of pridopidine revealed in preclinical trials
These unique activities of pridopidine indicate that its function might not be limited to regulating D_2R (Natesan et al., 2006; Nilsson et al., 2004; Rung et al., 2005; Sahlholm et al., 2018).

In preclinical trials, treatment with pridopidine had antipsychotic, procognitive and prosocial effects. In clinical research, limited but certain cognitive improvements were also observed in patients with schizophrenia (Carlsson & Carlsson, 2006) or PD (Tedroff J, 2004) after treatment with pridopidine. These findings suggest that pridopidine might have additional neuroprotective effects that are not typical of a D2R ligand **(Table 2)**.

Cognitive improvement is highly expressed in the prefrontal cortex and also underlies cortical up-regulation of the dopamine D1 receptor (D1R; (White et al., 1991). Stimulation of D1R could strengthen the induction of LTP in the murine hippocampus (Hansen & Manahan-Vaughan, 2014). D1R is also involved in visuospatial working memory formation in animal experiments (Muller et al., 1998). Pridopidine's actions on D1R are, however, rather indirect; for example, this interaction may occur through enhancing receptor gene transcription and trafficking though a sigma-1 receptor (S1R; (Kusko et al., 2018). In Section 1.3.3, these mechanisms are further discussed in detail.

1.3.3 Sigma-1 receptor (S1R) and its multifaceted neuromodulation

S1R is a 223-amino-acid-long 25 kD single transmembrane protein located in the mitochondrial-associated membrane (MAM) of the endoplasmic reticulum (ER). S1Rs are highly expressed in neuronal and glial cells in the brain.

Pons-raphe
Thalamus
Substantia nigra
Hypothalamus
Cerebellum
Cortex
Striatum

Table 3. Distribution of S1R in the brain (high to low; (Lepelletier et al., 2020)
An *in vivo* PET ligand-tracer experiment revealed in rodents and humans that S1Rs has anatomically different distribution rates in the brain.

An *in vivo* PET imaging experiment using radiotracer-ligands revealed that pridopidine displays up to 20-fold higher nanomolar-binding affinity for S1R compared to D_2R (Grachev et al., 2021; Sahlholm et al., 2015). Indirect dopaminergic effects of pridopidine might also be modulated by S1R: dopamine receptors and S1R can build heteromers in striatal neurons in rodents, restoring dopamine terminal density. However, this effect was diminished in S1R-knockout rodents (Francardo et al., 2014; Navarro et al., 2013).

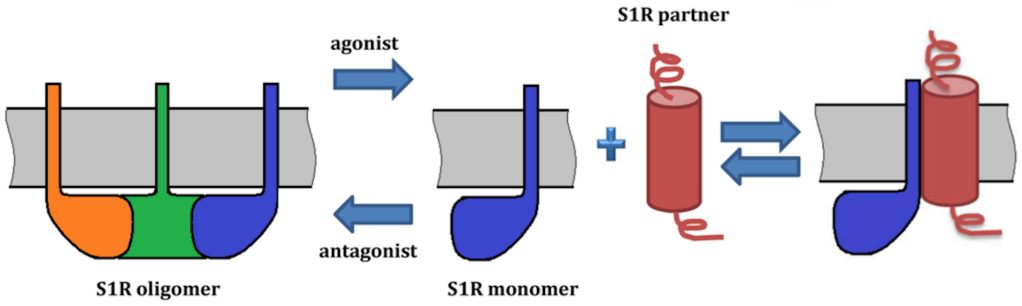

Figure 4. Simplified model of S1R (D. A. Ryskamp et al., 2019; Schmidt et al., 2016)

Many oligomeric forms (140–400 kDa) of S1R have been suggested in preclinical trials. There is limited evidence that monomeric states could represent an active form of S1R, since agonists such as pridopidine or pentazocine promote S1R dissociation into monomeric forms that build functional heteromers with other interaction partners.

Administration of S1R agonists increases cellular translocation of S1R monomers in various cell fractions such as nuclear envelopes or plasma membranes (Hayashi & Su, 2003; Meunier et al., 2006). In contrary, S1R antagonists such as haloperidol stabilize S1R oligomers. S1R oligomers stay linked to other ER-proteins such as binding immunoglobulin protein (BiP) chaperone. Therefore, S1R antagonists restrict the localization of S1R oligomers in ER fraction. The shifting balance of S1R between monomeric and oligomeric forms might define its activity state (Gromek et al., 2014; Mishra et al., 2015).

S1R density might not be a direct predictor of disease progress and cell loss because of its complex subcellular interactions. S1R functions as a 'ligand-operated molecular chaperone' through direct protein interaction in the ER-membrane **(Figure 4)**. The effects of S1R depend on its subcellular interaction partners and inner cell contexts. Indeed, the cortical and cerebellar expressions of S1R were decreased in *in vivo* and *in vitro* human AD and PD studies (Jansen et al., 1993; Mishina et al., 2005; Mishina et al., 2008). A PET ligand-tracer experiment revealed that S1R bindings were in fact increased in rodents with AMPA lesions (Lepelletier et al., 2020). In late HD brains, S1R protein levels were also more elevated than in early HD brains (Ryskamp et al., 2017).

1.3.4 Interactions between S1R and pridopidine

Initial studies on the potential role of pridopidine were conducted with animal models of HD. HD is a neurodegenerative disease characterized by extrapyramidal motor symptoms, such as hyperkinesia, initiated and accompanied by neuropsychological and cognitive decline. HD is an autosomal-dominant hereditary disease caused by a mutation in the Huntingtin gene that produces the Huntingtin protein (Htt). A mutated Huntingtin protein (mHtt) results from a trinucleotide repeat expansion of cytosine-adenine-guanine (CAG) with more than 40 repeats in the Huntingtin gene. This causes an increase in neuronal cell death, mostly in the basal ganglia (Kumar et al., 2015). Sporadic HD is another rare condition that occurs without family history. If parents have an intermediate range of CAG repeats, they are unlikely to

develop HD, but these CAG repeats can be passed to their children (Watanabe et al., 2000).

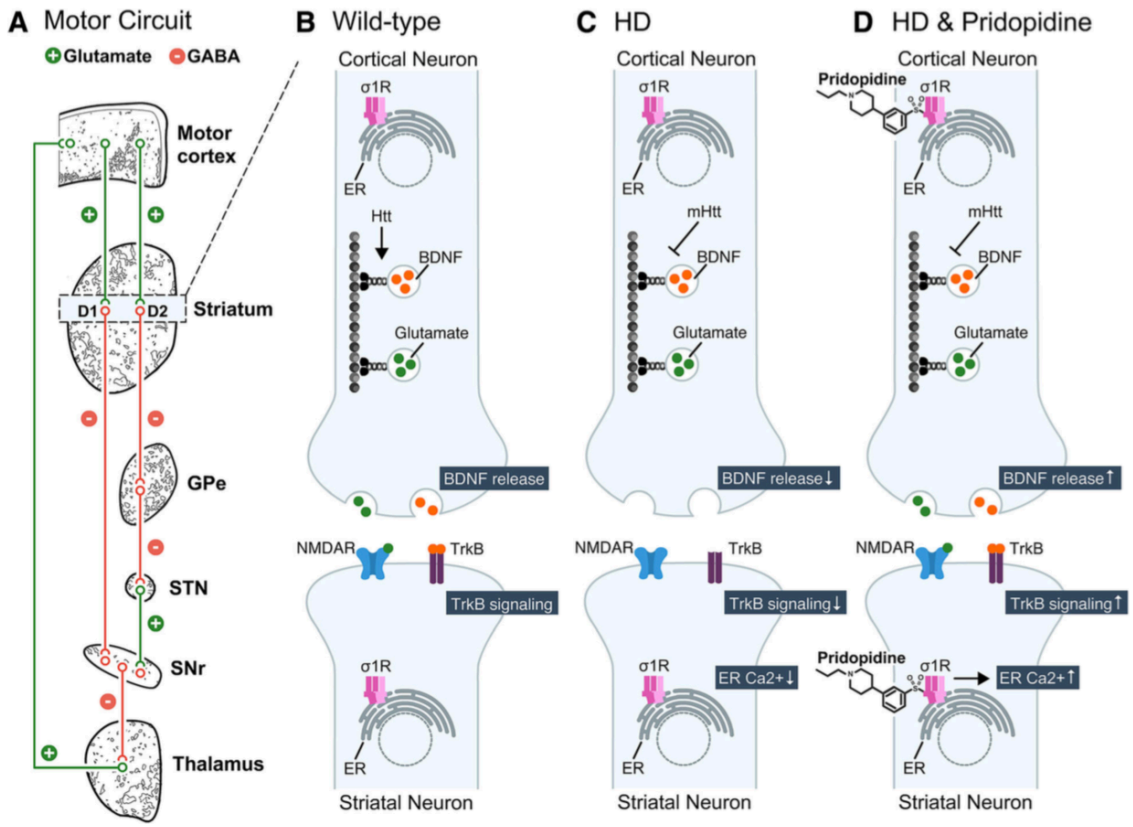

Figure 5. The signaling pathway promoted by pridopidine through S1R (Kusko et al., 2018)

A. a schematic representation of the corticostriatal pathway (A) in mammals' striatal and cortical co-culture

B. Htt promotes axonal transport of BDNF and glutamate for vesical release in the synaptic membrane in wild type striatum. Binding of BDNF and glutamate in postsynaptic density initiate tropomyosin receptor kinase B (TrkB) signaling in the striatum. TrkB acts as a catalytic receptor to mediate multiple functions of neurotrophic factors, such as neuronal survival and differentiation.

C. However, mHtt disrupts axonal transport of glutamate, BDNF and, consequently, TrkB signaling. Through decrease of BDNF levels in striatal neuron ER, Ca^{2+} homeostasis can be also disrupted.

D. An increase in striatal BDNF levels and restoration of ER Ca^{2+} levels can be achieved through direct activation of S1R through treatment with pridopidine

In preclinical animal models of HD, pridopidine relieved behavioral and motor symptoms through direct activation of S1R, mostly in ER in the striatum and cortex, by increasing gene transcription of several subcellular pathways, such as BDNF, D1R (Geva et al., 2016) and glucocorticoid receptor (GR) pathways (Maurice et al., 2001). Pridopidine upregulates through these neurotrophic pathway gene transcriptions, restores ER Ca^{2+} levels in striatal neurons, and stabilizes dendritic mushroom spines **(Figure 5**; (Ryskamp et al., 2017). Interestingly, a transgenic effect of pridopidine was more effective if treatment was initiated in early phases of disease; in this case, a robust gene expression was induced in a dose-proportional manner. Early treatment also improved neuropsychological symptoms of mice with HD, such as anxiety and depression. However, a late treatment only impacted depressive symptoms. However, neither early nor late treatment mitigated neuronal atrophy in the basal ganglia in late phases of HD, suggesting that pridopidine might be less effective as a disease-modifying substance in late phases of disease (Garcia-Miralles et al., 2017).

1.3.5 Clinical aspects of pridopidine

Loss of function mutations or polymorphisms of S1R may be related to various neurodegenerative diseases such as AD, amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD), PD and HD. Pridopidine, a S1R modulator, is currently undergoing several clinical trials to evaluate its efficacy in treating these diseases. In the USA, pridopidine was investigated in phase 2 clinical trials for the treatment of levodopa-induced dyskinesia in PD until July 2020, when trials were terminated due to the COVID-19 pandemic (McFarthing et al., 2019). Pridopidine is also currently in phase 2 and 3 clinical trials (HEALEY ALS platform; NCT04297683) for ALS in the USA and received orphan drug designation for ALS treatment in July 2021.

HD	***Phase 3**, ongoing* *Orphan drug designation in USA/Europe*
ALS	***Phase 3,** ongoing* *Orphan drug designation in USA*
PD	***Phase 2** for L-Dopa induced dyskinesia, terminated*
AD	*Preclinical cellular/animal trials*

Table 4. Clinical trials with pridopidine in neurodegenerative diseases

A clinical trial for schizophrenia was discontinued. There have also been preclinical trials for several eye disorders, including fragile X syndrome and Rett syndrome **(resource: https://clinicaltrials.gov**).

Two multicenter, randomized clinical trials for HD were sponsored by Teva pharmaceuticals: HART(Huntington Study Group, 2013) in the USA and MermaiHD (de Yebenes et al., 2011) in Europe. HART was a phase 2, randomized, double-blind, placebo-controlled clinical trial. Pridopidine was tested at three different doses: 20 mg, 45 mg, 90 mg or a placebo for 12 weeks; this trial involved 229 patients. In MermaiHD, 437 patients received either 45 mg, 90 mg or a placebo for 26 weeks. Neither studies reached primary endpoints due to insignificant improvement as measured by the Unified Huntington Disease Rating Scale (UHDRS) motor score **(Table 5)**. However, pridopidine was generally well tolerated at any dose without significant side effects. Moreover, patients who underwent an open-label extension study after HART and MermaiHD experienced slower decline of functional capacity in daily life, as measured by the Total Functional Capacity (TFC) scale (McGarry et al., 2017; Waters et al., 2018). The TFC scale measures a functional component of

patients' daily lives, including one's ability to manage tasks such as financing or managing their work. The scale ranges from 0 to 13 (Gibson et al., 2021).

Total motor score	***Cognitive functions***
Chorea, dystonia, oculomotor functions, dysarthria, gait, postural instability	Verbal fluency Symbol digit modalities Stroop interface test (color/word)
Total behavior score	***Functional checklist score***
Mood, behavior, psychosis, obsessive	Independence scale TFC

Table 5. UHDRS-TFC scale (Long et al., 2017)

The UHDRS-TFC scale measures the disease stage and functional status of HD patients. The UHDRS scale is an assessment tool for motor, cognitive, behavioral and neuropsychiatric symptoms of HD. Patients' functional capacity in daily life, for example, their ability to go work, to manage finances or household tasks to maintain their independency, can be further assessed using the TFC scale. Impairments to functional capacity might already be significant in early stages of HD.

Pridopidine is currently owned by Prilenia Therapeurics, which sponsored another phase 2 clinical study, PRIDE-HD (Reilmann et al., 2019). Doses of up to 112.5 mg (higher than previous studies) were investigated. Pridopidine did not yield statistically significant therapy effects, as measured by UHDRS-scores after 26 weeks of treatment. However, a slow disease progression was assumed in extended clinical trials after 52 weeks, as measured using the TFC-scale. Here, a dose of 90 mg per day was assumed to be most effective based on the UHDRS-score. At 45 mg twice-daily (b.i.d) doses, a PET-ligand tracer experiment indicated that pridopidine occupied up to 90% of the S1R in human

brains. (Grachev et al., 2021) No major side effects from pridopidine have been described. However, some rare but serious adverse events were reported: falls (in five patients), suicidal attempts and thoughts (in four and three patients, respectively), head trauma (three patients), and aspiration pneumonia (three patients).

Currently, pridopidine has an orphan drug status for HD and ALS treatment in the USA. In Europe, pridopidine only has an orthan drug status for HD. Since October 2020, pridopidine has been in the third phase of clinical trials for HD (PROOF HD; NCT04556656). PROOF HD is a multicenter, randomized, placebo-controlled trial in Europe, the USA and Canada that includes 480 patients aged 20 and older who are in the early stages of adult-onset HD. Patients will be treated with either 45 mg pridopidine or with a placebo b.i.d. UHDRS scores between placebo and treatment group will be compared from the baseline at week 1 to week 65. The expected completion day of this research is April 2023.

1.4 Aim of the study

In 2005, Marrazzo et al. first investigated the neuroprotective effects of S1R agonists against Aβ-induced toxicity in the brain (Marrazzo et al., 2005). Indeed, pridopidine reversed memory impairment in behavioral studies (Sahlholm et al., 2018) and also stabilized dendritic mushroom spines in murine CNS (D. Ryskamp et al., 2019). Pridopidine also prevented functional capacity decline in HD patients, possibly through its antiamnesic and procognitive effect. The aim of this study is to gain an understanding of the neuroprotective characteristics of pridopidine in attenuating $A\beta_{1-42}$ oligomer toxicity in hippocampal neurons. $A\beta_{1-42}$ oligomers were tested for detrimental effects on LTP in murine hippocampal slices. After confirming such detrimental effects, pridopidine was cotreated in an attempt to reverse LTP deficits. The methodological constraints of testing interactions of Aβ with any drug using LTP as a readout requires one to apply concentrations of drugs that do not interfere with LTP per se. Therefore, the

highest dose of pridopidine that did not inhibit LTP was used for the first set of experiments. Then, we tested whether pridopidine could restore LTP.

2. Material and Methods

2.1 Material

2.1.1 Chemicals (Table 6)

$A\beta_{1-42}$	Bachem, Bubendorf, Schwitzerland
Ethanol	Merck KGaA, Darmstadt, Germany
Aceton	Merck KGaA, Darmstadt, Germany
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
D-(+)-Glucose-Monohydrat	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Histoacryl Tissue Adhesive	B.Braun Melsungen AG, Tuttlingen, Germany
Isoflurane	CP-Pharma, Burgdorf, Germany
Calciumchlorid-Dihydrate	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Potassium Chloride	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Magnesiumchloride-Hexahydrate	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Sodium Chloride	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Sodium Bicarbonate	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Sodium Phosphate Monohydrate	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Pridopidine	Cambrex Karlskoga AB, Karskoga, Sweden

2.1.2 Equipment

2.1.2.1 Electrophysiology setup (Table 7)

Binocular Microscope Stemi 1000	Zeiss, Göttingen, Germany
Headstage CV20303BU	Axon Instruments, Sunnyvale, USA
Amplifier Multiplyer Axonpatch 200B	Axon Instruments, Sunnyvale, USA
Piezomanipulator PM 500-20	Axon Instruments, Sunnyvale, USA
Isolated Stimulator, Type 2533	Devices, Garden City, Hertfordshire, England
Panel Mounted Pump IS3124A Peristaltic Pump	Ismatec, Glattburg, Germany

Borosilicate Glass Electrodes (OD 1,5mm/ID 1,17mm)	Hugo Sachs Electronic-Harvard Apparatus GmbH, March-Hugstetten, Germany
Glass Electrode Puller -DMZ Universal	Zeitz Instruments, Munich Germany
Bipolar Tungsten Electrodes	Hugo Sachs Electronic-Harvard Apparatus GmbH, March-Hugstetten, Germany
Laboratory Interface Board, ITC-16	Instrutech Corp. NY, USA

2.1.2.2 Brain slice preparation (Table 8)

Vibratome Microm HM 650 V	Thermo Scientific, Walldorf, Germany
Water Bath ED (v.2)	JULABO GmbH, Seelbach, Germany

2.1.2.3 Software (Table 9)

Recording Software Win LTP v. 1.10	Anderson and Collingridge, University of Bristol, UK
Analysis Software WIN LTP Reanalysis v. 1.10	Anderson and Collingridge, University of Bristol, UK
Statistics IBM SPSS v. 24	International Business Machines Corporation (IBM), Armonk, New York, U.S.
Microsoft Office Excel 2016	Microsoft Corporation, Redmond, Washington, USA
Graphs Graph Pad Prism 6.0	Graph Pad Software, Inc, La Jolla, California, USA

2.2 Methods

2.2.1 Composition and preparation of media and solutions

2.2.1.1 Artificial cerebrospinal fluid (ACSF)

NaCl	KCl	D-glucose	NaHCO3	CaCl2	MgSO4	NaH2PO4
124 mM	3 mM	10 mM	26 mM	2 mM	1 mM	1.25 mM

Table 10. Composition of Ca2+-containing ACSF (later only referred to as ACSF)

In all experiments, ACSF was saturated with carbogen (95% O2 / 5% CO2) and maintained a pH of 7.3. To prevent Ca^{2+}-induced excitotoxicity, Ca^{2+}-free ACSF was used during the brain slice preparation.

2.2.1.2 $A\beta_{1-42}$ oligomers

To produce 100 µM $A\beta_{1-42}$ stock solutions, 50 µg of solid $A\beta_{1-42}$ was dissolved in 111 µl of DMSO. The solution was dissolved in an ultrasonic bath for 1–2 min and mixed in the vortex. This procedure was repeated until solid $A\beta_{1-42}$ and DMSO became visually indistinguishable. During the experiment with $A\beta_{1-42}$, $A\beta_{1-42}$ stock solution was added to the ACSF bath, resulting in a final concentration of 50 nM. After the application of the $A\beta_{1-42}$/DMSO solution, pH of the ACSF was maintained at a physiological level (7.4) throughout the experiment.

2.2.1.3 Pridopidine

First, 3.18mg of solid pridopidine was dissolved in 1ml distilled water using the ultrasonic bath and the vortex mixer. A pridopidine stock solution was produced with a concentration of 10 mM. The stock solution was aliquoted and stored at -4ºC. During the experiment, the stock solution was further diluted by application to the ACSF bath, resulting in final concentrations of 1 µM, 300 nM, 100 nM and 30 nM.

2.2.2 Brain slice preparation

The experimental protocols were approved by the ethical committee on animal care and use of the government of Bavaria, Germany. Eight-week-old male wild type C57Bl/6 mice were anesthetized with isoflurane and decapitated. After decapitation, further processing for brain slice preparation was performed in ice-cold Ca^{2+}-free ACSF, which was continuously saturated with carbogen (95% O2/5% CO2). The brain was immediately removed from the skull, and two hemispheres were separated with a razor blade, which was first cleansed with acetone, ethanol and distilled water. The hemispheres were fixed with

hystoacryl glue on the metal block and transferred into the vibratome, which was also filled with ice-cold ACSF saturated with carbogen **(Figure 6)**. The whole procedure until this point was completed within 1 min of decapitation. The cerebellum was next removed. Slices were continuously cut off until the hippocampi were clearly visible. We used preferentially hippocampal slices located medial since at this position, the neuroanatomical orientation of the dendrites lays parallel to the slice plane and ensures the retention of full length CA1 neurons with healthy physiological capacity.

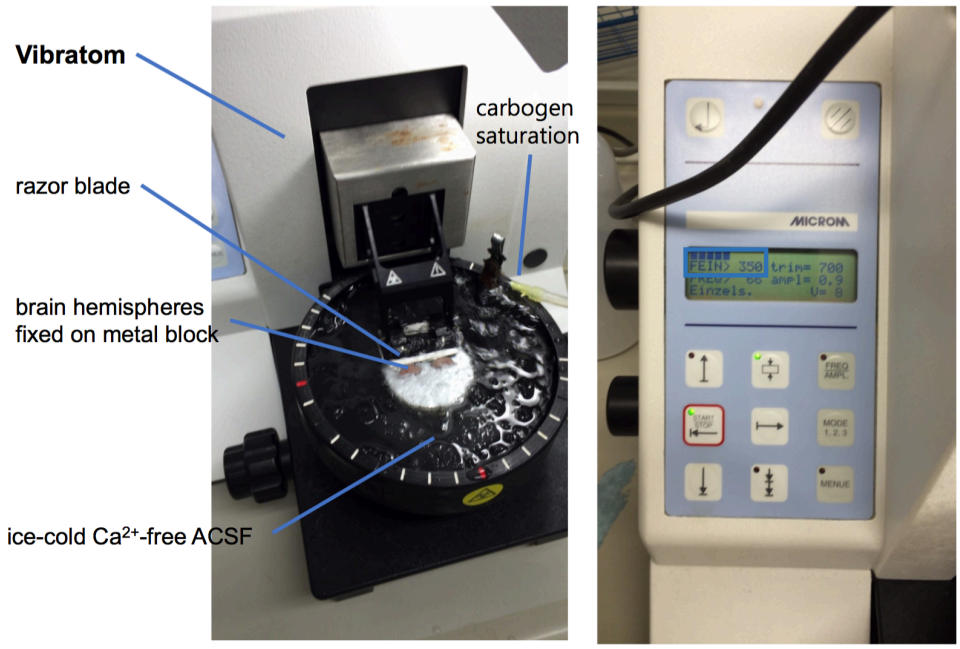

Figure 6. Vibratom preparing 350-um-thick hippocampal brain slices

A maximum of three 350-µm-thick slices with hippocampi were obtained from each hemisphere and stored in the holding chamber with carbogen-saturated ACSF **(Figure 7)**. For regeneration of the slices, the holding chamber with the brain slices was incubated in a water bath for 30 min and the temperature was gradually brought to 35ºC. During the next processes, the holding chamber was continuously carbogenated at room temperature (20–22ºC).

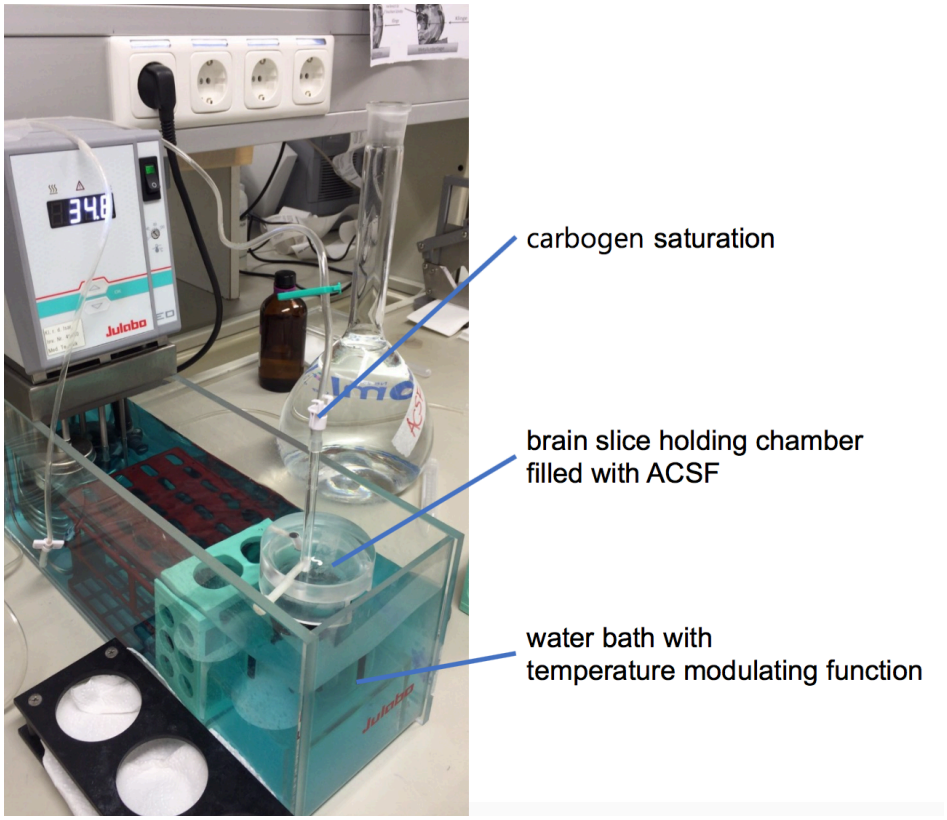

Figure 7. Brain-slice-holding chamber

2.2.3 Electrophysiology

2.2.3.1 Field excitatory postsynaptic potential (fEPSP)

Acute sagittal hippocampal slices were placed using a pipette in an immersion superfusing chamber (also a recording chamber; **Figure 9**) with an extracellular microelectrode setup. **(Figure 8)** The brain slices were fixed with a platinum grid in an ACSF-filled recording chamber. Carbogen-saturated ACSF was continuously provided in the recording chamber through a pump system in a closed circuit at a flow rate of 6 mL/min.

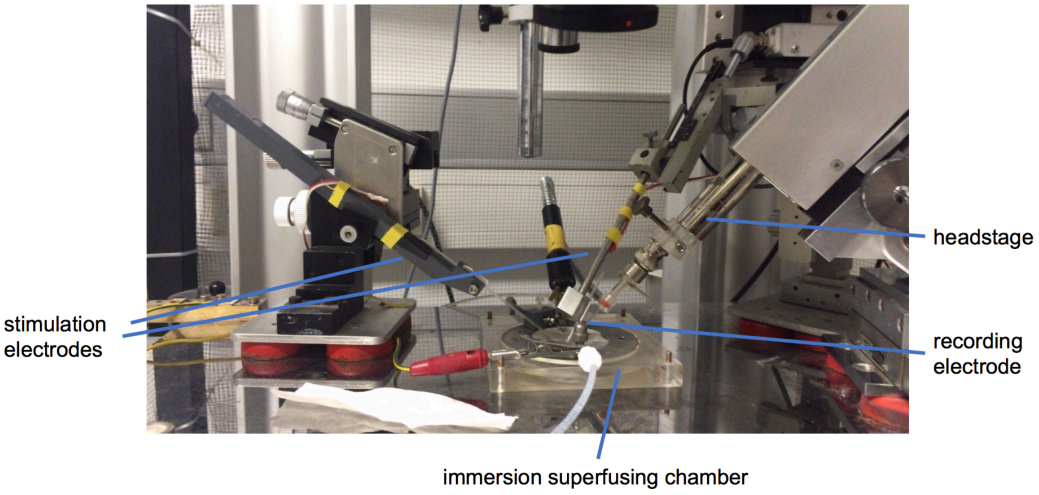

Figure 8. Extracellular microelectrode setup

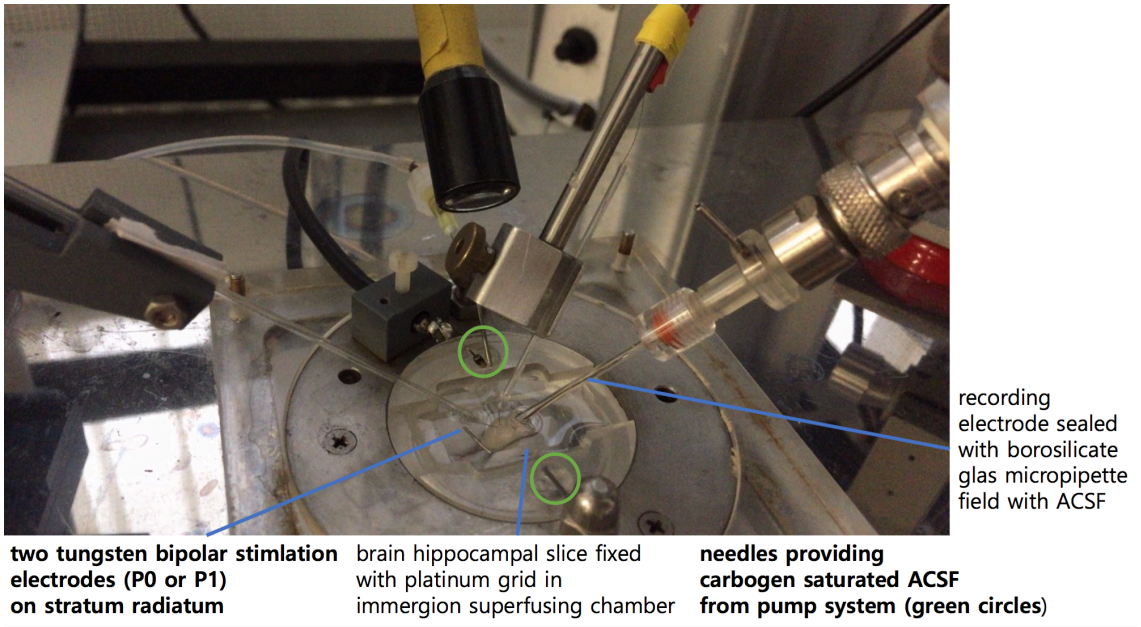

Figure 9. Immersion superfusing chamber

Both ends of SCCP from CA3 pyramidal cells were stimulated by two tungsten stimulation electrodes to generate nonoverlapping inputs, P0 and P1, representing four different experimental conditions (a control, only Aβ, only pridopidine, or both Aβ and pridopidine)

Extracellular recordings of fEPSPs were obtained by stimulating the SCCP passing through the hippocampal area CA1 of the stratum radiatum. Two tungsten bipolar microelectrodes (stimulation electrodes P0 and P1) were positioned at the anterior and the posterior end of the CA1 region using the binocular microscope and micromanipulators. Polarization of stimulus electrodes could be minimized using diphasic pulses through the twisted tungsten wire (insulated tips, a tip diameter of 50 μm). The recording electrode was a silver chloride (Ag/AgCl) electrode sealed with a borosilicate glass micropipette with an open tip (tip resistance of 1–2 MΩ) filled with ACSF. The recording electrode was positioned precisely between the two stimulus electrodes in the stratum radiatum.

Test stimuli were adjusted and applied (at a rate of 0.033 Hz, 4–5V, 20 μs per stimulating electrode) at intervals while slowly lowering the recording electrode onto the slice surface. The fEPSP amplitude was adjusted until it corresponded to 25–50% of the maximum response to avoid a population spike induction. Baseline fEPSPs were stimulated in an alternating manner in every 15 seconds via two inputs (stimulating electrodes P0 and P1). After the amplitude of the baseline fEPSPs became constant, they were recorded for at least 20 min. Then, LTP was induced through delivery of HFS (100 Hz/s) via one of the stimulation electrodes (P0 or P1). If the first LTP did not reach a minimum of 20% above the baseline level, the experiment was stopped to avoid false-negative values from hippocampal slices of poor quality.

After HFS, the stimulations from both inputs were further applied with the same frequency as before (0.033 Hz), and fEPSPs were recorded for another 60 min. The stimulation intensity was constant throughout the subsequent experiment periods.

2.2.3.2 Experimental setups

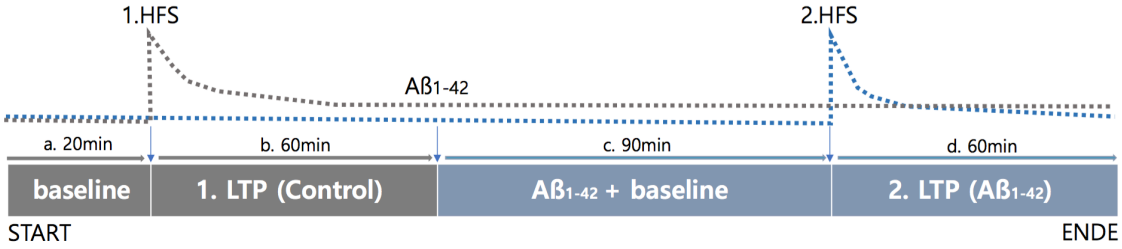

Figure 10. Effects of $A\beta_{1-42}$ oligomers on LTP of murine hippocampal slices

(Curves: schematics of fEPSP slope values that were plotted over time while recording with computer software)

a. Stable baselines were recorded for 20 min via both inputs (P0 and P1, 0.033 Hz)

b. An initial LTP (control) was induced through the HFS train (100 pulses/100 Hz) via one stimulus electrode (P1 or P0) and recorded for 60 min.

c. $A\beta_{1-42}$ (50nM) was applied to the ACSF bath and circulated for 90 min, until the $A\beta_{1-42}$ concentration reached a steady state between the circulating ACSF and the brain tissue.

d. To demonstrate synaptic toxicity of $A\beta_{1-42}$, a second LTP was induced via the other input (if the first input was P0, then with P1, and vice versa) and recorded for at least 60 min.

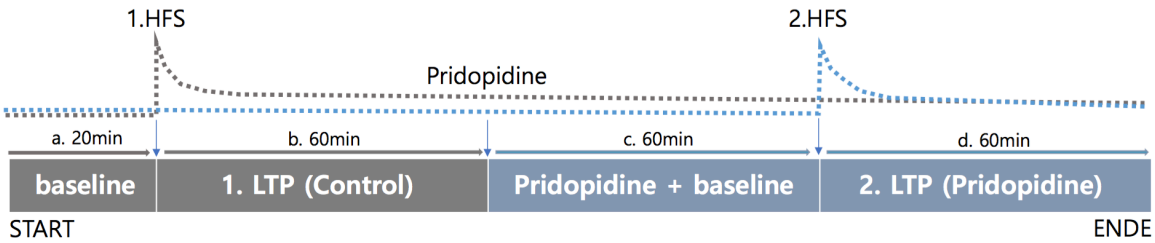

Figure 11. Effects of pridopidine on LTP of murine hippocampal slices

(Curves: schematics of fEPSP slope values that were plotted over time while recording with computer software)

a. Stable baselines were recorded for 20 min via both inputs (P0 and P1, 0.033 Hz)

b. An initial LTP (control) was induced (HFS, 100 pulses/100 Hz) via one input (P1 or P0) and recorded for 60 min.

c. Pridopidine (1 µM, 300 nM, 100 nM or 30 nM) was added to the ACSF bath and circulated for 60 min until the pridopidine concentration reached a steady state between ACSF circulation and the brain tissue.

d. A second LTP was induced via the other input and recorded for 60 min.

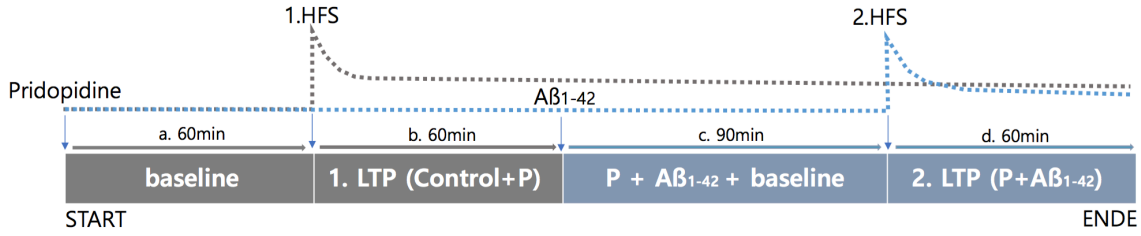

Figure 12. Effects of pridopidine on synaptic toxicity induced by $A\beta_{1-42}$ oligomers in murine hippocampal slices

(Curves: schematics of fEPSP slope values that were plotted over time while recording with computer software)

a. After the baseline fEPSPs stabilized, pridopidine (100 nM or 30 nM) was applied in the ACSF bath. The concentration of pridopidine reached a steady state while the baselines were recorded for 60 min.

b. An initial LTP (control) was induced (HFS, 100 pulses/100 Hz) via one input (P0 or P1) and recorded for 60 min.

c. $A\beta_{1-42}$ (50 nM) was applied to the ACSF bath. The concentration of $A\beta_{1-42}$ (50 nM) reached a steady state while recording fEPSPs for 90 min.

d. A second LTP was induced via the other input and recorded for 60 min.

2.2.4 Analysis and statistics

Using a laboratory interface board (ITC-16, Instrutech Corp., NY, USA) and Win LTP software, the recordings were amplified, filtered (3 kHz) and digitized (9 kHz). Two stimulation electrodes (P0 and P1) were used to apply inputs every 15 seconds in an alternating manner. For each input, two fEPSPs were averaged every minute. Data were analyzed with the software Win LTP. Slopes of the fEPSPs were measured at between 20 and 80% of the peak amplitude. The fEPSP slopes between 50 and 60 min after HFS were quantile normalized to the average baseline slopes over the last 5 min before HFS (the normalized

fEPSPs are, therefore, percent changes of LTPs in the last 10 min from the baseline). All statistical tests were performed using IBM SPSS. To test the normality of the data distribution, a Shapiro-Wilk test was used to obtain p-values. Using a paired t-test, the normalized fEPSPs of a group of slices before and after the same treatment were compared (control vs. treatment). An unpaired t-test was performed to compare two different treatments applied to groups of slices, since they were submitted to completely distinct experimental conditions. Results with $p < 0.05$ were considered statistically significant (*P ≤ 0.05, **P ≤ 0.01 and ***P ≤ 0.001).

3 Results

3.1 Aβ_{1-42} oligomers have a negative effect on LTP induction in the hippocampus

The level of control LTP induced via the first input was 147.5 ± 3.7%. After 90 min incubation of brain slices with Aβ_{1-42}, LTP was induced with the second input. In the presence of Aβ_{1-42} (50 nM), fEPSPs between 50 and 60 min after HFS could be only potentiated up to 115.4 ± 3.9%. Compared to control LTPs, the reduction was statistically significant ($p = 0.012$). Aβ_{1-42} did not affect the fEPSP before LTP induction.

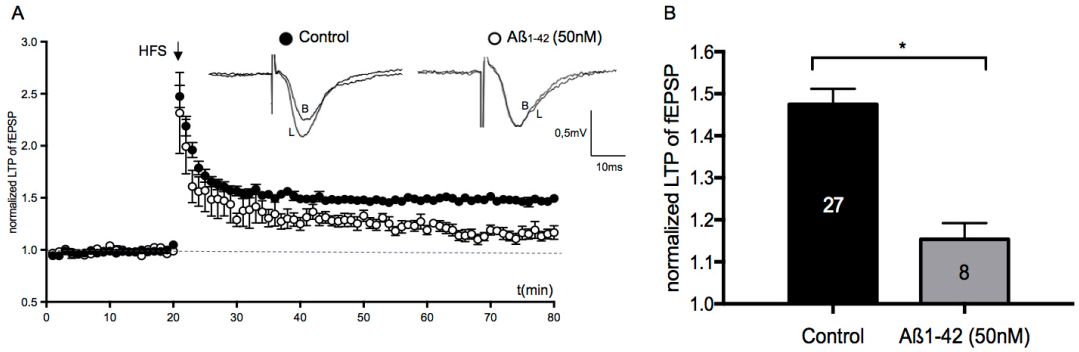

Figure 13. LTP impairment induced by Aβ_{1-42} (50nM) in murine hippocampus

A. Comparison of normalized LTPs under the control condition (black) and after the application of Aβ_{1-42} oligomers (white, 50 nM). The insets represent

normalized fEPSP traces before and after HFS (B: baseline, L: LTP, arrow: time of HFS delivery).

B. The histogram depicts averaged fEPSP values between 50 and 60 min after HFS under control conditions and after the application of $A\beta_{1-42}$. The values were quantile normalized to baseline. After 90 min of $A\beta_{1-42}$ exposure, fEPSPs in the last 10 min were significantly decreased from 147.5 ± 3.7% (n = 27) to 115.4 ± 3.9% (n = 8, p = 0.012). Data are displayed as means ± standard error of means (S.E.M.; the numbers in the histogram represent the number of experiments).

3.2 Pridopidine affects LTP induction differently depending on its concentration

To confirm the highest concentration of pridopidine that does not interfere with LTP per se, four different concentration levels of pridopidine were tested (1 µM, 300 nM, 100 nM and 30 nM). At all concentrations, pridopidine did not affect fEPSPs before HFS. Both 100 nM and 30 nM were effective concentrations, whereas 100 nM still inhibited LTP slightly compared to a control condition **(Figure 14)**. The reduction was, however, not statistically significant (147.5 ± 3.7%, n = 27 vs. 137.7 ± 7.8%, n = 9, p = 0.361). Pridopidine applied at 300 nM reduced LTP by more than half compared to control LTP. In the presence of 1 µM pridopidine, LTP was far under 20% and therefore was significantly reduced compared to a control (147.5 ± 3.7%, n = 27 vs. 110.6 ± 6.1%, n = 16, p = 0.001).

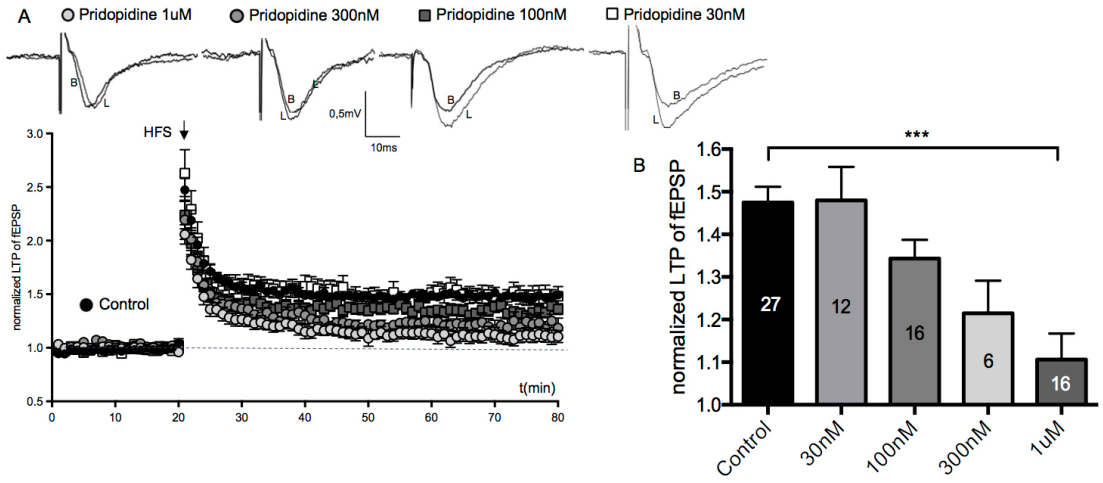

Figure 14. The effects of pridopidine applied at different concentrations upon LTP in murine hippocampus.

A. Comparison of normalized LTPs in the presence of different concentrations of pridopidine (1 μM, 300 nM, 100 nM and 30 nM). The insets represent normalized fEPSP traces before and after HFS (B: baseline, L: LTP, arrow: time of HFS delivery). Under the control condition, fEPSPs were potentiated to 147.5 ± 3.7% (n = 27). Compared to control LTP, pridopidine with a concentration of 1 μM (110.6 ± 6.1%, n = 16, p = 0.001, light grey circle) and 300 nM (121.5 ± 7.6%, n = 6, p = 0.239, dark grey circle) reduced LTP. However, 100 nM pridopidine had only a slight negative effect on LTP (134.3 ± 4.4%, n = 16, p = 0.361, dark grey square). Moreover, 30 nM of pridopidine did not block LTP (148.0 ± 7.9%, n = 12, p = 0.167, white square). Data are displayed as means ± S.E.M. and n represents the number of experiments.

B. Histogram representing the normalized fEPSPs between 50 and 60 min after HFS compared to baseline. After a brain slice was exposed to pridopidine at one of four different concentrations (1 μM, 300 nM, 100 nM and 30 nM), significant reduction of LTP could be only observed in slices incubated with 1 μM (p = 0.001).

3.3 Pridopidine (100 nM) reverses $A\beta_{1-42}$ oligomer-induced LTP deficits

Two effective concentrations of pridopidine (100 nM, 30 nM) that did not significantly reduce LTP were further tested to assess whether they could

restore the synaptotoxic effect of $A\beta_{1-42}$ (50 nM) on LTP. As mentioned above, 100 nM pridopidine alone inhibited LTP slightly; however, the effect was not statistically significant compared to a control case (p = 0.361). An $A\beta_{1-42}$-mediated inhibitory effect on LTP could be reduced through the application of 100 nM pridopidine. LTP recovery under 100nM pridopidine was statistically significant compared to LTP under $A\beta_{1-42}$ (137.7 ± 7.8%, n = 9, p = 0.034).

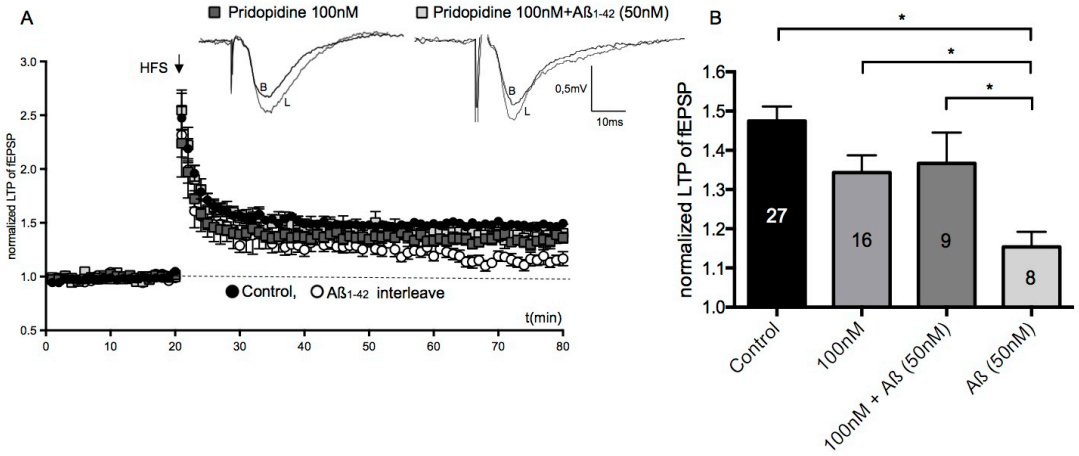

Figure 15. Effects of pridopidine (100 nM) on $A\beta_{1-42}$ (50 nM)-induced LTP impairment

A. 100 nM Pridopidine could reverse negative effects of $A\beta_{1-42}$ (50 nM) on LTP. The insets represent normalized fEPSP traces before and after HFS. (B: baseline, L: LTP, arrow: time of HFS delivery). After 60 min incubation of murine hippocampal slice with pridopidine (100 nM, dark grey square), the control LTP was evoked with HFS and monitored for 60 min. Subsequently, $A\beta_{1-42}$ was incubated for 90 min, and the second LTP was induced (light grey square). The negative effect of $A\beta_{1-42}$ on LTP could be reproduced through the interleaved experiments in a control condition (black circle) and via $A\beta_{1-42}$ application (white circle).

B. Histogram representing the normalized fEPSPs averaged between 50 and 60 min after HFS. 100 nM of pridopidine alone reduced LTP; however, the reduction was significantly minimal compared to the detrimental effect of $A\beta_{1-42}$ on LTP (134.3 ± 4.4%, n = 16 vs. 115.4 ± 3.9%, n = 8: p = 0.011). Notably, 100 nM pridopidine could restore LTP after the simultaneous

application of $A\beta_{1-42}$ (137.7 ± 7.8%, n = 9 vs. 115.4 ± 3.9%, n = 8: p = 0.034). Data are displayed as means ± S.E.M. and n represents the number of experiments.

3.4 Pridopidine (30 nM) reverses $A\beta_{1-42}$ oligomer-induced LTP deficits

Brain slices incubated with a lower concentration of pridopidine (30 nM), which did not interfere with LTP at all, produced LTP of 148.0 ± 7.9% (n = 12) on average. $A\beta_{1-42}$-mediated negative effects on LTP could be ameliorated through the application of 30 nM pridopidine with averaged LTP of 140.2 ± 8.7% (n = 8).

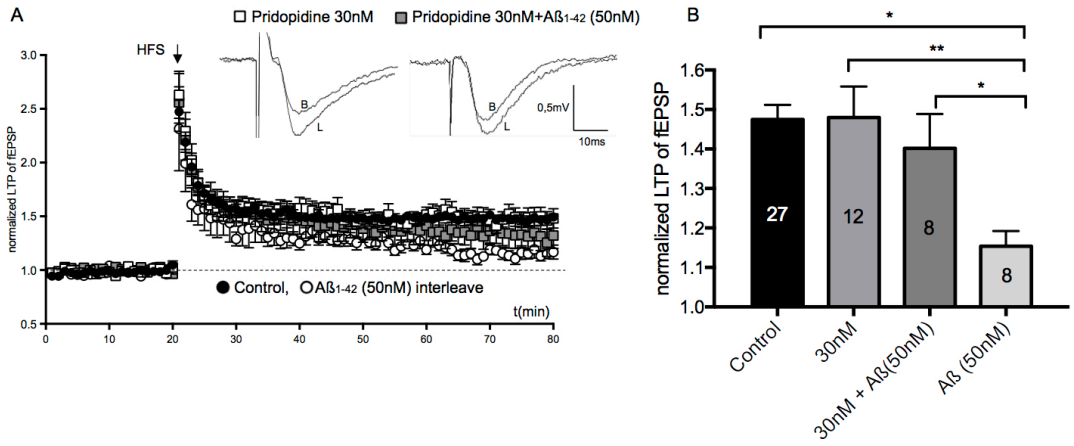

Figure 16. Effects of pridopidine (30 nM) on $A\beta_{1-42}$ (50 nM)-induced LTP impairment

A. 30 nM pridopidine could prevent $A\beta_{1-42}$-induced LTP impairment. The insets represent normalized fEPSP traces before and after HFS (B: baseline, L: LTP, arrow: time of HFS delivery). Brain slices were incubated for 60 min with 30 nM pridopidine (white square) followed by the first LTP. After 90 min of brain slice incubation with $A\beta_{1-42}$, a second LTP was evoked (grey square). The $A\beta_{1-42}$-mediated inhibitory effect on LTP could be reproduced through interleaved experiments under a control condition (black circle) and via $A\beta_{1-42}$ application (white circle).

B. Histogram showing the normalized fEPSPs averaged between 50 and 60 min after LTP induction. Compared to the control LTP, 30 nM pridopidine did not

interfere with fEPSPs at all (148.0 ± 7.9%, n = 12, p = 0.005) but restored LTP in the presence of $A\beta_{1-42}$ (140.2 ± 8.7%, n = 8, p = 0.021). Data are displayed as means ± S.E.M. and n represents the number of experiments.

4. Discussion

Pridopidine prevented LTP impairment by $A\beta_{1-42}$ oligomers in a concentration-dependent manner. Because of its higher affinity, the neuroprotective effects of pridopidine are likely mediated though S1R rather than D2R. However, subcellular mechanisms of S1R rescuing CA3-CA1 LTP impairment caused by $A\beta_{1-42}$ oligomers remain unclear. In the present experiments, S1R antagonists such as haloperidol or NE-100 were not used to confirm that S1R is required in the rescue of LTP by pridopidine.

4.1 S1R-mediated effects of pridopidine on synaptic plasticity

Researchers have suggested that S1R might improve synaptic plasticity by modulating NMDAr functions. NMDAr is known as a ligand-gated cation channel that allows the passage of nonselective cations such as Na^{2+}, Ca^{2+}-influx and K^{+}-efflux. NMDAr is blocked by Mg^{2+} at resting membrane potential and releases Mg^{2+} in a voltage-dependent manner. NMDAr, whose physiological actions are associated with the formation of LTP, is mostly localized in the postsynaptic membrane of excitatory synapses in various regions of the CNS (Kleshchevnikov, 1998). The NMDAr contains a glutamate and a glycine binding sites. NMDAr is localized not only in synaptic membranes but also in extrasynaptic compartments. Researchers have suggested that extrasynaptic NMDAr might instead be related to the formation of LTD (Papouin et al., 2012).

Martina and colleagues measured postsynaptic amplitudes of NMDAr in rat hippocampal slices using patch-clamp whole-cell recording (Martina et al., 2007). The amplitude of NMDAr was significantly increased through another

S1R-agonist, pentazocine, and this effect might be mediated by the blocking of a Ca^{2+}-dependent K^+ channel (SK channel). SK channels facilitate the hyperpolarization of dendritic spines of murine hippocampal slices. They are activated by Ca^{2+}-influx through postsynaptic NMDAr during synaptic membrane depolarization (Aydar et al., 2002; Ngo-Anh et al., 2005). Pentazocine could also enhance LTP significantly after tetanic stimulation in CA3-CA1 SCCP in hippocampal slices (Martina et al., 2007). Similarly, another synthetic SK-channel blocker was also found to potentiate LTP in CA3-CA1 synapses in murine hippocampal slices (Ngo-Anh et al., 2005).

4.2 S1R-mediated synaptogenetic effects of pridopidine

In vivo rat experiments conducted by Pabba et al. have indicated that S1R might also be related to synaptogenetic effects of S1R on NMDAr (Pabba et al., 2014). Through intraperitoneal injection of S1R agonist and antagonist, these researchers demonstrated that activations of S1R are related to an increase of protein synthesis of NMDAr subunits (GluN1A and GluN2B) and PSD-95. Pabba et al. also observed that S1R acted as a molecular chaperone to increase NMDAr traffic to the plasma membrane. Interestingly, S1R activation decreased interactions between GluN2B and PSD-95 in the synaptic membrane. As mentioned above, an extrasynaptic NMDAr that preferably contains GluN2B as at least one of its subunit may be associated with LTD for rapid homeostatic downregulation of synaptic activity (Delgado et al., 2018; Yasuda & Mukai, 2015). The homeostatic action of extrasynaptic NMDAr might indicate limited efficiency of pridopidine at certain high concentrations (1 μM, 300 nM, 100 nM), so S1R-mediated modulation of LTP might depend on cellular contexts involving different mechanisms of action.

4.3 Effects of pridopidine on ER Ca^{2+} homeostasis and Aβ formation

Ryskamp et al. demonstrated in 2017 and 2019 that pridopidine may stabilize hippocampal and striatal dendritic spines via activation of S1R through regulation of Ca^{2+} levels in ER (Ryskamp et al., 2017; D. Ryskamp et al., 2019). The stability of dendritic mushroom spines depends on intraluminal Ca^{2+} levels regulated by the neuronal-store-operated Ca^{2+} (nSOC) pathway (D. A. Ryskamp et al., 2019). In physiological states, S1R does not significantly change ER Ca^{2+} levels. S1R is, as formerly mentioned, restricted mostly to the MAM of the ER. In the ER MAM, S1R is responsible for the Ca^{2+} flux from the ER to the mitochondria (Watanabe et al., 2016).

Hippocampal and striatal cell types differ in how S1R affects Ca^{2+} homeostasis depending on S1R's interaction partners and subcellular contexts (Wu et al., 2016; Zhang et al., 2010). In murine hippocampal neurons, S1R increases nSOC and decreases ER Ca^{2+} levels through positive regulation of PS1 and PS2 proteins. Both PS1 and PS2 facilitate ER Ca^{2+}-leakage pathways in hippocampal neuronal cells. S1R increases trafficking of PS1 and PS2 into the ER membrane as a molecular chaperone (Nelson et al., 2007; Tu et al., 2006). As mentioned above, PS1 and PS2 comprise the catalytic site of γ-secretase and act as intramembrane proteases. This quality might also suggest that pridopidine and other S1R agonists could attenuate the formation of $A\beta_{1-42}$ oligomers by preventing catalytic cleavage of PS-holoproteins to produce a catalytic component of γ-secretase. Indeed, treating mice with a PS1-mutation using a synthetic S1R agonist decreased the formation of soluble and insoluble $A\beta_{1-40}$ and $A\beta_{1-42}$ in brain tissues (Fisher et al., 2016). In the present experiments, however, this effect could not be observed as $A\beta_{1-42}$ oligomers were directly applied to brain slices.

In contrast, pridopidine restored ER Ca^{2+} levels in mouse models of HD by decreasing nSOC, which is upregulated in HD (Ryskamp et al., 2017). In HD, mHtt aggregates sensitize Ca^{2+} efflux from the ER in medium spiny neurons

(MSNs; (Tang et al., 2003). MSNs are gamma-aminobutyric acidergic (GABAergic) inhibitory neurons mostly found in the basal ganglia. In the dorsal striatum, there are two types of MSNs: D1 for direct pathway and D2 for indirect pathway (Mansuri et al., 2020). These two pathways play a key role in locomotion.

4.4 Effects of pridopidine in synaptic plasticity in dose/time-dependent manners

Both 100 nM and 30 nM pridopidine reversed detrimental effect of $A\beta_{1-42}$ on LTP. Throughout the experiment, pridopidine did not change the basal fEPSPs. However, it is unclear why high concentrations of pridopidine (1 µM, 300 nM, 100 nM) reduced the amplitude of LTP compared to a control. Further experiments were not conducted to investigate if higher concentrations of pridopidine (1 µM, 300 nM) counteract the synaptotoxic effects of $A\beta_{1-42}$ oligomers on LTP. In behavioral studies of mice, the reversible effect of S1R agonists (for example, neurosteroids such as pregnenolone or dehydroepiandrosterone [DHEA] and pentazocine) on Aβ-induced amnestic deficit was found to be dose-dependent in a bell-shaped manner (Maurice et al., 1998). In contrary, a 16-hour-long treatment of murine hippocampal cultures with 100 nM or 1 µM pridopidine prevented mushroom spine loss; this effect persisted in higher doses of up to 1µM but resulted in a linear dose effect (D. Ryskamp et al., 2019). This finding indicates that the effect or pridopidine might not only be dose-dependent but also time-dependent (16 hours vs. 60 min). In oral treatment, pridopidine must cross the blood-brain barrier. Moreover, gene transcriptional effects of S1R via BDNF might be facilitated at different time courses. To investigate synaptogenetic effects of pridopidine, such as mushroom spine stabilization, a longer incubation of brain slices in pridopidine might be required. Indeed, effects of pridopidine in clinical trials based on TFC-scales could first be observed after 12 months. Since brain slices are unlikely to be healthy in our electrophysiological setups for such long periods, further investigations should be done *in vivo*.

5. Conclusion

Pridopidine is a small molecular substance that has been investigated for numerous neurodegenerative disorders such as HD, ALS, PD and AD. The aim of the present study was to explore potential therapeutic effects of pridopidine against AD symptoms by demonstrating its procognitive effect on synaptic plasticity in preclinical phases of drug development. We tested whether pridopidine could reverse detrimental effects of $A\beta_{1-42}$ oligomers on LTP. In our experiment, pridopidine restored murine hippocampal neurons from the synaptotoxic effects of $A\beta_{1-42}$ oligomers on LTP in dose- and time-dependent manners. Interestingly, pridopidine was initially assumed to be a 'dopamine stabilizer' in its pre-clinical developing phase as it improved motor functions in dual ways in animal experiments. Pridopidine was believed to target dopamine receptors such as D2R. Therefore, further investigations were initially widely performed under the hypothesis that pridopidine might improve motor functions of HD, until novel mechanisms were discovered suggesting that pridopidine might work through S1R even with higher binding affinity. Pharmacological studies have reported that S1R has generated much attention as a biomarker for the treatment of neurodegenerative diseases through its upregulation of numerous neuroprotective pathways. Given as a S1R agonist, pridopidine regulates key cellular pathways that are impaired in neurodegenerative diseases, such as release of BDNF, maintenance of Ca^{2+} homeostasis, improvement of neuronal plasticity and reduction of neuronal oxidative stress. Pridopidine has recently undergone numerous clinical trials to prove its safety. Although several clinical trials (HART, MermeiHD, PRIDE-HD) did not reach primary endpoints based on UHDRS scores because they did not significantly improve motor function of HD patients, pridopidine has been further investigated in open-label extension tests, and it indeed effected TFC-scales positively as a secondary endpoint. The efficacy of pridopidine based on TFC-scales is related to the neuroprotective and procognitive mechanisms of S1R. Pridopidine is currently in third, randomized, double blind, placebo-controlled

clinical study (PROOF HD); this work will evaluate both the motor features of patients and the efficacy of pridopidine based on TFC-scales in early stages of HD. Through PET-imaging experiments and phase 2 and 3 clinical trials, a dose with the best efficacy in early stages of HD (45 mg, b.i.d.) has also been well investigated. Further investigations should be done on the treatment of late-phase of HD *in vitro* and *in vivo* to test for a correlation between TFC-scales and biological parameters for neurodegeneration in CSF or brain tissues.

6. List of Figures

7. List of Tables

8. References

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9. Abstract

Memory impairment is one of the main symptoms of Alzheimer's disease (AD), a neurodegenerative disorder with increasing prevalence among elderly people. The pathophysiology of AD is characterized by chronic, progressive neurodegeneration which involves early synaptotoxicity. One of the most obvious pathological features of AD is the accumulation of β-amyloid (Aβ) peptides, especially $A\beta_{1-42}$, in the brain. Soluble $A\beta_{1-42}$ oligomers are thought to interfere with Ca^{2+} signaling by, for example, activating N-methyl-D-aspartic acid receptors (NMDAr) directly or increasing Ca^{2+} currents through voltage-gated calcium channels. Pridopidine is a novel drug that is currently being investigated in third clinical trials. These trials have demonstrated the neuroprotective and precognitive effects of pridopidine in neurodegenerative disorders such as Huntington's disease (HD), Parkinson's disease (PD), and AD. Pridopidine's neuroprotective effects are mediated through sigma-1 receptor (S1R). As a highly selective S1R agonist, pridopidine enhances neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and normalizes endoplasmic reticulum (ER) Ca^{2+} levels in neuronal cells. S1R might act as a molecular chaperone to potentiate synaptic plasticity by enhancing protein synthesis and trafficking NMDAr to the plasma membrane. This work explored whether pridopidine ameliorates synaptic deficits induced by $A\beta_{1-42}$. To address this question, we examined the effects of pridopidine on long-term potentiation (LTP) of the Schaffer collateral-commissural pathway **(**SCCP) in mice hippocampal slices. Specifically, we explored whether pridopidine could reverse LTP deficits in hippocampal slices in the presence of $A\beta_{1-42}$ oligomers. Since higher concentrations of pridopidine (1 μM, 300 nM) significantly reduced LTP, concentrations of 100 nM and 30 nM were further tested. Although 100 nM pridopidine slightly reduced LTP, both 100 nM and 30 nM concentrations of pridopidine restored the deficit induced by 50 nM of $A\beta_{1-42}$ oligomers. The positive synaptogenetic effects of pridopidine were demonstrated in hippocampal cell culture experiments; specifically, this treatment stabilized mushroom spines in mouse models of AD. The present data provide evidence

that pridopidine might be a potential disease-modifying therapeutic agent to treat AD, as it seems to counteract the synaptotoxic effects of $A\beta_{1-42}$ oligomers on LTP in a dose-dependent manner.

10. Publication

Pridopidine stabilizes mushroom spines in mouse models of Alzheimer's disease by acting on the sigma-1 receptor

Daniel Ryskamp, Lili Wu, Jun Wu, Dabin Kim, Gerhard Rammes, Michal Geva, Michael Hayden, Ilya Bezprozvanny

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11. List of abbreviations

A

Aβ **Amyloid-Beta**

AcH **Acetycholine**

ACSF **Artificial Cerebrospinal Fluid**

AD **Alzheimer's Disease**

ALS **Amyotrophic Lateral Sclerosis**

ApoE **Apolipoprotein E**

APP **Amyloid Precursor Protein**

AMPAr **α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor**

B

BDNF **Brain-derived neurotrophic factor**

b.i.d**. bis in die, twice a day**

BiP **Binding Immunoglobulin Protein**

C

CAG repeat **Cytosine-Adenine-Guanine repeat**

CAMKII **Calcium-calmodulin-dependent protein kinase II**

CERAD **the Consortium to Establish a Registry for Alzheimer's Disease**

CNS **Central Nervous System**

CREB **cAMP-Response Element Binding Protein**

CSF **Cerebrospinal Fluid**

CT **Computed Tomography**

CTF **C-Terminal Fragment**

D

DA **Dopamine**

DG **Dentate Gyrus**

DHEA **Dehydroepiandosterone**

DMSO **Dimethyl Sulfoxide**

D2L **the Long form of Dopamine D2 Receptor**

D1R **Dopamine D1 Receptor**

D2R **Dopamine D2 Receptor**

D2S **the Short form of Dopamine D2 Receptor**

E

eLTP **early phase of LTP**

ER **Endoplasmic Reticulum**

F

fAD **familiar Alzeimer's Disease**

fEPSP **field Excitatory Postsynaptic Potential**

FTD **Fronto-Temporal Dementia**

G

GABA **Gamma-Aminobutyric Acid**

GR **Glucocorticoid Receptor**

GSK-3α **Glycogen Synthase Kinase-3α**

H

HD **Huntington's Disease**

HDL **High-Density Lipoprotein**

HFS **High Frequency Stimulation**

5-HT **Serotonin**

Htt **Huntingtin Protein**

I

IGF **Insulin Growth Factor**

L

LRP **Low-density lipoprotein receptor-Related Protein**

LTD **Long-Term Depression**

LTP **Long-Term Potentiation**

lLTP **late phase of LTP**

M

MAM **Mitochondrial-Associated Membrane**

MAPK **Mitogen-Activated Protein Kinase**

mHtt **mutated Huntingtin Protein**

MRI **Magnetic Resonance Imaging**

mGluR **Metabotrophic Glutamate Receptor**

MMST **Mini-Mental-Status Test**

MSN **Medium Spiny Neuron**

N

nAChr **nicotinergic Acetylcholine Receptor**

NA **Noradrenaline**

NIA **National Institute of Aging**

NMDAr **N-methyl-D-aspartic acid receptor**

NSF **N-ethylmaleimide-Sensitive Fusion Protein**

nSOC **pathway neuronal-store-operated Ca^{2+} pathway**

P

PD **Parkinson's Disease**

PET **Positron Emission Tomography**

PICK1 **Protein Interacting with C Kinase-1**

PIN1 **Protein Interacting with NIMA 1**

PKA **Protein Kinase A**

PKC **Protein Kinase C**

PKMζ **Protein Kinase M-zeta**

PrP^c **cellular prion protein**

PSD-95 **Postsynaptic Density protein 95**

PSEN1 **Presenilin Gene 1**

PSEN2 **Presenilin Gene 2**

PS1 **Presenilin 1**

PS2 **Presenilin 2**

R

ROS **reactive oxygen species**

U

UHDRS **Unified Huntington Disease Rating Scale**

S

sAPPα **Soluble Amyloid Precursor Protein**

SCCP **Schaffer Collateral-Commissural Pathway**

S1R **Sigma-1 Receptor**

SK-channel **Ca^{2+}-dependent K^{+} Channel**

SPECT **Single Photon Emission Computed Tomography**

T

TFC **Total Functional Capacity**

TrkB **Tropomyosin receptor kinase B**

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