DOCTORAL THESIS

Toxicity of Amyloid Beta Peptides and the Effect of α-lipoic Acid in Cellular and Fruit Fly Models of Alzheimer's Disease

Kristel Metsla

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Declaration:
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for doctoral or equivalent academic degree.

Kristel Metsla

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Amüloid beeta peptiidide toksilisus ning α-lipoehappe mõju Alzheimeri tõve rakulistes ja äädikakärbse mudelites

KRISTEL METSLA

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Contents

List of Publications

The list of publications, on the basis of which the thesis has been prepared:

I Krishtal, J., Bragina, O., **Metsla, K.**, Palumaa, P., & Tõugu, V. (2017). In situ fibrillizing amyloid-beta 1-42 induces neurite degeneration and apoptosis of differentiated SH-SY5Y cells. *PLoS One*, *12*(10), e0186636. https://doi.org/10.1371/journal.pone.0186636

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III **Metsla, K*.**, Kirss, S.*, Laks, K., Sildnik, G., Palgi, M., Palumaa, T., Tõugu, V., & Palumaa, P. (2022). α-Lipoic Acid Has the Potential to Normalize Copper Metabolism, Which Is Dysregulated in Alzheimer's Disease. *Journal of Alzheimer's disease: JAD*, *85*(2), 715–728. https://doi.org/10.3233/JAD-215026

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Author's Contribution to the Publications

Contribution to the papers in this thesis are:

I Cell growth and differentiation, Aβ40 and Aβ42 toxicity experiments, measurement of caspase activity, preparation of microscopy slides, preparation of the manuscript

II Cell growth and differentiation, Aβ42 toxicity experiments, immunocytochemistry and microscopy, preparation of the manuscript

III Cell growth and differentiation, toxicity experiments, sample preparation for ICP-MS experiments, *Drosophila melanogaster* maintenance and husbandry, feeding of *Drosophila* with lipoic acid, *Drosophila* negative geotaxis experiments, preparation of the manuscript

Introduction

Alzheimer's disease (AD), the most common type of dementia, is the 7th leading cause of mortality in the USA and one of the costliest diseases for society. AD is a global public health concern and unfortunately, there is no treatment to cure it, making its research particularly important. *In vitro* experiments, especially those employing neuronal cell lines, play an essential role in advancing our understanding of neurodegenerative diseases, including AD. Human neuroblastoma SH-SY5Y cells are one of the most used cellular models of neurodegenerative diseases. Although widely used, they are commonly used in the undifferentiated state, which is not representative of mature neurons. The major advantages of using SH-SY5Y cells are their human origin and ability to differentiate into various types of neuron-like cells resembling the neurons in the human brain. Moving beyond cell culture studies, *Drosophila melanogaster* is a widely used, cost-effective, simple model organism for studying human diseases, including AD, at the organismal level. Accumulation of amyloid beta (Aβ) peptides and metal ion imbalance, especially copper, are characteristic features of AD. However, the role of Aβ and the exact mechanism of toxicity are largely unknown. Restoration of copper homeostasis has also not been successful so far. The current thesis included the development of cell culture models with different neuronal phenotypes suitable for studying the effects of Aβ peptides and potential drug candidates. Cell viability measurements with WST-1 and membrane permeability tests with propidium iodide demonstrated that cholinergic and noradrenergic cells were susceptible to Aβ toxicity, whereas dopaminergic cells were nearly resistant. In addition to the investigation of Aβ on neuron-like cells, one of the obtained differentiated cultures was successfully used to show that a natural compound, α-lipoic acid was able to redistribute copper from the extracellular to the intracellular environment. Moreover, it rescued AD model flies from developing an AD phenotype. The differentiated cell culture models obtained can be used to study more precise mechanisms of Aβ toxicity and to screen potential drug candidates at the cellular level. The results also point to α-lipoic acid as a compound that may help improve some of the underlying copper-related pathological mechanisms of this devastating disease.

Abbreviations

Aβ	Amyloid beta
APP	Amyloid precursor protein
ACh	Acetylcholine
ACH	Amyloid cascade hypothesis
ACH2.0	Amyloid cascade hypothesis 2.0
AChE	Acetylcholinesterase
AD	Alzheimer's disease
AICD	Amyloid precursor protein intracellular domain
α-LA	Alpha lipoic acid
BACE1	β-site APP cleaving enzyme 1
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
ChAT	Choline acetyltransferase
CSF	Cerebrospinal fluid
CTR1	Copper transporter 1
DA	Dopamine
dbcAMP	Dibutyryl cyclic adenosine monophosphate
DHLA	Dihydrolipoic acid
DMT1	Divalent metal transporter 1
EOAD	Early-onset AD
FAD	Familial AD
GWAS	Genome-wide association study
LC	Locus coeruleus
LOAD	Late-onset AD
MD	Menkes disease
NFT	Neurofibrillary tangle
NA	Noradrenaline
PD	Parkinson's disease
PSEN1	Presenilin 1
PSEN2	Presenilin 2
RA	Retinoic acid
ROS	Reactive oxygen species
SMVT	Sodium-dependent multivitamin transporter
SNP	Single nucleotide polymorphism
SOD1	Superoxide dismutase 1
TH	Tyrosine hydroxylase
TPA	12-O-tetradecanoylphorbol-13-acetate
VAChT	Vesicular acetylcholine transporter
VTA	Ventral tegmental area
WD	Wilson disease
WHO	World Health Organization

1 Review of the Literature

1.1 Alzheimer´s Disease

AD is a progressive, multifactorial neurodegenerative disease that slowly destroys memory, thinking, behaviour, and eventually the ability to carry out simple tasks (Selkoe, 2001). According to the World Health Organization (WHO), over 55 million people worldwide live with dementia, and AD contributes to 60-70% of these cases (World Health Organization, 2023). Moreover, 22% of all persons aged 50 and above have some form of AD, ranging from preclinical to late stages (Gustavsson et al., 2023).

AD is pathologically defined by extensive neuronal loss and the combined presence of extracellular metal-enriched amyloid plaques composed of Aβ peptides and intracellular neurofibrillary tangles (NFT) composed of hyperphosphorylated tau proteins (Alzheimer et al., 1995). Other abnormalities linked to AD include altered levels of neurotransmitters, metal ions, and overproduction of reactive oxygen species (ROS) (Yang et al., 2023; Babic Leko et al., 2023; Manoharan et al., 2016).

AD can be divided into different types based on genetic predisposition and the age of onset. Based on the age of onset AD can be divided into early-onset (EOAD) and late-onset (LOAD) forms. EOAD is estimated to account for only 5-10% of all disease cases (Ayodele et al., 2016). Some cases of EOAD are caused by gene mutations that can be passed from parent to child, also known as familial AD (FAD). FAD is caused by mutations in amyloid precursor protein (APP), presenilin 1 (PSEN1), or presenilin 2 (PSEN2). Although very rare, these mutations have extremely significant implications. In total, ∼330 pathogenic or likely pathogenic mutations have been reported in these three genes (Xiao et al., 2021). Several APP mutations have been identified within the Aβ sequence, e.g., Flemish (A692G), Dutch (E693Q), Arctic (E693G), and Iowa (D694N) (Grabowski et al., 2001; Cras et al., 1998). Most people have the late-onset sporadic form, in which symptoms become apparent after the age of 65. There is no specific gene that directly causes LOAD, however, the apolipoprotein E ε4 allele (ApoE4) is the strongest known genetic risk factor that increases the risk of developing AD up to 15-fold (Corder et al., 1993; Troutwine et al., 2022). In addition, many AD-related single-nucleotide polymorphisms (SNPs) have been discovered by genome-wide association studies (GWASs) (Escott-Price, Hardy 2022; Andrews et al., 2023). Although SNPs have a smaller impact, they may be helpful in early disease prediction.

As AD exerts a huge economic burden on society and a psychological burden on patients and caregivers, there is an urgent need to find cost-effective strategies for both the prevention and treatment of this multifactorial disease.

1.2 Amyloid Beta Peptides

Amyloid plaques, one of the two lesions required for the neuropathological diagnosis of AD, are mainly composed of Aβ peptides but also contain high concentrations of metal ions (Rajendran et al., 2009). Aβ peptides are generated from APP by enzymes that cleave it into smaller fragments. APP is a single-pass transmembrane protein that is especially abundant in neurons. APP can be cleaved by amyloidogenic or non-amyloidogenic pathways (**Figure 1, top**), both are present also in healthy individuals (O´Brien & Wong, 2011). In the non-amyloidogenic pathway, APP is cleaved by α-secretase within the Aβ sequence, producing the neuroprotective sAPPα and preventing Aβ formation. Subsequent γ-secretase cleavage generates the p3 peptide. In contrast, the amyloidogenic pathway is the major source of Aβ peptides. In the amyloidogenic pathway, the first cleavage of APP is usually performed by an

enzyme called β-site APP cleaving enzyme 1 (BACE1, also known as β-secretase), which cuts the protein into two fragments: a large extracellular portion, sAPPβ, and a smaller piece C99 that remains in the cell membrane. The remaining fragment is subsequently cleaved by an enzyme called γ-secretase, a multi-subunit complex consisting of four different proteins (PSEN1 or PSEN2, nicastrin, APH-1, PEN-2), that releases Aβ peptide from the membrane (Zhang et al., 2014). Depending on the cleavage sites, the length of the peptides can vary from 37 to 43 amino acids. Of these peptides, the two most abundant Aβ species found in amyloid plaques are composed of 40 and 42 amino acid residues – Aβ40 and Aβ42, respectively. Although less abundant, Aβ42 is more amyloidogenic and toxic due to two additional hydrophobic amino acids in the C-terminus (Mrdenovic et al. 2022) (**Figure 1, bottom**).

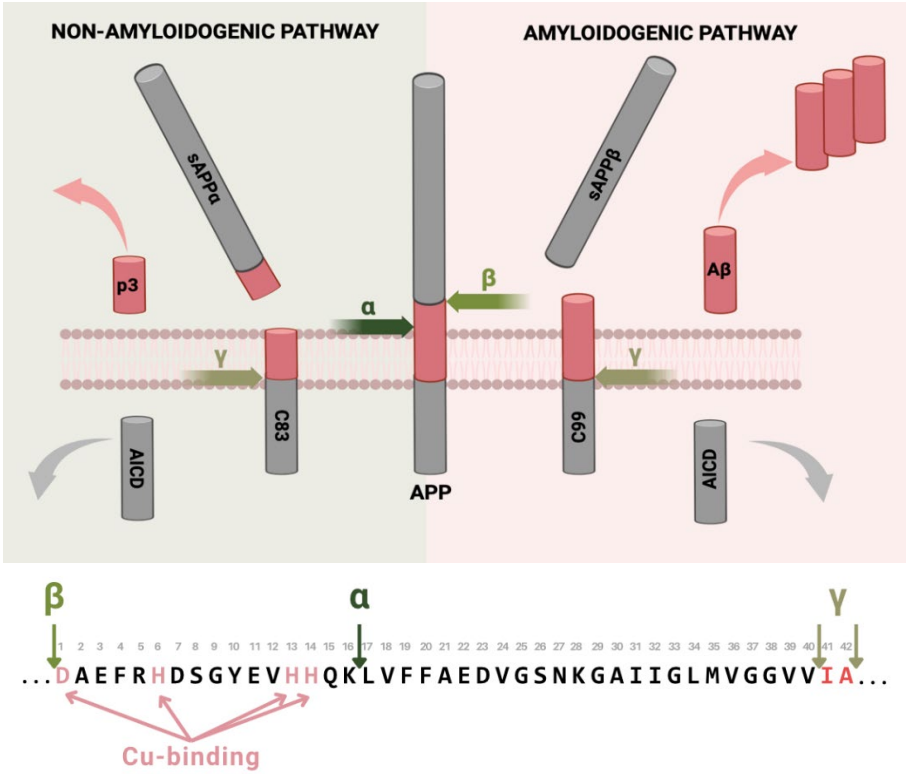

Figure 1. Top: *Schematic representation of APP processing pathways. APP undergoes proteolytic cleavage by α-, β-, and γ-secretases (abbreviated as α, β, and γ), resulting in either the non-amyloidogenic or amyloidogenic processing. Cleavage products include soluble APP fragments (sAPPα, sAPPβ), C-terminal fragments (C83, C99), Aβ or p3, and the APP intracellular domain (AICD). The figure was created with BioRender.* ***Bottom:*** *Amino acid sequence of Aβ showing the main α-, β-, and γ- secretase cleavage sites and amino acid residues participating in binding of Cu(II) ions (light pink). Two extra C-terminal amino acid residues making Aβ42 more prone to aggregation, are marked in red. The figure was created with BioRender.*

In addition to the well-known α-, β-, and γ-secretases, some other secretases have been identified that also contribute to the processing of APP. These include η-secretase, which generates longer APP fragments with potential neurotoxic effects, δ-secretase, which enhances amyloidogenic processing by facilitating β-secretase cleavage, and meprin-β, a metalloprotease capable of cleaving APP at an alternative β-like site (Willem et al., 2015; Zhang et al., 2015; Bien

et al., 2012). While their exact roles are still being elucidated, these additional secretases may also impact APP metabolism and AD progression.

The exact role of Aβ is unclear, but in physiological conditions, it may be important for normal development, neuronal growth and survival, synaptic function, and, in low concentrations, may even exhibit neuroprotective properties against oxidative stress, toxins, and pathogens (Bishop & Robinson, 2004; Bernabeu-Zornoza et al., 2019). In pathological conditions, overproduction, misfolding, and impaired clearance of Aβ occur, the ratio of Aβ42/Aβ40 changes, and the levels of toxic Aβ42 increase (Mrdenovic et al. 2022). An increased Aβ42/Aβ40 ratio is associated with enhanced neurotoxicity in cultured mouse hippocampal neurons and affects behaviour and learning in mice *in vivo* (Kuperstein et al., 2010). In humans, Aβ42/Aβ40 ratio is widely used as a biomarker in cerebrospinal fluid (CSF) and plasma for the diagnosis of AD. A decreased ratio is usually observed in AD patients reflecting the deposition of Aβ42 in plaques and the relative abundance of Aβ40 in circulation (Pérez-Grijalba et al., 2019; Doecke et al., 2020).

Aβ can exist in multiple assembly states – monomers, oligomers, protofibrils, fibrils. Soluble monomers can aggregate into oligomers, protofibrils and fibrils. Fibrils are large and insoluble and can assemble into plaques (Hardy & Selkoe, 2002). Monomeric Aβ binds metal ions, e.g. Cu(II), Zn(II), Fe(II), and Pb(IV), in the N-terminal region (Wärmländer et al., 2019). Amino acid residues involved in Cu(II) coordination are Asp1, His6, His13, and His14 (Faller & Hureau, 2009) (**Figure 1, bottom**). The most toxic form of Aβ is still a subject of debate. While monomers and fibrils are generally considered non-toxic, oligomers and protofibrils are widely regarded as the most neurotoxic species (Chen et al., 2023; Tolar et al., 2021). In addition, toxic effects have been attributed to Aβ-copper complexes (Smith et al., 2006). Aβ accumulation does not always correlate with neuronal loss and cognitive decline (Aizenstein et al., 2008), and later studies have shown that even the extent of brain damage has a weak correlation with cognitive symptoms, likely due to large individual differences in baseline cognitive reserve (Morbelli & Nobili, 2014). Moreover, Aβ deposition has even been suggested to play a protective role (Rischel et al., 2023).

Aβ is one of the potential biomarkers to indicate early stages of the disease and can be detected using imaging technologies, blood plasma, or CSF test (Schindler et al., 2019; Hansson et al., 2018). Studies have shown that Aβ42 is lower in AD CSF compared to healthy controls, and the decline is evident even 25 years before the appearance of the symptoms (Motter et al., 1995; Bateman et al., 2012). On the other hand, high CSF Aβ42 levels predict normal cognition in amyloid-positive individuals with *APP*, *PSEN1*, or *PSEN2* genetic mutations (Sturchio et al., 2022).

Numerous studies have focused on understanding the toxicity of Aβ and its role in AD progression, but the precise mechanism by which Aβ neurotoxicity is mediated is unclear. However, it is known that Aβ interacts with plasma membrane and cellular organelle membranes and may change their properties (Sasahara et al., 2013). In addition, Aβ may form pores in the membrane that allow a dysregulated influx of Ca^{2+} ions into the cells that may ultimately lead to cell death (Di Scala et al., 2016; Dhaouadi et al., 2023). Accumulation of Aβ in the mitochondria has also been observed to precede extracellular Aβ deposition. Intramitochondrial Aβ can interact with mitochondrial proteins and cause their dysfunction (Caspersen et al., 2005). By targeting mitochondria, Aβ42 has been shown to induce apoptotic cell death by activating the caspase signalling pathway (Han et al, 2017; Keil et al., 2004).

The events preceding Aβ accumulation play an important role in AD pathogenesis, however, their exact contributions and the mechanisms underlying Aβ toxicity are unclear and need thorough investigations.

1.3 Hypotheses of AD

Since the description of AD in 1906, several hypotheses have been proposed to describe the cause of the disease, and these hypotheses have also been the basis for drug development. Some of the most popular hypotheses are shown in **Figure 2** and important hypotheses in the context of this thesis are described in more detail in the following chapters.

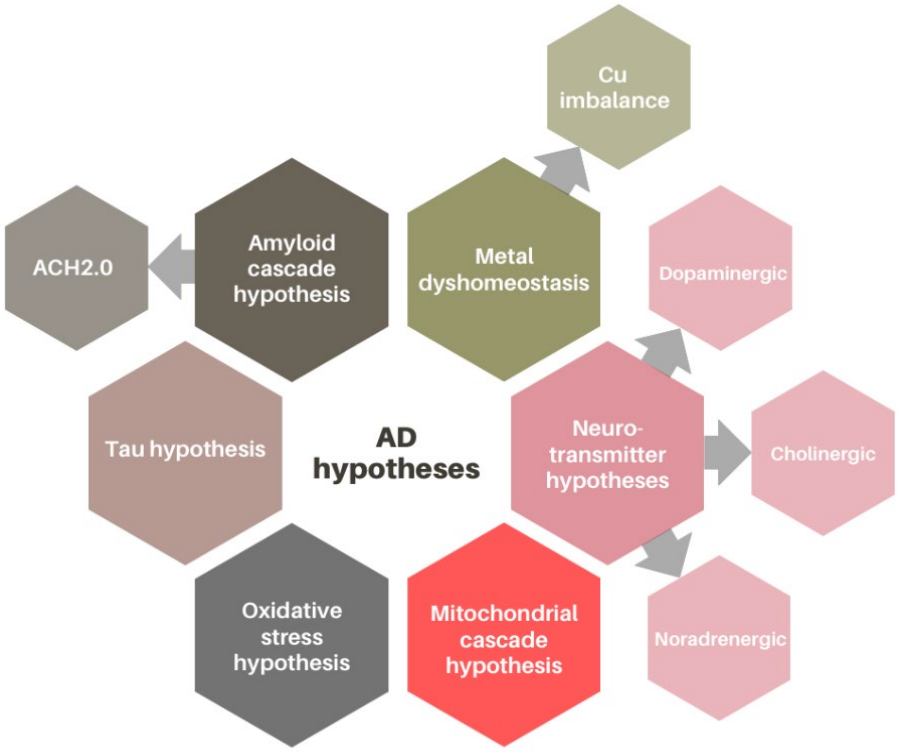

Figure 2. *Some of the hypotheses of AD. The figure was created with Canva.*

1.3.1 Amyloid Cascade Hypothesis

Amyloid cascade hypothesis (ACH), described by Hardy and Higgins in 1992, posits that the deposition of Aβ is the central event in AD pathology, and that hyperphosphorylation of tau, the formation of NFT, cell damage and dementia follow as a direct result of this deposition (Hardy & Higgins, 1992). The hypothesis has been modified throughout the years. In 2002, Hardy and Selkoe proposed that soluble Aβ oligomers, but not monomers or insoluble fibrils, are the toxic entities responsible for synaptic dysfunction in AD (Hardy & Selkoe, 2002).

Recently, another reformulation of ACH was proposed called amyloid cascade hypothesis 2.0 (ACH2.0) (Volloch & Rits-Volloch, 2023). Compared to the original ACH, ACH2.0 suggests that toxicity is driven by intracellular rather than extracellular Aβ. Although APP-derived Aβ is normally secreted from the cell membrane after proteolytic cleavage, there are two ways it can end up in the cell. First, the cellular uptake of secreted Aβ constitutes its conversion to intracellular Aβ. A prerequisite to the uptake of Aβ appears to be the formation of Aβ aggregates from "stickier" Aβ42, which are taken up twice as efficiently as other Aβ species. The cellular uptake of Aβ is mediated by multiple receptors and depends on ApoE isoforms, with ApoE4

being significantly more efficient than the other variants. Another source of APP-derived intracellular Aβ is its retention within neurons. Although the vast majority of APP-derived Aβ results from the γ-cleavage of APP on the plasma membrane and is secreted, a small fraction undergoes γ-cleavage on the internal membranes, and the derived Aβ is retained as intracellular Aβ (Volloch & Rits-Volloch, 2023). Intraneuronally-accumulated, APP-derived intracellular Aβ triggers a relatively benign cascade that activates the APP-independent intracellular Aβ-generating pathway, which, in turn, initiates the second devastating cascade that includes tau pathology and leads to neuronal loss (Volloch & Rits-Volloch, 2022). The important role of intracellular Aβ is also indicated by the fact that in AD patients, intraneuronal Aβ precedes both extracellular Aβ deposition and intracellular NFT formation (Gouras et al., 2000).

ACH has been considered the central and most influential hypothesis in AD research for the past three decades. Therefore, Aβ has been the most extensively investigated and commonly targeted molecule for the development of AD therapy.

1.3.2 Neurotransmitter Hypotheses

Beyond the well-established amyloid and tau hypotheses, alterations in neurotransmitter systems have been implicated in AD. Brain changes may start more than 20 years before the AD symptoms appear, and brain atrophy may start 13 years before the expected onset of symptoms (Gordon et al., 2018). The brain contains various types of neurons that are often defined by the identity of the neurotransmitters they release. Studies have shown that multiple neurotransmitter systems, e.g. cholinergic, dopaminergic, glutamatergic, serotonergic, noradrenergic, and GABAergic, are involved in the development and progression of AD (Chakraborty et al., 2019; Carello-Collar et al, 2023; Gannon et al., 2015; Conway, 2020; Pan et al., 2019; Ferreira-Vieria et al., 2016), further reflecting the heterogeneity of the disease. Alterations to the neurotransmitter systems and corresponding hypotheses relevant to the context of this thesis will be described in more detail.

1.3.2.1 The Cholinergic Hypothesis

The cholinergic system plays an important role in memory and learning (Hasselmo, 2006). Cholinergic neurons are mainly located in the striatum and basal forebrain, including the nucleus basalis of Meynert (Ahmed et al., 2019).

The cholinergic hypothesis is the earliest theory explaining the pathogenesis of AD (Francis et al., 1999). The theory arose when decreased cholinergic activity in AD patients' brains was discovered. Degeneration and reduction of cholinergic neurons and severe deficiency of acetylcholine (ACh), a neurotransmitter that plays a vital role in memory, attention, learning, and neuroplasticity, are characteristic of AD pathogenesis (Chen et al., 2022). Additionally, AD patients exhibit reduced activity of choline acetyltransferase (ChAT), an enzyme responsible for ACh synthesis in the hippocampus, temporal, and frontal cortex (Rinne et al., 1988). A large body of evidence collected over the decades suggests that cerebral loss of cholinergic innervation in AD patients' cerebral cortex is correlated with cognitive impairment (Hampel et al., 2019).

Three of the currently used symptom-relieving drugs, donepezil, galantamine, and rivastigmine, were developed based on this hypothesis. These are acetylcholinesterase inhibitors that slow down the hydrolysis of ACh, thus increasing the availability of ACh in synapses. Unfortunately, their effect appears to be temporary, and efficacy in improving cognition is low (Moreta et al., 2021).

1.3.2.2 The Noradrenergic Hypothesis

It was described already in the early 1980s that locus coeruleus (LC), the major noradrenergic nucleus of the brain, degenerates early in AD before the appearance of pathological features

(Mann et al., 1980). LC is a small nucleus in the pons of the brainstem where most of the central nervous system (CNS) noradrenaline (NA) is produced, and it is crucial for regulating attention, arousal, and cognitive functions. It has also been demonstrated that the overall neuronal loss is the greatest in LC and exceeds the cholinergic neuronal loss in the nucleus basalis of Meynert (Zarow et al., 2003). This is noteworthy as neuronal loss in the nucleus basalis is generally considered a hallmark sign of AD.

It has been hypothesized that early loss of noradrenergic neurons and reduction of NA in the brain lead to cognitive dysfunction and neurodegeneration. The involvement of NA in AD was first suggested when a marked loss of noradrenergic neurons in the LC of patients with AD was identified (Bondareff et al., 1982). Atrophy of LC has been detected in mild cognitive impairment as well as in the case of AD. In addition, volumetric reduction of LC was seen in cognitively normal people who later progressed to a diagnosis of AD dementia (Dutt et al., 2020). Moreover, postmortem analysis has shown that LC is among the first brain regions where Aβ is deposited and NFT start to accumulate (Thal et al., 2002; Braak et al., 2011).

1.3.2.3 The Dopaminergic Hypothesis

Another catecholamine neurotransmitter, dopamine (DA), is also linked to the pathology of dementia. Dopaminergic neurons are mainly located in the substantia nigra and ventral tegmental areas (VTA), but can be found in raphe nuclei, hypothalamus and olfactory bulb (Fu et al., 2016; Pignatelli & Belluzzi, 2017; Fontaine et al., 2015; Lin et al., 2020).

More than 50% of AD patients have concomitant parkinsonism with DA system disorders, e.g. rigidity, resting tremor, and reduced DA transporter availability (Sasaki, 2018). Evidence from both an early-stage AD genetic mouse model (Tg2576) and AD patients suggests that DA neurons in the VTA undergo degeneration (Nobili et al., 2017; De Marco & Venneri, 2018), which reduces hippocampal dopaminergic innervation (Spoleti et al., 2024). It has been hypothesized that diminished dopaminergic VTA activity may be pivotal for the earliest pathological features of AD (De Marco & Venneri, 2018). Behavioural and psychological symptoms of dementia are present since the earliest AD stages, and dopaminergic dysfunction likely plays a role in their manifestation (Serra et al., 2010). Dopaminergic system has a seizure-modulating effect (Bozzi & Borrelli, 2013), and it has been demonstrated that seizures can occur early in AD, even before memory problems become apparent (Vossel et al., 2013).

Additionally, reduced levels of DA and its receptors have been reported in the brains of AD patients and meta-analyses indicate that DA depletion is associated with faster cognitive decline (Storga et al., 1996; Karrer et al. 2017; Pan et al., 2019). Interestingly, a protective effect of DA and its derivatives on Aβ aggregation and toxicity has been demonstrated (Nam et al., 2018).

1.3.3 Metal Ion Hypothesis

The dyshomeostasis of metal ions has been implicated in a wide range of diseases, such as AD, Parkinson disease (PD), Wilson disease (WD), Menkes disease (MD), cardiovascular disease, and metabolic syndrome (Bush, 2003; Pyatha et al., 2022; Bandmann et al., 2015; Kaler, 2011; Ding et al., 2022). In the CNS, copper, iron, and zinc ions are essential enzyme cofactors, and are required for mitochondrial and neuronal function. In healthy brains, levels of free metal ions are strictly regulated, and their concentrations are very low. The metal ion hypothesis proposes that disruptions in the homeostasis and distribution of metal ions, especially copper, iron and zinc, contribute to AD by promoting Aβ deposition, tau hyperphosphorylation, neuronal death and neuroinflammation (Bush & Tanzi, 2008; Chen et al., 2023).

Recently, a new copper imbalance hypothesis has been proposed by Squitti et al. (Squitti et al., 2021). Although the dysregulation of Cu has been known to participate in AD for a long time, the authors theorize that the imbalance of copper due to a gradual shift from strongly protein-

bound intracellular pools to extracellular pools of loosely bound toxic metal ions is among the contributing early drivers of AD. Supporting evidence includes findings of reduced total copper in AD brain tissue, elevated levels of non-ceruloplasmin-bound copper in serum and CSF, and correlations between increased labile copper and cognitive decline (James e al., 2012; Squitti et al., 2021).

1.4 The Role of Metals in the Brain and AD

Although various metals play essential roles in the human body, the dysregulated homeostasis of essential metals and exposure to non-essential metals may contribute to AD pathologies. Increased metal concentrations in the brain may lead to oxidative stress, neuroinflammation, Aβ aggregation, and tau hyperphosphorylation (Wallin et al., 2017; Ashok et al., 2015; Pathak & Sriram, 2023). Non-essential heavy metals such as lead, cadmium, mercury, and an essential heavy metal, manganese, affect cognitive function and may contribute to cognitive decline (Lee et al., 2022; Li et al., 2018; Xu et al., 2018; Menezes-Filho et al., 2011).

Studies have shown that the homeostasis of essential metals, especially iron, zinc, and copper, is altered in AD, and high concentrations of these metal ions have been found in amyloid plaques of AD patients' brains (Miller et al., 2006). Many regions of Aβ and domains of APP bind metal ions, especially zinc and copper ions, with high affinity (Faller & Hureau, 2009; Bush et al., 1993; Hesse et al., 1994). In addition, calcium and magnesium ions have also been implicated in AD pathogenesis (Cascella & Cecchi, 2021; Du et al., 2022).

Redox active metal ions, iron and copper, can catalyse the production of ROS, and especially of hydroxyl radical (HO·), via Fenton or Haber-Weiss reactions. The Fenton reaction describes the formation of the most toxic and reactive HO· by a reaction between Fe^{2+} and H_2O_2. In the Haber-Weiss reaction, iron catalyses the generation of HO· and OH^- from the reaction of H_2O_2 and $O_2^{\cdot-}$. Although these reactions were initially discovered with the participation of iron, they also proceed with other metals, including copper, but may sometimes be named slightly differently, such as Fenton-like (Meyerstein, 2021). In general, the brain is more vulnerable to oxidative stress than other organs due to its high lipid content, low levels of antioxidants, and extremely high energy requirements (Lee et al., 2020). Even though the brain represents about 2% of total body weight, it accounts for about 20% of the body's oxygen consumption (Koeppen, 1999).

Iron is the most abundant trace element in the human body. In the brain, iron is involved in oxygen transport, mitochondrial respiration, DNA synthesis, myelin synthesis, and both the synthesis and metabolism of neurotransmitters (Ward et al., 2014). The concentration of iron in the brain is approximately 0.72 mM. Brain iron levels increase with aging and abnormal iron accumulation has been observed in different regions of the AD brain (Acosta-Cabronero et al., 2016). In amyloid plaques, the concentration of iron is approximately 0.94 mM (Lovell et al., 1998). Iron dyshomeostasis may cause oxidative stress in the brain, leading to cellular damage. Studies have found intracellular iron accumulation before the formation of amyloid plaques (Smith et al., 1997). In 2012, a novel iron-dependent cell death termed ferroptosis was discovered by Dixon et al. (Dixon et al., 2012), and it has been hypothesized to have a role in AD progression (Bao et al., 2021).

Zinc is the second most abundant trace element in the body. In the brain, zinc is a structural or functional component of many essential proteins and an important regulator of synaptic activity and neuronal plasticity (Li et al., 2022). The brain contains approximately 1.5% of the total body's zinc. The concentration of Zn in serum is approximately 15 μM, while in the brain it is 10 times higher, reaching 150 μM (Takeda, 2000). In amyloid plaques the concentration of zinc can be as high as 1 mM (Lovell et al., 1998). Zinc protects cells from oxidative stress by

increasing the synthesis of a powerful antioxidant glutathione (Cortese et al., 2008). On the other hand, increased intracellular zinc levels may induce neuronal death by inhibiting mitochondrial energy production (Sheline et al., 2000; Brown et al., 2000). More than 2-fold increase in zinc levels has been detected in AD brains compared with normal control subjects (Religa et al., 2006).

Copper is the third most abundant trace element in the body, and the brain contains approximately 9% of the total body's copper (Zatta et al., 2008). In the brain, it is crucial for both general and more brain-specific metabolic processes, acting as a cofactor for enzymes involved in energy metabolism (cytochrome c oxidase), neurotransmitter biosynthesis (dopamine β-hydroxylase), iron metabolism (ceruloplasmin), antioxidant defence (SOD1), etc (Arredondo & Núñez, 2005). The concentration of copper ions in amyloid plaques is approximately 0.4 mM (Lovell et al., 1998).

1.4.1 The Role of Copper in AD

Copper is an essential trace element that is vital for the proper functioning of all organisms. Copper is mainly present in two redox states, Cu(I) and Cu(II). It is closely associated with the oxidative stress hypothesis of AD, which proposes that the imbalance between ROS production and antioxidant defence is a key driver of neurodegeneration (Butterfield & Halliwell, 2019; Roy et al., 2023). As a redox-active metal, copper can catalyse Fenton-like reactions that convert H_2O_2 into highly reactive HO·, thereby exacerbating oxidative damage and promoting Aβ aggregation, tau hyper-phosphorylation, and mitochondrial dysfunction (Barnham et al., 2004; Bagheri et al., 2018). Copper metabolism must be tightly regulated to provide a sufficient supply for the metalation of cuproenzymes and, on the other hand, to prevent copper-induced oxidative stress. Generally, optimal copper levels are necessary for normal brain functioning, and imbalance may lead to disease. Two of the best-studied disorders related to impaired copper metabolism are MD and WD, of which MD is caused by ATP7A mutations leading to copper deficiency and WD is caused by ATB7B mutations leading to copper overload (Bull et al., 1993). AD is somewhere in between, as AD patients show decreased levels of copper in the brain tissues and increased levels of copper in blood serum and CSF (Squitti et al., 2021; Grotto & Glaser, 2024; Hozumi et al., 2011).

In humans, copper is unevenly distributed in the brain, and its levels generally decline with age (Bonilla et al., 1984). Highest copper levels are found in the LC, substantia nigra, and hippocampus (Warren et al., 1960). In AD patients, reduced copper levels have been observed in the hippocampus and amygdala, which are associated with learning and memory and usually show severe histopathological alterations (Deibel et al., 1996). Some studies even suggest that higher brain copper levels may protect from cognitive decline (Agarwal et al., 2022).

Copper enters the brain through the blood-brain barrier (BBB) and blood-cerebrospinal fluid barrier (BCB). BBB cells express copper transporters CTR1, ATP7A, and ATP7B. CTR1 is responsible for copper uptake. The efflux of copper from brain cells is mainly mediated by ATP7A and ATP7B, which are regulated by copper levels. The role of ATP7B is less clear; it may also be involved in copper buffering (Roy & Lutsenko, 2024). Copper uptake can be alternatively mediated by non-specific divalent metal transporter 1 (DMT1). Because free copper ions can generate ROS, excess cellular copper in the brain is safely stored through binding to copper chaperones and metallothioneins (Tapia et al., 2004). In the extracellular space, copper can be taken up by neighbouring cells or cleared into the CSF (Ohrvik & Thiele, 2014; Monnot et al., 2012). Under physiological conditions, the concentration of free copper ions is very low - less than one per cell (Rae et al., 1999).

Historically, the prevailing view was that copper bound to Aβ in amyloid plaques contributed to AD pathogenesis mainly through redox cycling between Cu(I) and Cu(II), generating ROS and promoting oxidative stress (Opazo et al., 2002, Cheignon et al., 2018). However, more recent studies have refined this understanding. It has been shown that in plaques, copper preferentially binds to N-terminally truncated Aβ species such as $A\beta_{4\text{-}40}$ or $A\beta_{4\text{-}42}$, which contain a high-affinity ATCUN binding site. These truncated peptides exhibit sub-picomolar affinity for Cu(II) and form redox-inactive complexes that significantly suppress ROS generation compared to full-length Aβ (Stefaniak & Bal, 2019). This suggests that the plaque-bound pool of copper is largely redox-silent, and that the redox-active, potentially toxic copper species in AD are more likely to reside in soluble or loosely bound pools rather than in the fibrillar plaque core.

Copper is implicated in multiple disease processes and pathological mechanisms. In AD brains, increased levels of loosely bound labile copper have been reported (James et al., 2012). As evidence points to Aβ oligomers as key neurotoxic species, Cu(II) may stabilize these oligomeric states and modulate their generation (Sharma et al., 2013). Copper is also involved in tau pathology as it can bind tau and potentially initiate tau hyperphosphorylation and fibril formation, leading to synaptic failure and neuronal death (Bacchella et al., 2020). Additionally, decreased levels or activity of several copper-binding proteins, including superoxide dismutase 1 (SOD1), cytochrome c oxidase, and metallothionein-3 have been reported in AD (Murakami et al., 2011; Morais et al., 2021; Yu et al., 2001). Presenilins, which mediate the proteolytic cleavage of APP, can promote cellular uptake of copper (Southon et al., 2013; Greenough et al., 2011). Furthermore, excessive dietary copper intake from drinking water and supplements has been proposed as a contributing factor to AD development (Brewer, 2019).

1.5 Risk Factors and Prevention of AD

While some risk factors like age and genetics cannot be changed, other factors can be managed to help reduce the risk of getting AD. As with many other diseases, a healthy lifestyle helps decrease the risk of developing AD. A 2024 report published in The Lancet, found that nearly half of global dementia cases could be delayed or prevented by eliminating, treating, or reducing 14 risk factors (Livingston et al., 2024). These risk factors are a lower level of education, hearing loss, hypertension, physical inactivity, diabetes, social isolation, excessive alcohol consumption, air pollution, smoking, obesity, traumatic brain injury, depression, high LDL cholesterol, and untreated vision loss.

In addition, various nutritional interventions may improve cognitive functions and slow the progression of AD. Healthy eating, especially Mediterranean diet, appears to reduce the risk of cognitive decline (Carcía-Casares et al., 2021). Gut microbiota is also associated with cognitive functioning through the gut-brain axis (González Cordero et al., 2022). Studies show that AD patients appear to have lower plasma levels of carotenoids, vitamins A, C, and E (Qu et al., 2021; Mullan et al., 2018; Hamid et al., 2022). Vitamin B supplementation is associated with slowing cognitive decline (Wang et al., 2022) and vitamin D supplementation may also reduce dementia and AD risk (Zhang et al., 2024). Moreover, levels of some micronutrients are significantly lower even in the brains of AD patients, further indicating the importance of a healthy diet and proper supplementation (Dorey et al., 2023).

Genetic testing and personalized medicine are also emerging and may prove to be useful early preventive measures. Recent findings indicate that approximately 95% of APOE4 homozygotes exhibit preclinical signs of AD that were evident nearly 20 years before clinical AD diagnosis (Fortea et al., 2024).

1.6 Treatment of AD

To date, there is no effective treatment to prevent or halt the progression of AD. Thus, drugs that can prevent AD or cease its progression are urgently needed. As of January 1, 2024, there were 164 clinical trials assessing 127 drugs (Cummings et al., 2024).

Currently used drugs are divided into two groups, symptom-relieving drugs and drugs that change the progression of the disease (**Table 1**). Three of the five symptom-relieving drugs are cholinesterase inhibitors: donepezil, rivastigmine, and galantamine. The fourth, memantine, is an NMDA receptor antagonist that regulates glutamate activity, and the fifth, Namzaric, is a combination of cholinesterase inhibitor donepezil and glutamate regulator memantine (2024 Alzheimer's disease facts and figures, 2024).

***Table 1**. Currently approved and used AD drugs and their mechanisms of action.*

Drug	Target	Description	References
Symptom-relieving drugs			
Donepezil	Acetylcholinesterase	Increases ACh levels by inhibiting its breakdown in the synaptic cleft	Rogers & Friedhoff, 1996
Rivastigmine	Acetylcholinesterase, butyrylcholinesterase	Increases ACh levels by inhibiting both acetylcholinesterase and butyrylcholinesterase	Nordberg et al., 2013
Galantamine	Acetylcholinesterase, nicotinic receptors	Increases ACh levels and enhances nicotinic receptor activity	Coyle & Kershaw, 2001
Memantine	NMDA receptor	Antagonizes NMDA receptors to regulate glutamate activity, reducing excitotoxicity	Tampi et al., 2007
Namzaric	Acetylcholinesterase, NMDA receptor	Combination drug of Donepezil & Memantine targeting cholinergic dysfunction and glutamate activity	Deardorff & Grossberg, 2016
Anti-amyloid therapies			
Aducanumab*	Aggregated forms of Aβ	Monoclonal antibody (mAB) binding to aggregated soluble and insoluble forms of Aβ	Dhillon, 2021
Lecanemab	Soluble Aβ protofibrils	mAB targeting soluble Aβ protofibrils to reduce neurotoxic aggregation	Van Dyck et al., 2023
Donanemab	Aβ plaques	mAB targeting a specific Aβ epitope (N3pG) to promote plaque clearance	Shukla & Misra, 2023

*Although aducanumab is no longer in clinical use since 2024, it played a pivotal role as the first disease-modifying therapy to receive FDA approval for AD.

The use of drugs that change disease progression started in June 2021, when an accelerated approval was given to a new drug, aducanumab, which helps to reduce amyloid deposits in the brain and may therefore help to slow the progression of AD. This was the first treatment to affect the underlying cause of the disease, although it has not been shown to affect clinical outcomes, e.g., cognitive decline (Dhillon, 2021). Lecanemab is the second amyloid-targeting drug approved by the FDA in 2023 that is used to treat early AD or mild dementia due to AD with elevated brain Aβ (van Dyck et al., 2023; U.S. Food and Drug Administration, 2023). In 2024, the third anti-amyloid drug donanemab received approval from the FDA. Poor clinical improvement of anti-amyloid therapies and unknown long-term side effects suggest that amyloid may not be the optimal target (Piller, 2022; Dantas et al., 2024).

In addition to pharmacological treatments, non-drug interventions such as physical activity, memory exercise, music and art-based therapies, and dancing have been shown to support cognitive function and improve daily living in individuals with AD (2024 Alzheimer's disease facts and figures, 2024; De la Rosa et al., 2020).

1.6.1 Metalloregulatory Clinical Trials

The restoration of metal homeostasis may be one way to treat AD and various metalloregulatory approaches have already been tested. One approach is taking metal supplements. Although several studies have proposed a positive effect of Cu and Zn supplementation on cognitive performance, the improvement of cognitive function has not been confirmed (Bagheri et al., 2018). A pilot phase 2 clinical trial did not show any effect of oral copper supplementation on cognition in mild AD patients (Kessler et al., 2008). In a six-month randomized, double-blind trial of a novel zinc formulation, participants aged 70 years or older showed slower cognitive decline and reduced serum free copper compared with placebo group (Brewer & Kaur, 2013; National Library of Medicine, 2011). A phase II trial registered in the EU Clinical Trials Register evaluated zinc sulphate in individuals with prodromal AD but was ended prematurely without published results (European Medicines Agency, 2019).

Another approach is the chelation of excess Zn, Cu, or Fe ions. Although high-affinity chelators may be very effective, they may also remove metals from essential metalloproteins and pathways. Because metals are critical to many vital cellular processes, the chelator must be carefully selected, and metal ionophores are preferred. Several copper ionophores have been tested, but unfortunately, they are not effective enough without severe side effects. Clioquinol, a moderate chelator of Cu, Zn, and Fe, has been tested in phase 2 clinical trials (Ritchie et al., 2003). Although it decreased Aβ aggregation and improved the cognitive behaviour of mice, it was banned due to neurotoxicity. PBT2, a derivative of clioquinol, showed improvement in the learning and memory capacity of APP transgenic mice and was proposed as a candidate disease-modifying drug. Unfortunately, it did not show significant results in phase 2 trials either (Adlard et al., 2008; Villemagne et al., 2017).

Recently, a phase 2 clinical trial studied the effect of iron chelator deferiprone on cognitive decline in AD (U.S. National Library of Medicine, 2017). Results presented at Alzheimer's Association International Conference in August 2024 revealed that deferiprone treatment caused significant acceleration of cognitive decline. It could be discussed whether the dose was inappropriate, or iron is important for cognition in AD (Alzheimer's Association, 2024).

Considering the inefficacy of synthetic chelators and the occurrence of adverse effects, it is necessary to identify better alternatives for the restoration of metal homeostasis in AD.

1.6.2 Alpha-Lipoic Acid

Alpha-lipoic acid (α-LA), is a naturally occurring antioxidant that is endogenously synthesized, found in several foods, and available as a dietary supplement. It has been considered a promising substance for the prevention and treatment of neurodegenerative disorders, including AD. The antioxidant properties of α-LA include ROS scavenging, regeneration of endogenous antioxidants, repair of oxidative damage, and metal chelating capacity (Biewenga et al., 1997). α-LA shows antioxidant, anti-inflammatory, antiapoptotic, glioprotective, and metal chelating properties in both *in vivo* and *in vitro* studies (Kaur et al., 2021). Furthermore, α-LA has been shown to reverse age-associated loss of neurotransmitters, e.g. DA, NA, serotonin, and their receptors (Arivazhagan & Panneerselvam, 2002).

α-LA functions as a cofactor in several mitochondrial multienzyme complexes, including the glycine decarboxylase complex, pyruvate dehydrogenase complex, α-ketoglutarate dehydrogenase complex, branched-chain α-ketoacid dehydrogenase complex (Solmonson & DeBerardinis, 2018). This role depends on lipoylation, a process in which α-LA is covalently attached to specific lysine residues of these enzymes (Rowland et al., 2018). Structurally, α-LA contains two sulfhydryl groups, which can be oxidized to form a disulfide bond (**Figure 3**). Its reduced form, dihydrolipoic acid (DHLA), predominates in the intracellular reducing environment and exhibits stronger antioxidant and metal-chelating activity compared to α-LA. While both α-LA and DHLA can scavenge ROS, only DHLA can repair oxidative damage and regenerate endogenous antioxidants such as vitamins C, E, and glutathione (Biewenga et al., 1997).

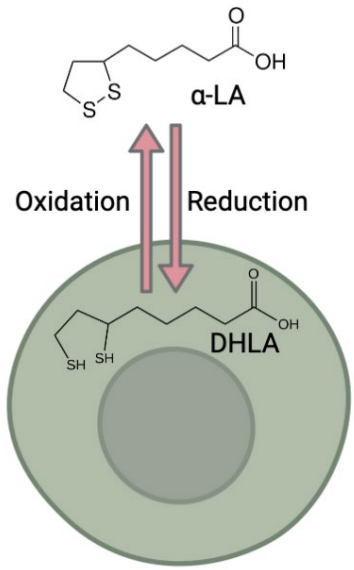

Figure 3. *The oxidized and reduced forms of lipoic acid. The figure was created with BioRender.*

α-LA exists as two optical isomers: the R- and S- enantiomers. Only the R-enantiomer is endogenously synthesized and can be covalently bound to proteins via lipoylation. Most commercial supplements contain a racemic mixture of R- and S-lipoic acid (Shay et al., 2009). α-LA is absorbed from the diet and can easily cross the BBB (Biewenga et al., 1997). Although the exact mechanisms of its cellular uptake remain unclear, α-LA is believed to be transported via several pathways, including the proton-linked monocarboxylic acid transporter and the sodium-dependent multivitamin transporter (SMVT) (Takaishi et al., 2007; Zehnpfennig et al., 2015).

Beyond its antioxidant properties, α-LA role in mitochondrial metabolism also links it to copper homeostasis. Recent study by Tsvetkov et al. demonstrated that copper binds with very high affinity to the lipoyl groups of TCA cycle enzymes, causing their aggregation, disruption of iron-sulfur cluster proteins, and mitochondrial dysfunction, leading to a distinct form of cell death termed cuproptosis. This mechanism highlights the central role of lipoylation in copper biology and mitochondrial integrity, both of which are altered in AD (Tsvetkov et al., 2022).

α-LA has been studied for its potential therapeutic applications across a range of health conditions. It was first proposed as an effective antioxidant due to its ability to prevent the deficiency symptoms of vitamins C and E (Rosenberg & Culik, 1959). It is currently used in the treatment of diabetic polyneuropathy (Ziegler et al., 2004) and has also been used in managing hepatic diseases (Berkson, 1999). A 2018 meta-analysis found that α-LA supplementation significantly reduced fasting glucose, insulin, hemoglobin A1c, triglycerides, total- and low-density lipoprotein-cholesterol levels in individuals with metabolic disorders (Akbari et al., 2018). Another clinical trial demonstrated beneficial effects of α-LA supplementation on blood pressure (Vajdi et al., 2023). In a 16-month trial in patients with mild to moderate AD, 600 mg/day α-LA was associated with slower cognitive decline, with the greatest improvements seen in patients with insulin resistance (Fava et al., 2013). In a small, randomized, placebo-controlled pilot trial, a combination of omega-3 fatty acids and α-LA was shown to slow cognitive and functional decline in patients with AD (Shinto et al., 2014). In animal models, dietary supplementation with α-LA improved memory and learning and reduced hippocampal-dependent memory deficits of aged Tg2576 mice, without affecting Aβ levels (Quinn et al., 2007).

Despite these findings, the exact mechanism by which α-LA may influence copper metabolism and neurodegenerative processes remain unclear.

1.7 Models to Study AD

1.7.1 Cellular Models of AD

A variety of *in vitro* cellular models are widely used to investigate the pathology and progression of AD, as well as for drug screening. Among them are primary neuronal cultures, induced pluripotent stem cells (iPSCs), 3D-cell models, and cancer cell lines, including rat PC12 cells, mouse Neuro-2A cells, and human neuroblastoma SH-SY5Y cells (Choi et al., 2016).

The SH-SY5Y cell line is a thrice-cloned subline of SK-N-SH cell line originally derived from a metastatic bone marrow biopsy of a female neuroblastoma patient. It serves as a good model for the study of neurodegenerative disorders since the cells can be differentiated into various neuronal phenotypes in response to specific chemical compounds. Neuroblastoma cell lines are generally comprised of three different cell

types: neuroblastic N-type, non-neuronal epithelial- or glial-like S-type, and intermediate I-type cells (Ross et al., 1995; Campos Cogo et al., 2020). The SH-SY5Y line is primarily derived from N-type cells, but there are also several other cells present, making it a heterogeneous culture (Bell et al., 2013).

Undifferentiated SH-SY5Y cells exhibit low expression of ChAT and tyrosine hydroxylase (TH), markers of cholinergic and dopaminergic neurons, respectively (Kovalevich & Langford, 2013). However, various studies have managed to differentiate SH-SY5Y cells toward cholinergic, noradrenergic, dopaminergic, and more recently, glutamatergic phenotypes (Martin et al., 2022). Cell treatment with retinoic acid (RA) followed by BDNF increases the expression of ChAT and VAChT, and activity of acetylcholinesterase (AChE), indicative of cholinergic differentiation (**Figure 4**) (Goldie et al., 2014). Sequential treatment with RA followed by phorbol esters (TPA, MPA) induces a dopaminergic phenotype, as evidenced by increased TH and dopamine transporter (DAT) expression, and elevated dopamine D2 and D3 receptor levels (Avola et al., 2018; Presgraves et al., 2004). Dibutyryl cyclic adenosine monophosphate (dbcAMP) treatment promotes noradrenergic differentiation, indicated by increased TH expression and NA content (Kume et al., 2008).

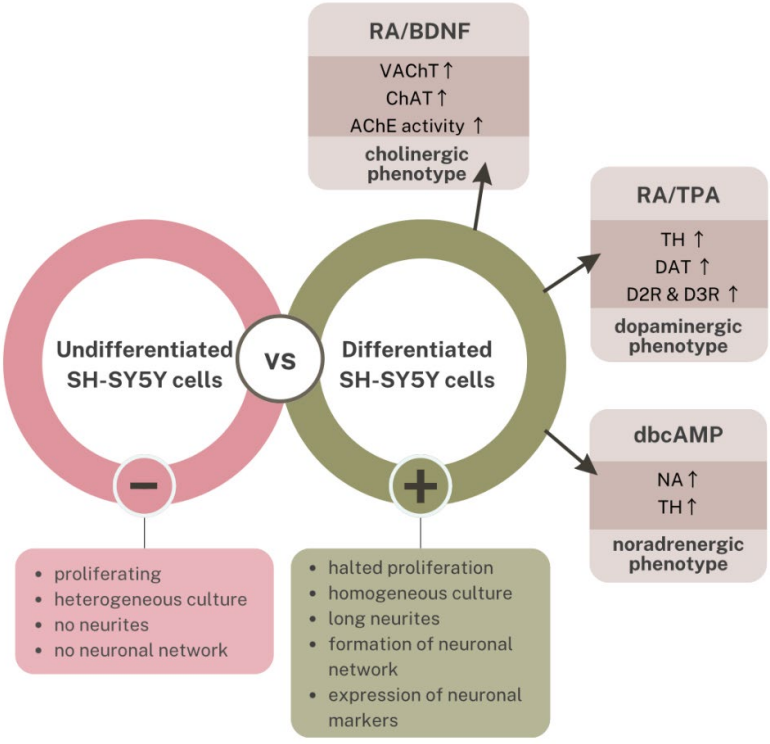

Figure 4. *Comparison of undifferentiated and differentiated SH-SY5Y cells. The figure was created with Canva.*

Although SH-SY5Y cells differentiated into different neuronal phenotypes using previously mentioned agents have been used in various experiments (Pifferi et al., 2024; Presgraves et al., 2004; Arun et al., 2006), their susceptibility to Aβ has not been thoroughly studied or compared.

1.7.2 Drosophila Melanogaster as a Model Organism

The fruit fly *Drosophila melanogaster* has been used as a model organism for genetic studies longer than any other. There are many advantages of using *Drosophila* as a model organism. It has a short lifespan, requires only 9 days to progress from a fertilized egg to an adult, and the maintenance and breeding are cheap and easy. More than 76% of the genes that cause human diseases have a homolog in fruit flies, and mutants can now be obtained for essentially any gene (Reiter et al., 2001). Neurodegenerative diseases, including AD, can be easily modeled using *Drosophila*, and several different transgenic lines have been generated that can be bought from stock centers, e.g., Bloomington Drosophila Stock Center. The most widely used AD models are flies expressing human Aβ, which are generated using UAS-GAL4 system (Nitta & Suggie, 2022). This two-component system is based on crossing a driver line that provides cell- or tissue-specific GAL4 expression with a responder line carrying human Aβ under the control of Upstream Activation Sequence (UAS) that binds GAL4. When these two lines are crossed, GAL4 binds to UAS and Aβ is expressed in the desired cells or tissues in the progeny (Brand & Perrimon, 1993).

Different phenotypes and markers can be measured in AD fly models. Longevity measurement is a great indicator of a fly´s overall viability and neurological health. Behavioral assays, e.g. negative geotaxis assay (**Figure 5**), and aversive phototaxis suppression assay, represent a good method to measure locomotor activity, learning, and memory functions in *Drosophila* (Ali et al., 2011).

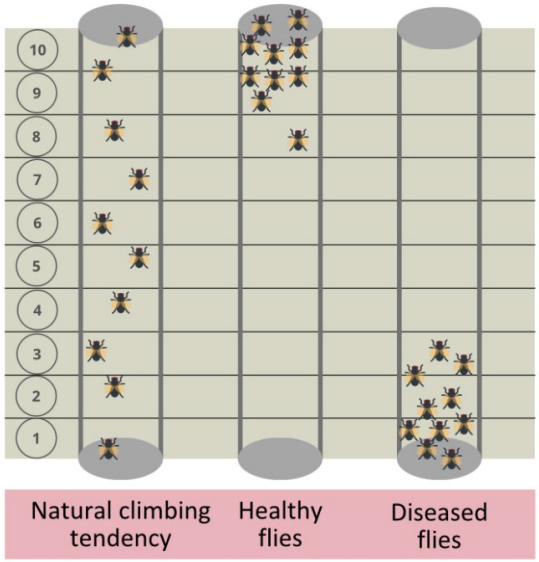

Figure 5. *Negative geotaxis experiment setup. Fruit flies have a natural upward climbing behaviour after being tapped to the bottom of a test tube. Healthy flies climb up quickly, but diseased flies remain at the bottom of the test tube. The figure was created with Canva.*

Drosophila eye is also an excellent model system. While the normal compound eye of a fruit fly consists of regularly arranged ommatidia, mutations and toxic compounds can disrupt it and lead to different eye phenotypes, e.g. rough or smooth (Finelli et al., 2004).

AD model flies have been used in various types of experiments. It has been shown that the expression of Aβ42 in the brain reduces the lifespan of flies and induces a rough eye phenotype that worsens with age (Finelli et al., 2004). *Drosophila* AD models have been used to study the influence of metal ions on Aβ-induced neurodegeneration (Ott et al., 2015; Hua et al., 2011; Singh et al., 2013; Rival et al., 2009). Singh et al showed that feeding flies expressing human Aβ42 with copper-supplemented food caused severe eye degeneration with dark patches and a novel copper chelator, which they called compound L, rescued this severe phenotype (Singh et al, 2013). Rival et al. showed that flies expressing Aβ42 were more sensitive to oxidative stress and had also higher levels of oxidative damage compared to control flies and flies expressing Aβ40 (Rival et al., 2009). They also showed that iron-binding protein ferritin suppressed the toxicity of Aβ42 and restored the survival and locomotion function to normal. In their experiments, clioquinol increased the lifespan of Aβ42-expressing flies and reduced iron levels in the brain. Hua et al showed that dietary supplementation with zinc and iron exacerbated eye damage caused by eye-specific Aβ42 expression, but the administration of metal chelators DP-109 or bathocuproine sulphonate (BCS) were able to ameliorate it (Hua et al., 2011).

Aims of the Study

1. To develop a differentiated neuron-like SH-SY5Y cell culture suitable for the studies of Aβ toxicity and screening of potential therapeutic compounds.
2. To compare the susceptibility of three different neuron-like cell types, cholinergic, noradrenergic, and dopaminergic, to Aβ toxicity.
3. To investigate the effect of α-lipoic acid on copper translocation in neuron-like SH-SY5Y cell culture.
4. To study the effect of α-lipoic acid in *Drosophila melanogaster* AD model.

Materials and Methods

The following methods, described in more detail in the respective articles, were used in the study:

- Cultivation and differentiation of SH-SY5Y cell line (Publications I, II, III)
- WST-1 assay for cell viability assessment (Publications I, II)
- Propidium iodide (PI) staining for the detection of dead/permeabilized cells (Publication I, II, III)
- Measurement of caspase activity (Publication I)
- Preparation of Aβ peptides for experiments (Publications I, II)
- Immunocytochemistry (Publications I, II)
- Microscopy (Publications I, II, III)
- Assessment of neurite abnormalities through image analysis (Publication I)
- ICP-MS analysis (Publication III)
- Breeding and crossing of *Drosophila melanogaster* (Publication III)
- Feeding and maintenance of *Drosophila melanogaster* (Publication III)
- Negative geotaxis experiments with *Drosophila melanogaster* (Publication III)
- Scanning Electron Microscopy (SEM) for evaluation of eye phenotype in *Drosophila melanogaster*

Results

Publication I

- The toxicity of Aβ40 and Aβ42 was compared in undifferentiated and RA/BDNF-differentiated SH-SY5Y cells after 48 and 72 hours of incubation. The results are as follows:
  - 20 μM Aβ40 had no toxic effect on undifferentiated cells at either 48 or 72 hours, as assessed by the WST-1 cell viability assay and PI staining. In fact, after 48 hours, cell viability increased by 11%.
  - 20 μM Aβ42 reduced viability of undifferentiated cells by 16% after 48 hours, but did not affect cell permeability. However, after 72 hours, 20 μM Aβ42 did not alter cell viability but increased cell permeability by 30%.
  - 20 μM Aβ40 had no statistically significant effect on RA/BDNF-differentiated cells at either 48 or 72 hours.
  - 20 μM Aβ42 did not significantly affect RA/BDNF-differentiated cells after 48 hours. However, the highest toxicity was observed after 72 hours, where Aβ42 caused a 42.7% reduction in cell viability and a 50% increase in cell permeability.
- Overall, RA/BDNF differentiation of SH-SY5Y cells increased susceptibility to Aβ peptide toxicity compared to undifferentiated cells.
- Aβ42 induced apoptosis of differentiated SH-SY5Y cells, whereas Aβ40 did not, as determined with caspase-3/7 activity measurement.
- Both Aβ40 and Aβ42 induced neurite beading and fragmentation in differentiated cell culture, but Aβ42 had a much more pronounced effect.
- Aβ42 aggregates covered both cell bodies and neurites, whereas the less amyloidogenic Aβ40 partially covered only the cell bodies, as determined by immunocytochemistry.
- Fibrillar aggregates were present in the cell culture exposed to Aβ42 after 48 hours but not in the cell culture exposed to Aβ40.
- The addition of preformed Aβ42 fibrils to differentiated cells did not result in toxicity.

Publication II

- Differentiation of SH-SY5Y cells using three different protocols (RA/BDNF, RA/TPA, dbcAMP) induced β-III tubulin positive neurite outgrowth and network formation.
- The RA/BDNF-differentiated culture was the most homogenous, whereas dbcAMP and RA/TPA-differentiated cultures contained small populations of undifferentiated cells.
- The toxicity of 10 μM and 20 μM Aβ42 after 48 hours of incubation was compared in undifferentiated and differentiated SH-SY5Y cells. The results are as follows:
  - 10 μM Aβ42 did not affect cell viability or membrane permeability in undifferentiated SH-SY5Y cells, as determined by the WST-1 cell viability assay and PI staining, respectively. However, 20 μM Aβ42 decreased cell viability by 43% and increased dead cell count by 1.8-fold.
  - Noradrenergic dbcAMP-differentiated cells were most sensitive to Aβ42 toxicity in a concentration-dependent manner. At 10 μM Aβ42, viability decreased by 49%, and cell permeability increased by 1.37-fold. At 20 μM Aβ42, viability

decreased by 63%, and dead cell count increased by 1.94-fold compared to control.

- Cholinergic RA/BDNF-differentiated cells were also sensitive to Aβ42 toxicity after 48 hours, although the effect was independent of concentration. At 10 μM Aβ42, viability decreased by 41%, and cell permeability increased by 1.84-fold. At 20 μM Aβ42, viability decreased by 23%, and dead cell count increased by 1.61-fold.
- Dopaminergic RA/TPA-differentiated cells were resistant to Aβ42 toxicity after 48 hours, with no statistically significant effect observed at either 10 μM or 20 μM concentrations.

- Although sensitivities of the differentiated cultures to Aβ42 varied, the peptide uniformly covered cell bodies and neurites in all cell cultures.

Publication III

- α-lipoic acid facilitated the distribution of copper from the extracellular to the intracellular space in SH-SY5Y cell cultures in a concentration-dependent manner.
- Two copper chelators used in the treatment of WD were also studied, but did not exhibit similar results with α-lipoic acid:
  - D-penicillamine did not affect intracellular copper concentration
  - Trientine decreased intracellular copper concentration
- The ability of α-lipoic acid to redistribute copper was more pronounced in RA/BDNF-differentiated, neuron-like SH-SY5Y cells compared to undifferentiated cells.
- α-lipoic acid and trientine did not exhibit toxicity alone or in the presence of copper ions as determined with PI staining. However, D-penicillamine was toxic in the presence of copper ions.
- Supplementation with α-lipoic acid protected a *Drosophila melanogaster* AD model expressing Aβ with the Iowa mutation from developing the AD phenotype, but it did not reverse an already established phenotype.
- α-lipoic acid slightly alleviated copper-induced smooth eye defects in *Drosophila melanogaster* AD model.

Discussion

In the current study, SH-SY5Y cells were used to explore various aspects related to AD at the cellular level. The cells were induced to become more neuron-like by treatment with agents that have previously been shown to promote different neuronal phenotypes.

SH-SY5Y cells are commonly used in neurodegenerative disease research, however, they are typically utilized in their undifferentiated state, which does not accurately replicate the characteristics of real neurons. First, in Publication I, the effects of Aβ40 and Aβ42 were studied in both undifferentiated and differentiated SH-SY5Y cells. Differentiation was achieved using RA and BDNF, which are known to induce a cholinergic neuronal phenotype (de Medeiros et al., 2019). The differentiation resulted in a neuron-like culture with long beta-III tubulin-positive neurites and inhibited proliferation. RA/BDNF-differentiated SH-SY5Y cells were more sensitive to Aβ toxicity compared to undifferentiated cells, as evidenced by cell viability measurement with WST-1 and PI staining. The fact that undifferentiated cells continue to proliferate may also affect viability measurements. Aβ42 was found to exhibit higher toxicity than Aβ40, inducing apoptotic cell death as measured by caspase 3/7 activity. Similar results have been observed in primary cultured mouse cerebral cortical neurons by Han et al. who demonstrated that Aβ42 induces apoptosis via the caspase pathway (Han et al., 2017). However, in primary cultured rat cortical neurons, Boland et al. showed that Aβ40 also induces apoptotic cell death, but through a Ca^{2+}-dependent protease, calpain, suggesting that Aβ40 may activate a different apoptotic pathway (Boland et al., 2003). Although both peptides, Aβ40 and Aβ42, induced neuronal abnormalities, such as beading and fragmentation, the effect was more pronounced with Aβ42.

The question of which form of Aβ is the most toxic has been the subject of long debate. In the 1990s, when the amyloid cascade hypothesis was proposed, neurotoxicity was primarily attributed to large, insoluble Aβ fibrils (Hardy & Higgins, 1992). Later, Aβ oligomers were thought to be the main culprits of neuronal damage (Lambert et al., 1998), and until now, toxicity is primarily attributed to Aβ oligomers and protofibrils. Some studies suggest even a protective role for amyloid accumulation and deposits (Rischel et al., 2023; Huang et al., 2021). In our experiments, mature fibrils did not show acute toxicity toward cultured cells, whereas toxicity emerged during fibril formation (Publication I). Nevertheless, these results do not exclude the possibility that mature fibrils could have other, potentially harmful effects *in vivo*.

Since Aβ peptides tend to self-assemble, the method used to prepare these peptides for toxicity experiments has a significant impact on the results. In Publication II, a slightly modified peptide preparation protocol was implemented, which resulted in increased toxicity. In contrast to the initial protocol used in Publication I, where HFIP-pretreated peptides were dissolved at neutral pH, the protocol in Publication II involved incubating the HFIP-treated aliquots in 10 mM NaOH on ice. Such alkali conditions were applied to reduce pre-aggregates in the solution and achieve a more uniform monomeric peptide composition. Previous studies have shown that NaOH pretreatment increases the proportion of low molecular weight Aβ species, such as monomers and dimers (Fezoui et al., 2000), which may explain the increased toxicity.

Considering the complexity of AD and knowing that various types of neurons are destroyed in the disease, in Publication II we aimed to model different neuronal phenotypes to investigate the effect of Aβ on these distinct cell types. Our experiments focused on Aβ42, as it exhibited higher toxicity in our initial cell culture

model used in Publication I. Several agents can be used to differentiate SH-SY5Y cells into more mature neuronal cells. In addition to the RA/BDNF differentiation method used in Publication I, we employed two additional differentiation protocols to study the effects of Aβ42. DbcAMP was used to induce a noradrenergic neuronal phenotype, while RA followed by TPA was used to induce a dopaminergic neuronal phenotype in SH-SY5Y cells. We found that cholinergic and noradrenergic cultures were more vulnerable to Aβ toxicity, which aligns with the observation that noradrenergic and cholinergic neurons are among the first to degenerate in AD (Mann et al., 1980; Zarow et al., 2003; Ferreira-Vieira et al., 2016). Interestingly, 10 μM Aβ42 exhibited greater toxicity than 20 μM Aβ42 in RA/BDNF-differentiated cholinergic cells, whereas in other differentiation routes, toxicity followed a concentration-dependent pattern. This effect may be attributed to differences in Aβ42 aggregation dynamics, cellular receptor expression, and membrane interactions specific to RA/BDNF differentiation. Differentiation agents may affect the cell's gene expression and function in distinct ways, which can influence their susceptibility to Aβ toxicity. DbcAMP has been shown to enhance Aβ-induced nitric oxide release, a known mediator of neurodegeneration (Pyo et al., 1999). In addition, it has been shown that dbcAMP increases the expression of APP, which could potentially lead to an increase in Aβ production (Sagy-Bross et al., 2015). However, other studies have suggested that dbcAMP can protect neurons from degeneration by promoting the production of BDNF (Abd-El-Basset & Rao, 2018). BDNF may enhance NMDA receptor function, and Aβ oligomers are known to interact with NMDA receptors, potentially mediating the toxic effects (Caldeira et al., 2007; Afonso et al., 2019; Ortiz-Sanz et al., 2022; Taniguchi et al., 2022). Looyenga et al. showed that RA/TPA differentiation of SH-SY5Y cells enhances resistance to the neurotoxic compound 6-OHDA toxicity compared to undifferentiated cells, which may help explain the results observed with Aβ (Looyenga et al., 2013). Some studies have shown that dopaminergic cells are generally more resistant to oxidative stress, which may account for their increased resistance to Aβ toxicity (Kweon et al., 2004; Nakamura et al., 2001). Better tolerance to oxidative stressors may mean that a longer incubation time is required to achieve a toxic effect with Aβ. A deeper understanding of how Aβ affects different neuronal cell types may provide insights into the mechanisms of neurodegeneration and lead to improved treatments for AD. Since different patients experience different symptoms based on the specific neurotransmitter system that is most affected, a one-size-fits-all approach may not be effective, and a more personalized or multi-targeted treatment strategy could improve the treatment outcomes.

Aβ has been the primary target for recent drug developments. One of the anti-amyloid drugs, lecanemab, targets preferentially soluble Aβ protofibrils. Other anti-amyloid drugs have been developed to target mainly amyloid plaques. However, the effectiveness of anti-amyloid therapies remains uncertain, as the link between amyloid removal and cognitive improvement has not been fully established. These therapies also have side effects, such as brain swelling and microhemorrhages, and their long-term efficacy is still under investigation (Salloway et al., 2022; Honig et al., 2024). Therefore, it is crucial to identify and study the upstream events preceding amyloid accumulation.

Given the increasing evidence implicating the role of copper in the pathogenesis of AD, we next investigated the effect of copper in cell culture. Our group has previously shown that a natural compound, reduced dihydro-lipoic acid, has a substantial Cu(I)-

binding affinity (Smirnova et al., 2018). Since copper is misdistributed in AD, with deficiencies in the brain tissue and excess in plasma and serum (Squitti et al., 2021), we aimed to explore whether α-LA could restore the normal copper balance and facilitate the transport of copper from the extracellular to the intracellular environment (Publication III). For that purpose, we used undifferentiated and cholinergic RA/BDNF-differentiated SH-SY5Y cell cultures. First, to exclude a potential toxicity of α-LA in the presence of copper ions, PI-staining was performed on differentiated cells. The study also included two copper chelators, DPA and TETA, commonly used in the treatment of WD. Individually, all tested agents and copper ions were non-toxic. Furthermore, α-LA and TETA remained non-toxic in the presence of copper ions. However, DPA exhibited toxicity at higher concentrations in the presence of copper. Interestingly, DPA treatment has been associated with neurological worsening in WD patients (Brewer et al., 1987; Kumar et al., 2022; Antos et al., 2023). Next, we studied the effect of α-LA and other chelators on the distribution of copper ions in cell culture and found that α-LA promoted the relocation of copper from the extracellular to the intracellular space in a concentration-dependent manner. The effect was more pronounced in neuron-like RA/BDNF-differentiated cells compared to undifferentiated cells. Unlike α-LA, other chelators did not exhibit similar effect. DPA had no impact on intracellular copper content, while TETA reduced intracellular copper levels.

Although technological advancements are progressing rapidly, the demand for accurate cell culture models remains high. Differentiated SH-SY5Y cells have become an essential model for studying AD due to their ability to mimic various neuronal phenotypes, including cholinergic, noradrenergic, and dopaminergic cells. Such models allow to explore the disease's complex molecular and cellular mechanisms and continue to play an important role in basic mechanistic research and early-stage drug screening, complementing more advanced models.

We also aimed to investigate the effect of α-LA *in vivo* using a *Drosophila* AD model. We conducted negative geotaxis locomotor activity experiments and found that α-LA supplementation helped to rescue *Drosophila melanogaster* AD models with the overexpression of human Aβ carrying the Iowa mutation from developing AD-like phenotype. However, α-LA had no effect on the phenotype once it had already developed, indicating only a preventive effect. Additionally, α-LA slightly alleviated the copper-induced smooth eye phenotype in a *Drosophila* AD model where APP and BACE1 were overexpressed in the eye photoreceptor cells. It is known that high doses of Cu negatively impact *Drosophila* development, viability, and locomotor behavior (Ding & Wang, 2006). Furthermore, Cu exposure has been shown to reduce memory retention in *Drosophila* (Zamberlan et al., 2020). Previous studies with α-LA in *Drosophila* have demonstrated its positive effects, including improved lifespan and resistance to oxidative stress in wild-type *Drosophila* (Chattopadhyay et al., 2024). Moreover, α-LA has been shown to improve motor activity and lifespan in a mutant hSOD1 *Drosophila* model of amyotrophic lateral sclerosis (Wang et al., 2018).

It is crucial to identify easily measurable biomarkers that can help detect AD in its early stages, as once the brain has already undergone significant degeneration, it becomes irreversible. Genetic testing and personalized medicine may also enable easier identification of individuals at risk. Studies have shown that a subset of AD patients with elevated copper levels carry a specific ATP7B haplotype, which may predispose them to copper imbalance (Squitti et al., 2021). Recently, the FDA approved a blood test for AD that offers a less invasive and more accessible diagnostic option. By measuring the key

biomarkers, phosphorylated tau (pTau217) and Aβ42, the test enables accurate detection of AD pathology in individuals aged 55 and older with cognitive symptoms, supporting earlier diagnosis and timely intervention.

As the Danish philosopher Desiderius Erasmus wisely stated in the 1500s, “Prevention is better than cure” (Erasmus, circa 1500). This timeless insight emphasizes the importance of proactive measures in alleviating the onset and progression of AD. Considering the growing prevalence of AD and its multifactorial aetiology, it is crucial to prioritize strategies aimed at preventing or delaying its onset. A comprehensive approach to prevention, encompassing not only healthy lifestyle modifications but also appropriate nutritional and supplement interventions, plays a pivotal role in alleviating both the economic and societal burdens associated with this devastating disease. Given that α-LA is safe and already used as a dietary supplement, it could be suitable for AD prevention and could potentially benefit individuals with a genetic predisposition to copper imbalance or those in the early stages of AD. α-LA is a well-researched compound with a favorable safety profile and low toxicity, even at relatively high doses (Fogacci et al., 2020; Derosa et al., 2020). It has been widely used in the treatment of various conditions, such as diabetic peripheral neuropathy, where its antioxidant and neuroprotective properties are well documented (Han et al., 2012; Pingali et al., 2024). Given its established clinical use, α-LA is a promising repurposing drug candidate for the treatment of AD. Although several clinical studies have investigated the effect of α-LA on AD, the current evidence remains limited and inconclusive (Hager et al., 2007; Shinto et al., 2014; Galasko et al., 2012). Most trials have been small-scale, open-label or lacked accurate controls, making it difficult to make clearly defined conclusions about the efficacy of α-LA. To better evaluate the therapeutic effect of α-LA in AD, larger, well-designed, placebo-controlled clinical trials are needed.

Conclusions

- Human neuroblastoma SH-SY5Y cells can be differentiated into various types of neuron-like cells that resemble mature neurons both in appearance and chemical properties.
- Differentiation route of SH-SY5Y cells affects susceptibility to Aβ42 toxicity: RA/BDNF-differentiated cholinergic cells and dbcAMP-differentiated noradrenergic cells are highly susceptible to Aβ toxicity, whereas RA/TPA-differentiated dopaminergic cells are resistant to Aβ.
- Experiments in cell culture and *Drosophila melanogaster* revealed that a natural compound α-lipoic acid has the potential to normalize dysregulated copper metabolism characteristic of AD:
  1) α-lipoic acid can translocate copper from extracellular environment to the cells in a concentration-dependent manner both in undifferentiated and RA/BDNF-differentiated cholinergic SH-SY5Y cells, however, the effect was greater in differentiated cells.
  2) α-lipoic acid protected AD model flies from developing AD-like phenotype.
  3) α-lipoic acid slightly weakened copper-induced eye defects in AD model flies.
- Differentiated SH-SY5Y cells and *Drosophila melanogaster* provide good models to study different aspects of AD and to screen new potential therapeutic agents at the cellular and organismal level.

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Abstract

Toxicity of amyloid beta peptides and the effect of α-lipoic acid in Cellular and Fruit Fly Models of Alzheimer's Disease

Alzheimer's disease is the most common form of dementia, with devastating economic and social impacts. The neuropathological features of the disease – amyloid plaques and neurofibrillary tangles – have long been considered central to its aetiology. Although amyloid plaques, composed of amyloid beta peptides and neurofibrillary tangles, formed by hyperphosphorylated tau protein, are frequently observed in the brains of AD patients, the precise role these features play in the initiation and progression of the disease remains unclear. Recently, there has been an increased focus on the processes preceding amyloid accumulation, and it has been found that dysregulation of metal ions plays a role in the development of the disease. The association of metal ions with AD has also led to the investigation of various metal-binding compounds as potential therapeutic agents.

Cellular models, including immortalized cell lines, have become essential tools for investigating the molecular and cellular mechanisms underlying AD in a cost-effective and reproducible manner. One of the most widely used immortalized cell lines in AD research is the human neuroblastoma SH-SY5Y cell line. Most research has used these cells in their undifferentiated form, but they have the ability to differentiate into multiple neuronal phenotypes, including cholinergic, dopaminergic, and noradrenergic cells, which resemble neurons found in the human brain. It is known that various neurotransmitter systems are implicated in AD and a better understanding of these systems helps personalize treatment approaches, which is crucial for managing the complex nature of AD. In this thesis, human neuroblastoma SH-SY5Y cells were differentiated into cholinergic, noradrenergic, and dopaminergic phenotypes, and their sensitivities to Aβ42 were compared. Cell viability measurements and cell membrane permeability assays showed that noradrenergic and cholinergic cells were susceptible to Aβ42 toxicity, whereas dopaminergic cells were resistant. Using cholinergic SH-SY5Y cells, Aβ42 was shown to induce apoptotic cell death. Aβ can exist in multiple assembly states, and the most toxic form has long been debated. Our results showed that cellular toxicity was raised during the fibrillization process, as mature fibrils were not toxic to the cells.

Since it is known that copper is misdistributed in the brains of AD patients, and that the natural compound α-lipoic acid (α-LA) has previously been found to have copper-binding properties, it was investigated whether α-LA could help to restore copper homeostasis. Using undifferentiated and cholinergic SH-SY5Y cells, ICP-MS measurements showed that α-LA was able to promote translocation of copper from the extracellular space into the cells. The effect of α-LA was also studied *in vivo*, and negative geotaxis locomotor experiments with *Drosophila melanogaster* demonstrated that it protected AD model flies from developing AD-like phenotype. SEM experiments further showed that α-LA alleviated copper-induced eye defects in AD model flies.

In conclusion, differentiated SH-SY5Y cells represent a valuable model for investigating various aspects of AD and for supporting the development of novel therapeutic strategies. Furthermore, the natural compound α-LA shows promise in modulating copper metabolism and may be beneficial in the prevention or early stages of AD.

Lühikokkuvõte

Amüloid beeta peptiidide toksilisus ning α-lipoehappe mõju Alzheimeri tõve rakulistes ja äädikakärbse mudelites

Alzheimeri tõbi (AT) on neurodegeneratiivne haigus, mida iseloomustavad ajurakkude degeneratsioon ning kognitiivsete funktsioonide järk-järguline halvenemine. Haiguse täpseid molekulaarseid mehhanisme ei ole siiani täielikult mõistetud. AT neuropatoloogilised tunnused – amüloidsed naastud ja neurofibrillaarsed kämbud – on pikka aega olnud haiguse etioloogia keskseks osaks. Kuigi amüloidsed naastud, mis koosnevad amüloid-beeta (Aβ) peptiididest ja neurofibrillaarsed kämbud, mis moodustuvad hüperfosforüleeritud tau valgust, esinevad sageli AT patsientide ajus, on nende täpne roll haiguse tekkes ja arengus endiselt ebaselge. Seetõttu on hakatud viimastel aastatel rohkem tähelepanu pöörama amüloidide kogunemisele eelnevatele protsessidele ning on leitud, et metalliioonide regulatsiooni häired mängivad haiguse arengus olulist rolli. Metalliioonide seos AT-ga on viinud ka erinevate metalle-siduvate ühendite uurimiseni, mida oleks võimalik kasutada potentsiaalsete terapeutiliste agentidena.

AT molekulaarsete ja rakuliste mehhanismide uurimisel on erinevad rakumudelid muutunud olulisteks tööriistadeks. Üks enim kasutatavaid rakuliine AT uurimisel on inimese neuroblastoomi SH-SY5Y rakuliin. Enamik teadusuuringutest on kasutanud neid rakke nende diferentseerimata vormis, kuid neil on ka võime diferentseeruda erinevateks neuronisarnasteks fenotüüpideks, sealhulgas koliinergilisteks, dopamiinergilisteks ja noradrenergilisteks, mis meenutavad neuroneid. Käesolevas doktoritöös diferentseeriti inimese neuroblastoomi SH-SY5Y rakud koliinergilisteks, noradrenergilisteks ja dopamiinergilisteks fenotüüpideks ning võrreldi nende tundlikkust Aβ42 suhtes. Rakkude elulemuse mõõtmised WST-1 testiga ning rakumembraani läbilaskvuse katsed propiidiumiodiidiga näitasid, et noradrenergilised ja koliinergilised rakud olid Aβ42 toksilisuse suhtes tundlikud, samas kui dopamiinergilised rakud olid resistentsed. See on kooskõlas varasema leiuga, et AT ajus on just koliinergilised ning noradrenergilised neuronid need, mis esimestena hukuvad. Koliinergiliste SH-SY5Y rakkudega näidati, et Aβ42 põhjustas apoptootilist rakusurma, Aβ40 aga rakkudele toksiline ei olnud. Aβ võib esineda erinevates molekulaarsetes vormides ning kõige toksilisema vormi üle on pikka aega arutletud. Antud töö tulemused näitasid, et toksilisus tekkis Aβ fibrillisatsiooni protsessi käigus ning küpsed Aβ fibrillid rakkudele toksilised ei olnud.

On leitud, et vask on AT-ga patsientide ajus valesti jaotunud – rakust väljaspool on seda liiga palju ning raku sees liiga vähe, jättes olulised vaskensüümid vase puudusesse. Kuna varasematest uuringutest on teada, et looduslik ühend α-lipoehape (α-LH) on vaske-siduvate omadustega, uuriti, kas α-LH võiks aidata normaliseerida vase homöostaasi. Nii diferentseerimata kui ka koliinergiliste SH-SY5Y rakkudega tehtud ICP-MS mõõtmised näitasid, et α-LH suudab viia vase ekstratsellulaarsest ruumist rakkudesse kontsentratsioonist-sõltuval viisil, samas kui kaks Wilsoni tõves kasutatavat ravimit trientiin ja D-penitsillamiin sellist efekti ei omanud. Kõrgematel kontsentratsioonidel muutus D-penitsillamiin vase juuresolekul hoopis toksiliseks. α-LH mõju uuriti edasi ka *in vivo* tingimustes kasutades selleks äädikakärbseid. Äädikakärbestega tehtud negatiivse geotaksise katsetes näidati, et α-LH manustamine omas ennetavat mõju, kaitses AT

mudelkärbseid AT-sarnase fenotüübi tekkimise eest. SEM-katsed näitasid, et α-LH leevendas ka vase põhjustatud silmakahjustusi AT mudelkärbestel.

Kokkuvõttes võib öelda, et diferentseeritud kujul pakuvad SH-SY5Y rakud sobilikku mudelit erinevate AT-ga seotud aspektide uurimiseks ning on abiks uute ravimeetodite väljatöötamiseks. Lisaks omab looduslik ühend α-LH potentsiaali vase metabolismi reguleerimiseks ning sellest võiks eelkõige kasu olla AT ennetusel või varajases staadiumis.

Appendix 1

Publication I

Krishtal, J., Bragina, O., Metsla, K., Palumaa, P., & Tõugu, V. (2017). In situ fibrillizing amyloid-beta 1-42 induces neurite degeneration and apoptosis of differentiated SH-SY5Y cells. *PloS one*, *12*(10), e0186636. https://doi.org/10.1371/journal.pone.0186636

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RESEARCH ARTICLE

In situ fibrillizing amyloid-beta 1-42 induces neurite degeneration and apoptosis of differentiated SH-SY5Y cells

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Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; ATCC, American Type

Abstract

The progression of Alzheimer's disease is causatively linked to the accumulation of amyloid-β aggregates in the brain, however, it is not clear how the amyloid aggregates initiate the death of neuronal cells. The *in vitro* toxic effects of amyloid peptides are most commonly examined using the human neuroblastoma derived SH-SY5Y cell line and here we show that differentiated neuron-like SH-SY5Y cells are more sensitive to amyloid peptides than non-differentiated cells, because the latter lack long neurites. Exogenous soluble amyloid-β 1–42 covered cell bodies and whole neurites in differentiated cells with dense fibrils, causing neurite beading and fragmentation, whereas preformed amyloid-β 1–42 fibrils had no toxic effects. Importantly, spontaneously fibrillizing amyloid-β 1–42 peptide exhibited substantially higher cellular toxicity than amyloid-β 1–40, which did not form fibrils under the experimental conditions. These results support the hypothesis that peptide toxicity is related to the active fibrillization process in the incubation mixture.

Introduction

Alzheimer's disease (AD), a complex neurodegenerative disorder, is the most prevalent cause of dementia worldwide. Although the disease was first described more than 100 years ago, the etiology of AD is still elusive. Amyloid plaques in the patient's brain are the primary hallmark of AD and the evidence for the central role of amyloid beta (Aβ) peptides–the main component of amyloid plaques- in the pathogenesis of AD is very strong [1, 2]. For more than twenty years, the amyloid cascade hypothesis has served as the dominant framework for AD studies, however, a clear understanding and description of the molecular events leading to neurodegeneration is still missing and several alternative explanations for disease progression are under discussion [3–6]. It has been shown that various aggregated forms of Aβ peptides are neurotoxic in animal models, primary neuronal cultures and immortalized cell lines [7–9]. However, the results of Aβ toxicity studies are often controversial and have not yet provided a clear understanding of the disease mechanism or the molecular events underlying Aβ toxicity. Since mainly neuronal cells die during neurodegeneration, it is likely that Aβ acts via a specific mechanism to induce neuronal cell death. Previous studies on primary neurons have shown

Culture Collection; Aβ, amyloid beta; BDNF, brain derived neurotrophic factor; CalceinAM, calcein-acetoxymethylester; DAPI, 4',6-diamidino-2-phenylindole; DIC, differential interference contrast; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EDTA, Ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; LSM, laser scanning microscope; MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RA, retinoic acid; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Sy, Synaptophysin1; TUJ-1, beta III tubulin; WST, water soluble tetrazolium.

that Aβ causes neuritic abnormalities in neuronal cultures [10, 11], which are also initial signs of dying neurons in AD. Therefore, it is important to use relevant cellular models for the study of the neuron-specific effects of Aβ peptides. The human SH-SY5Y cell line is widely used as a model for different neurodegenerative diseases including AD [12]. The phenotype of SH-SY5Y cells can be manipulated by inducing different programs of neural differentiation, however, in most (81.5%) publications non-differentiated cells are used [12]. Due to their dopaminergic character, SH-SY5Y cells are generally considered as a model for Parkinson's disease, however, they can be differentiated to dominantly cholinergic phenotype suitable for AD studies by treatment with retinoic acid (RA) and brain-derived neurotrophic factor (BDNF) [13]. Aβ toxicity on SH-SY5Y cells has been determined in a large number of studies, however, there are only a few examples examining Aβ-induced toxicity in SH-SY5Y cells where cell proliferation has been suppressed and preliminary differentiation initiated by RA [14–16]. Additionally to the best of our knowledge, there are currently no available data investigating whether Aβ is toxic for RA/BDNF differentiated SH-SY5Y cells.

Another important yet understudied area within the framework of the amyloid hypothesis concerns the exact nature of the toxic form(s) of Aβ. In the AD brain, the "extra" amyloid in developing plaques is in the form of amyloid fibrils. The fibrillation is an autocatalytic process —once the fibrils are formed they start to grow by trapping monomers. Due to the relatively low toxicity of Aβ monomers and preformed Aβ fibrils for cell cultures, the pathogenic entities of the peptide are intensively searched for and the toxic effects have been attributed to a wide variety of species, including oligomers, intermediate aggregates and peptide-copper complexes [17–20]. In many cases the peptide formulations have been pretreated in conditions entirely different from those that can occur in living organisms. For instance, a popular oligomerization procedure involves fast dilution of concentrated peptide solutions in an organic solvent to form a supersaturated solution [21, 22]. In 1994 Lambert and colleagues demonstrated the toxic effect of Aβ42 on RA pretreated SH-SY5Y cells and attributed this effect to the peptide oligomers (DMSO-induced) [23]. Recent studies have demonstrated that the toxic entities of the peptide can be the metastable particles that form during the natural fibrillization of Aβ [20, 24], and serve to highlight that these more natural fibrils should be preferred over artificially generated oligomers.

Here we used RA and BDNF differentiated human neuroblastoma SH-SY5Y cells, a simple model suggested for neuronal screening [25–27], to study the effects of Aβ-peptides. The differentiation of SH-SY5Y cells increased their susceptibility to Aβ and allowed the description and quantification of pathological changes associated with primary neuronal cultures and patients with AD [28]. The obtained results support the hypothesis that neuron-specific Aβ toxicity may be caused by the intermediate amyloid aggregates that form during the fibrillization of Aβ-peptides [29]. In our opinion, further study of the differentiated SH-SY5Y cells will aid our understanding of the molecular mechanisms responsible for the pathological processes induced by amyloid peptides in cells of human origin.

Materials and methods

Chemicals and reagents

Cell culture associated reagents were purchased from Gibco, Thermo Fisher: Dulbecco's Modified Eagle's Medium (DMEM), 0.25% Trypsin-EDTA solution. Penicillin/Streptomycin solution was from PAA, Cambridge, UK. Brain derived neurotrophic factor was obtained from Alomone Labs, Jerusalem, Israel. Recombinant human Aβ40 and Aβ42 peptides (TFA salts, purity >97%) were from rPeptide, Bogart, GA 30622, USA. Tween-20 (Ferak, Berlin, Germany) WST-1 cell viability assay was purchased from Roche, Switzerland, and the Caspase-

Glo assay kit from Promega Co, Madison, WI, USA. 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), poly-L-lysine, retinoic acid, CalceinAM (calcein-acetoxymethyl ester), sodium chloride; goat serum; staurosporine, 4',6-diamidino-2-phenylindole (DAPI), PBS were obtained from Sigma Aldrich.

Antibodies against a microtubule component βIII-tubulin TUJ-1 were obtained from Abcam, Cambridge, UK; anti-APP/Aβ antibody—from Millipore, Darmstadt, Germany. Guinea pig anti-Synaptophysin1 was from Synaptic Systems, Goettingen, Germany. Secondary antibodies were Alexa-488 and Alexa-568 from Invitrogen.

Cell culture

SH-SY5Y cells (ATCC, VA 20110, USA) were cultured in DMEM without Phenol Red and supplemented with 10% FBS and 1X Penicillin/Streptomycin solution at 37˚C and 5% CO_2, to allow fluorescence measurements (Gibco, Thermo Fisher). The medium was changed every 2–3 days and cells were split using 0.25% Trypsin-EDTA solution.

SH-SY5Y differentiation

A differentiation protocol from Ref. [25, 26] with several changes was applied. The cells were seeded onto microtiter plates (Greiner Bio-one) coated with poly-L-lysine to allow neurite outgrowth and differentiation. Cells were grown for 1 day prior to differentiation. The next day, 10 μM RA in DMEM with 10% FBS was applied; the medium with RA was changed every day. After a 4-day incubation with RA, BDNF at the final concentration of 50 ng/ml was applied in DMEM without serum for 2 days. After 6 days of differentiation the cells were used for the experiments. (S1 Fig).

Preparation of amyloid-β peptide solutions

Lyophilized Aβ40 and Aβ42 peptides were dissolved in HFIP to get a homogeneously monomeric preparation, vortexed briefly and incubated for 1 hour at room temperature. Next, defibrillized peptide solutions were aliquoted and dried in a vacuum desiccator overnight. Peptide aliquots were stored at -80˚C until usage. Peptide quality was assessed by ^{1}H NMR, MALDI-MS and SDS–PAGE (S2 Fig). For experiments, Aβ40 and Aβ42 aliquots were dissolved in 20 mM HEPES buffer containing 100 mM NaCl at pH 7.3 to the final concentration of 160 μM and vortexed for 10 sec. The prepared peptide solution was immediately applied to the serum free cell culture. Preformed fibrils were prepared as described in Ref. [30].

Evaluation of neurite degeneration

Cells were grown in 6 cm cell culture plates on 47 mm glass coverslips coated with poly-L-lysine and differentiated as described above. Photomicrographs of at least 12 random areas of neurites were taken using a Zeiss Duo 510 META microscope with a 20X objective or a 63X objective with oil immersion. Neurites were stained with CalceinAM for the analysis. General morphology of neurites was obtained with the differential interference contrast (aperture DIC2) technique. All microscopy experiments were performed in an incubation chamber at 37˚C in the presence of 5% CO_2.

Photomicrographs of neurites in randomly selected areas were stored and processed using LSM Image Browser software. The method to evaluate neurite degeneration was adopted from Ref. [31] with small modifications. The number of beads per total length of measured neurites was counted. Medium or thin neurites in captured regions were chosen for this purpose. The number of beadings/50 μm length was counted and averaged over at least 8 neurites for each

area. The proportion of fragmented neurites was counted for each area and expressed as a percentage of the total amount of counted neurites longer than 100 μm (S3 Fig). The procedures were repeated on three different samples (vehicle, Aβ40 and Aβ42). The results of three independent experiments were averaged and presented with SEM without normalization. The photomicrographs were coded for the evaluator by random codes.

Immunofluorescence

The neuronal phenotype of differentiated SH-SY5Y was established by immunocytochemical staining with antibodies against a microtubule component βIII-tubulin TUJ-1 (1:2000). For the determination of Aβ location in the cell culture, the cells were stained with anti-APP/Aβ antibody (1:2000) and guinea pig anti-Synaptophysin1 (1:2000). Samples were fixed for 15 min at 4˚C in methanol (for microtubule visualization) or 4% paraformaldehyde (anti-APP/Aβ staining). Blocking was performed with 3% goat serum. The samples were washed with PBS to remove excess protein before incubation with primary monoclonal antibodies in 0.25% Tween-20 solution 1:2000 at 4˚C overnight. Samples were washed with PBS for 5 min before incubation with secondary Alexa-488 or Alexa-568 conjugated goat anti-mouse antibody 1:2000 or goat anti-guinea pig antibody 1:2000 in PBS for 1 hour at room temperature. Nuclei were stained with DAPI for 5 min at room temperature. Cells were investigated using a confocal Zeiss Duo 510 META microscope with a 20X objective or a 63X objective with Zeiss oil immersion.

Cell cytotoxicity assays

The effects of peptides on the cells were determined using the WST-1 cell viability assay and the membrane permeability assay with propidium iodide (PI). 20 mM HEPES buffer containing 100 mM NaCl at pH 7.3 was used as a negative control (hereafter Vehicle) added to serum-free medium in equal amounts with the peptide sample. 5 μl/well of WST-1 reagent was added to 100 μl cell culture medium, incubated at 37˚C for 2 h and the absorbance measured at 450 nm. PI in PBS (0.5 mM) was added to 100 μl cell culture 1.5 μl/well and incubated for 2 h at 37˚C. Fluorescence was measured using a TECAN Genios Pro microplate reader (Tecan, Switzerland) (excitation 540 nm, emission 612 nm). Data from at least three independent experiments, all experimental points in triplicates, were normalized taking Vehicle as 100%.

Measurement of caspase activity

The differentiated SH-SY5Y cells were plated in 96-well plates. The activity of caspase-3/7 was measured using a Caspase-Glo assay kit. In this kit a substrate for luciferase is released when the colorimetric substrate, containing the tetrapeptide sequence DEVD, is cleaved by caspase-3/7. After cell treatment with the buffer (the vehicle), staurosporine or Aβ (20 μM), Caspase Glo-3/7 reagent was added to the culture medium and incubated at room temperature for 1 h. The intensity of the chemiluminescence was measured using a TECAN Genios Pro microplate reader.

Statistical analysis

The differential significance of the results obtained was determined by one-way ANOVA followed by a Bonferroni's multiple comparisons test at the 0.05 level. All values are presented with means ± SEM, except where otherwise indicated. Raw data values can be seen in S1–S7 Tables. The number of experiments is represented by *n*. p-values of post-hoc test are

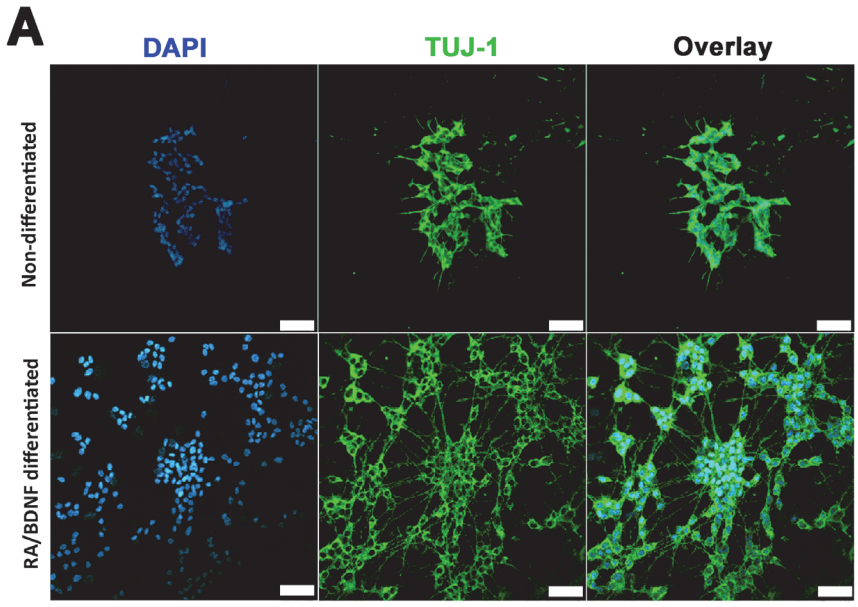

Fig 1. RA/BDNF differentiated cells establish a neuron-like phenotype with long neurites. (A) Immunocytochemistry of non-differentiated and RA/BDNF differentiated SH-SY5Y cells for DAPI (blue; left), anti-TUJ-1 (green; middle). Scale bar 50 μm.

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denoted by asterisks (* $p \leq 0.01$;** $p \leq 0.005$). Statistical analysis was carried out using GraphPad Prism 6.

Results

RA/BDNF differentiation increases the susceptibility of SH-SY5Y cells towards Aβ toxicity

Neuron-like SH-SY5Y human neuroblastoma cells were generated using a differentiation procedure modified from previously descried protocols [25–27]. The cells were pre-treated for 4 days with 10 μM *all-trans* RA to induce the expression of TrkB receptors and increase their biological responsiveness to neurotrophic factor treatment [32]. After the sequential treatment with RA and BDNF, the cells developed long beta III tubulin (TUJ-1)-positive neurites that formed networks characteristic to neurons (Fig 1A).

Aβ-peptides had a small effect on the viability of undifferentiated SH-SY5Y cells in serum-free DMEM. The influence of serum withdrawal on cell viability during 3 days was not significant (S4 Fig). In the presence of Aβ42 the cell viability decreased to 84 ± 5%, after a 48-hour incubation, whereas Aβ40 tended to increase the viability (111 ± 2%) (Fig 2A, left panels; S1 and S2 Tables). No changes in cell viability according to the WST-1 test were detected after a 72 h incubation with either peptide at a concentration of 20 μM. At the same time, the membrane permeability test with PI showed a statistically significant increase in cell death after a 72 h incubation with Aβ42 (130 ± 6%). Aβ40 also increased cell death, however, the results varied remarkably between independent repeats (124 ± 16%) and the effect was statistically insignificant. Thus, both the cell viability and the membrane permeability tests showed that the toxic

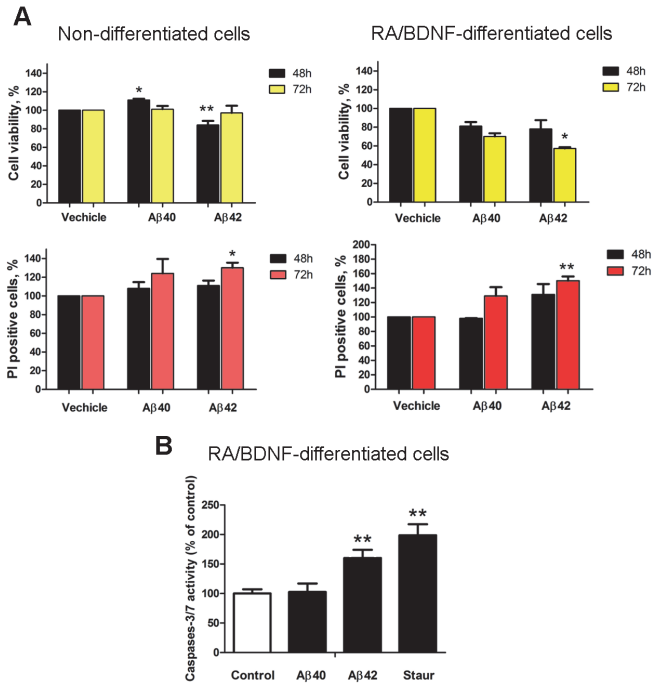

Fig 2. Aβ reduces viability and activates caspases-3/7 in differentiated SH-SY5Y cells. (A) Cell viability was measured with the WST-1 test and membrane integrity was measured using propidium iodide 48h and 72h after incubation with 20μM peptides. The figure displays the mean± SEM; at least *n = 3* independent experiments in case of Aβ42 and *n = 5* experiments in case of Aβ40; **p≤0.005; *p≤0.01; (B) Effect of Aβ42 on the activity of caspase-3 and/or 7. Caspase activity was determined by measuring DEVD-AFC hydrolysis in lysates from SH-SY5Y cells treated with 20μM Aβ42 for 48h. The figure displays the mean ± SD; *n = 5*; **p≤0.005. One-way ANOVA followed by a Bonferroni's multiple comparisons test at the 0.05 level was used to determine the difference between the conditions.

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influence of Aβ-peptides on non-differentiated SH-SY5Y cells is relatively small. Aβ-peptides could eventually affect a small subpopulation of non-differentiated SH-SY5Y cells (S5 Fig), however the majority of the cells continued to proliferate, which complicates the interpretation of viability measurements. Nevertheless, the non-differentiated SH-SY5Y cells with a low sensitivity to Aβ and heterogeneous RA-differentiated cells cannot be used as a reliable model for the study of the toxic effects of native Aβ.

Aβ-peptides were more toxic to the RA/BDNF neuronally differentiated SH-SY5Y cells according to both the WST-1 and the PI tests (Fig 2A, right panels; S3 and S4 Tables). In the WST-1 test, cell viability started to decline after a 48-hour incubation (81 ± 5%for Aβ40 and 78 ± 9% for Aβ42), however, statistically significant effects (70 ± 3% and to 57.3 ± 1.3%) in the presence of Aβ40 and Aβ42, respectively, were observed after 72 h. A statistically significant increase in the membrane permeability was observed after 72 h only in the case of Aβ42 (150 ± 6%). The PI fluorescence also slightly increased after a 72 h incubation with Aβ40 (129 ± 12%) and after 48 h with Aβ42 (131 ± 15%). The neuron-like RA/BDNF differentiated SH-SY5Y cells appeared to be more susceptible to Aβ-peptides than the non-differentiated SH-SY5Y cells.

Aβ42, but not Aβ40, induces apoptosis of differentiated SH-SY5Y

To prove the apoptotic nature of Aβ-induced cell death, we examined the activation of caspase-3/7. After a 48 h incubation, Aβ42 significantly increased caspase-3/7 activity (160±14%) compared to that of the vehicle (Fig 2B; S5 Table). Staurosporine (a positive control for the induction of apoptosis) increased the activation of caspases-3/7 by 199±19%. Incubation with Aβ40, which only slightly increased the number of PI-permeable cells, had no effect on caspase activity (103±14%). We can conclude that Aβ42, but not Aβ40, induced apoptotic cell death.

Aβ peptides induce pathological changes in neurite morphology

For the detection of Aβ-induced abnormalities in differentiated SH-SY5Y cells, the culture was stained with a CalceinAM dye that reveals viable cells and their extensions [33]. The morphological changes in neurites after incubation with Aβ peptides (Fig 3A) appeared considerably earlier than the Aβ mediated decrease in cell viability (Fig 2A). Aβ peptides induced beading and the subsequent fragmentation of neurites (Fig 3A, red arrows), whereas no CalceinAM signal was detected in fragmented neurites (Fig 3B). Quantitative analysis of the morphological changes in neurites showed that both peptides induced beading and fragmentation, but the influence of Aβ42 were considerably stronger than that of Aβ40 (Fig 3C; S6 and S7 Tables). The analysis of the early signs of microtubule disruption indicated that Aβ42 significantly induced bead formation. The amount of beads in the presence of Aβ42 per 50μm of neurite length increased to 1.12±0.08 compared to the vehicle 0.40 ± 0.03. Aβ40 did not induce statistically significant beading: 0.78 ± 0.11 beads per 50μm neurite length. Both peptides caused a substantial increase in the neurite fragmentation: 22.4±1.0% and 37.6±0.4% of neurites were fragmented in the presence of Aβ40 and Aβ42 respectively, whereas the fragmentation was not significant (0.57±0.57%) in the absence of the peptides.

Aβ42 aggregates cover cell bodies and neurites

Since highly amyloidogenic Aβ-peptides can form fibrils on the cell surface in cell cultures [34–36], we studied the distribution of Aβ40 and Aβ42 using an anti-APP/Aβ antibody (Fig 4A). Panels presenting "Vehicle" demonstrate the endogenous APP/Aβ pattern in RA/BDNF differentiated cells. When cells were incubated with 20 μM peptide (lower panels) the presence of large extracellular Aβ aggregates appeared, which almost overshadowed the endogenous signals in the case of Aβ42. Three-dimensional reconstruction (Fig 4B) demonstrated that the cell bodies and neurites were fully covered with Aβ42 aggregates after 24 hours of incubation (Fig 4B, medium panels), whereas the less amyloidogenic Aβ40 did not cover the neurites and only random aggregates were present near and on the cell bodies after 48h (Fig 4B, right panels). Furthermore, this is in accordance with the fact that only Aβ42 induces caspase3/7 activation (Fig 2C). It could also be concluded that covering of neurites does not cause rapid cell death since a significant decrease in viability was observed only after incubation for 72 hours (Fig 2A). The non-differentiated cells were only partially covered with Aβ42 after 24h (Fig 4B, left panels). These results indicate that the Aβ42-induced toxic effects are related to the "Aβ cover" of neurites, because the non-differentiated cells lack long neuron-like extensions.

The presence of fibrillar aggregates in the cell medium containing Aβ42 after 48 hours of incubation was confirmed by TEM (S6 Fig), whereas no fibrils were detected in the medium containing Aβ40 under similar conditions. The fibrils formed in the cell culture were comparable to those previously generated in a test tube using peptides obtained from the same source [37]. Thioflavin T test showed that the aggregation of Aβ42 in serum free cell medium was completed within 24h (S7 Fig). In contrast to the significant toxic effect of Aβ42 fibrillizing in the solution surrounding the cells, the matured Aβ42 fibrils (preformed according to the

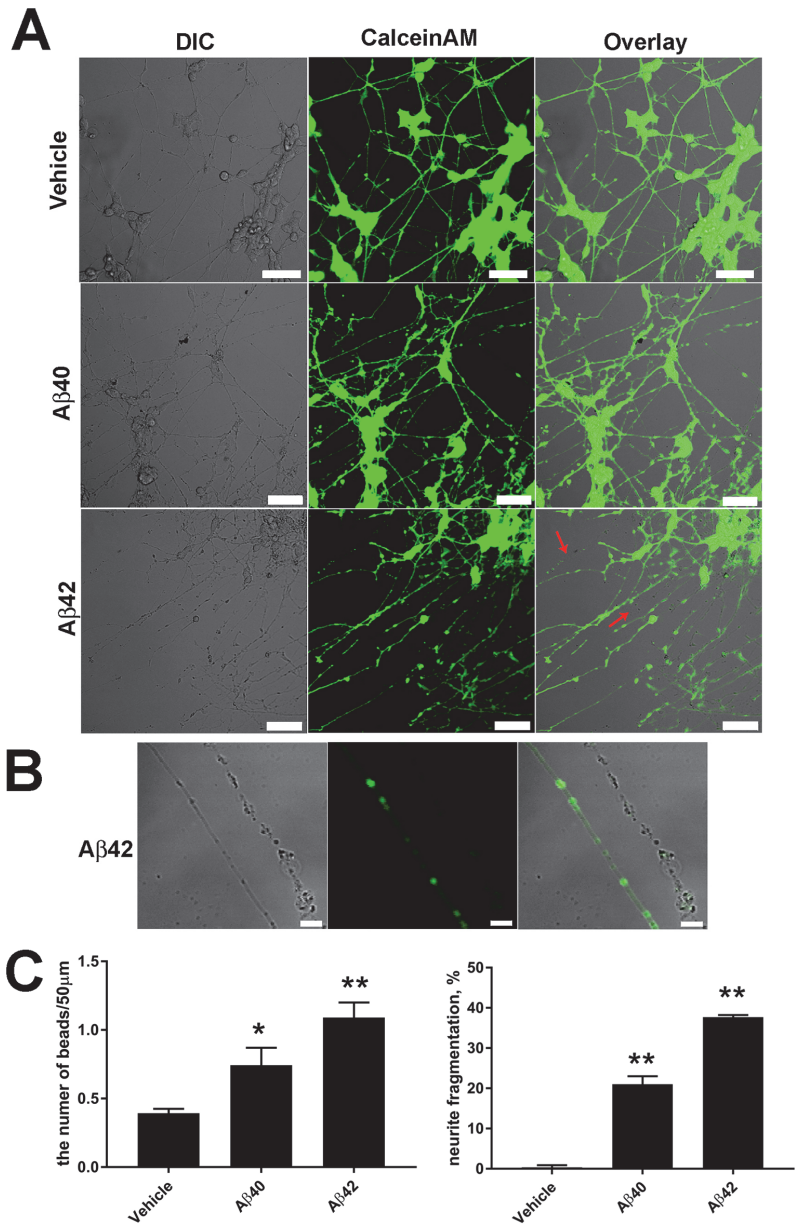

Fig 3. Aβ40 and Aβ42 induce pathological changes in neurite morphology after 72 h. (A) RA/BDNF differentiated live cell imaging for differential interference contrast (right), CalceinAM fluorescence (green; middle). Red arrows indicate fragmented neurites. Scale bar 20µm. (B) Greater magnification of a neurite with beads *versus* a fragmented neurite. Scale bar 5 µm. (C) Quantification of pathological changes in RA/BDNF differentiated cell culture. The figure displays the mean± SEM; at least $n = 3$ independent experiments; **$p \leq 0.005$; *$p \leq 0.05$, One-way ANOVA followed by a Bonferroni's multiple comparisons test at the 0.05 level was used to determine differences between the conditions.

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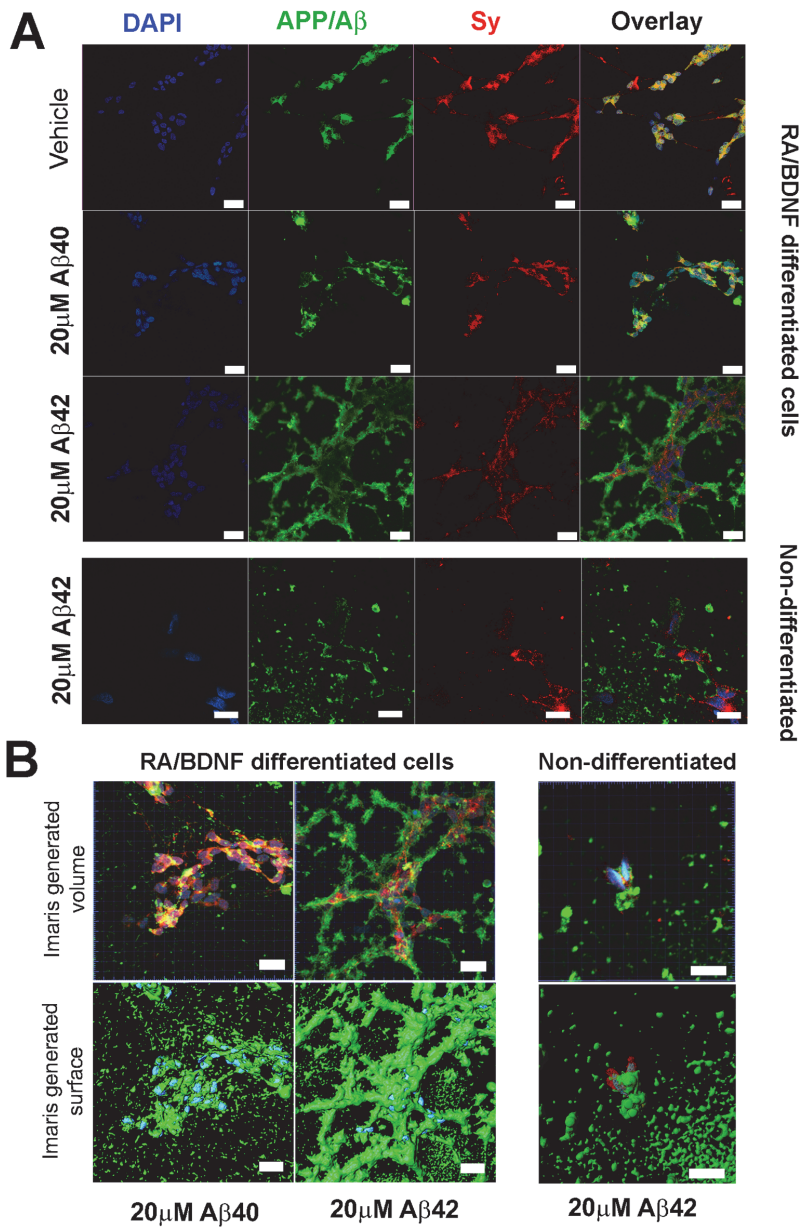

Fig 4. Aβ42 covers cell bodies and neurites. (A) Immunocytochemistry of non-differentiated and RA/BDNF differentiated SH-SY5Y cells after a 24h (Aβ42) and a 48h incubation (Vehicle; Aβ40) for DAPI (blue; left), anti-APP/Aβ (green; left middle), anti-Synaptophysin1 (red; right middle). Scale bar 20μm. (B) Three-dimensional reconstruction of differentiated cells incubated with 20μM Aβ40 and Aβ42 for 48h, and non-differentiated cells incubated with 20μM Aβ42 for 24h, stained for DAPI, anti-APP/Aβ, anti-Synaptophysin1. Confocal microscope images were obtained with optical section separation (z-interval) of 0.303 μm. Upper

panels represent the virtually generated volume of chromophores and the lower panels represent the surface. Scale bar 20μm.

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protocol in Ref [38]) added to the culture medium had no toxic effect on the differentiated SH-SY5Y cells (S8 Fig). These data support the hypothesis that the toxic amyloid species may form during the fibrillization process [39].

Discussion

During the last decades, when the amyloid hypothesis has been the prevailing concept in AD, research scientists have used various cellular and animal models to establish the toxic effects of Aβ-peptides and to search for possible "antidotes" [18, 40, 41]. Despite multiple promising candidates proposed as the result of these studies, none of the drug candidates have proved to be useful for AD patients. Although multiple examples of high Aβ toxicity can be found in the literature [15, 42–44], the toxicity of biologically more common peptide forms, e.g., monomers and fibrils, is often too low for use in test systems. The high toxicity values appear only under particular experimental conditions and are sometimes observed only when using particular test methods. For instance, Aβ peptides show high toxicity when cell viability is estimated by the MTT test. However, Aβ peptides have been shown to interfere with the MTT test, as the decrease in optical density is considerably larger than the decrease in the number of viable cells in the presence of Aβ [45–47]. Generating supersaturated Aβ solutions using organic solvents also enhance the peptide toxicity [21, 48, 49], however, this process has no analogue in living organisms. Aβ25–35 peptide, a fragment of Aβ consisting of amino acid residues only present in the non-amyloidogenic P3 peptide, also shows higher toxicity on cells than Aβ42. Aβ25–35 has not been found in the brains of AD patients and its high toxicity is related to the methionine in its C-terminal: the peptide variant with an additional amino acid in C-terminus is practically nontoxic under the same conditions [50]. Thus, Aβ25–35 is not a relevant substitution for Aβ-peptides in AD related studies [50]. It is desirable to use native peptides and biologically relevant procedures for the preparation of potentially toxic biomolecules in toxicological studies. Aβ peptides showed very low toxicity on non-differentiated SH-SY5Y cells, however the subsequent RA and BDNF treatment increased the susceptibility of the resulting neuron-like cells to the level applicable for toxicity studies and screening of putative protecting agents.

The most debatable topic in the amyloid hypothesis is which aggregation form of Aβ is toxic. Originally the amyloid fibrils were considered the neurotoxic species [23, 51]. Later on, interest concentrated on the more toxic Aβ oligomers generated by fast dilution of supersaturated Aβ solutions [22, 52]. Recently it has been proposed that the toxicity is related to the active fibrillization process: most likely the cells are affected by some metastable particles forming during the fibrillization [53]. In our experiments, only Aβ1–42 added in a predominantly monomeric form and fibrillizing in the cellular medium, induced apoptotic cell death. Both peptides Aβ1–40 and Aβ1–42 fibrillize within a half of an hour when the solution is vigorously agitated, whereas under quiescent conditions the fibrillization can take days to begin [54]. Once the fibrillization is initiated by adding fibrillary seeds or by agitation, Aβ1–42 retains a high fibrillization rate under non-agitated conditions, whereas the fibrillization rates of Aβ40 decrease substantially [55]. In our experiment, only Aβ42 that fibrillized within 24 hours in cellular medium was toxic for the cells, thus, relating the toxicity of amyloid to a myriad of dynamic intermediates present in the solution during the fibrillization. According to the relatively new concept that secondary nucleation mechanisms prevail in amyloidogenesis, the growing fibrils are partially converted to small amyloid species, which are considered to be

toxic and also responsible for propagation of plaques [39]. Our results support the general idea of this hypothesis, because pre-formed mature fibrils were not toxic for the cells.

Matsuzaki et al. demonstrated that amyloid fibrils growing on cells cause membrane deformation [35]. In our experiments Aβ1–42, but not Aβ1–40, covered the cell neurites and cell bodies with a dense fibrillary coating, which lead to a significant increase in membrane permeability as demonstrated by the PI test. The mechanism of how the intermediate Aβ1–42 aggregates induce apoptosis is not clear, for instance, they could form pores in cell membranes [56]. It is important to note that the non-differentiated cells are also partially covered with Aβ1–42, however, their viability was not significantly affected. The non-differentiated cells lack long neurites that have an especially tight amyloid cover and have a smaller surface area, thus, their contact area with Aβ is substantially smaller. Recently it was demonstrated that small fibrillary aggregates of Aβ consisting of 50–70 monomers are always present in Aβ1–42, but not in Aβ1–40 solutions right after the dilution [55]. This data can be invaluable in understanding the peptide toxicity–only the longer peptide containing fibrillary seeds and capable of forming fibrils in the cell culture medium had a substantial toxic effect on the cells.

RA/BDNF-differentiated SH-SY5Y cells with neuron-like morphology were more susceptible to Aβ than the non-differentiated cells. We demonstrated that Aβ1–42 impairs neurites and this is followed by apoptotic cell death. Similar results were obtained with primary cultures [10, 11], supporting the suggestion that RA/BDNF-differentiated SH-SY5Y cells are an appropriate model for studying the effects of amyloids on neuronal cells. The peptides showed relatively low toxicity on the RA/BDNF-differentiated cells *in vitro*, which is in agreement with the slow progression of AD, however, a question may arise about the biological relevance of the effects observed at extremely high concentrations of these effectors. Considering that in the AD brain neurons nearby the plaques are dying [57], it is the contact with peptide aggregate and not the bulk peptide concentration that is the trigger for cell death. The high concentration used in cell experiments ensures that all cells are influenced by the toxic entity–growing fibrils. The main plaque component Aβ42 was more toxic on the differentiated SH-SY5Y cells than its less malignant counterpart Aβ40, most likely because of its stronger ability to form fibrils and cover neurites with a dense coating. Taken together, our findings show that amyloid fibrils, formed *in situ* in the cell culture, induce beading and neurite fragmentation in differentiated human neuron-like SH-SY5Y cells. The RA/BDNF- differentiated SH-SY5Y cells can be used in further detailed studies of the Aβ toxicity on neuronal cells and *in vitro* screening of putative drugs that suppress the Aβ toxicity [58, 59].

Conclusion

The current study showed that the RA/BDNF-differentiated human SH-SY5Y cell line is substantially more sensitive to amyloid peptides than non-differentiated cells. Aβ42 fibrils forming spontaneously in the culture had clear toxic effect on the cells and caused neuritic abnormalities and caspase activation similar to the processes in the brain of patients with neurodegeneration, whereas Aβ40 was non-toxic. It can be concluded that the RA/BDNF- differentiated SH-SY5Y cells can serve as a reasonably good *in vitro* model for the study of neuronal death in AD. Further studies are needed to describe the putative mechanism of pathology in this model.

Supporting information

S1 Fig. Representative photograph of non-differentiated and RA/BDNF differentiated cells in phase contrast.
(PDF)

S2 Fig. Amyloid beta quality control by NMR, MALDI-MS and SDS-PAGE.
(PDF)

S3 Fig. Representative snapshot of the process of the evaluation of neurite degeneration.
(PDF)

S4 Fig. WST-1 test results on non-differentiated cells without serum.
(PDF)

S5 Fig. Representative photograph of RA-differentiated SH-SY5Y cells.
(PDF)

S6 Fig. Transmission electron microscopy of cell medium after a 48 h incubation with amyloid peptides.
(PDF)

S7 Fig. Detection of amyloid aggregation by Thioflavin T in the cell medium.
(PDF)

S8 Fig. Cell viability after a 72 h incubation with 20 μM previously formed fibrils measured with the WST-1 test and membrane integrity counted with the propidium iodide permeabilization tests.
(PDF)

S1 Table. Non-differentiated SH-SY5Y cells, cell viability WST-1 test.
(PDF)

S2 Table. Non-differentiated SH-SY5Y cells, propidium iodide test.
(PDF)

S3 Table. RA/BDNF-differentiated SH-SY5Y cells, cell viability WST-1 test.
(PDF)

S4 Table. RA/BDNF-differentiated SH-SY5Y cells, propidium iodide test.
(PDF)

S5 Table. Effect of Aβ42 on the activities of caspase-3 and/or 7 on RA/BDNF differentiated cells.
(PDF)

S6 Table. The number of beads per 50 μM of neurite length after 72 h with 20 μM peptide.
(PDF)

S7 Table. Proportion of fragmented neurites per area after 72 h with 20 μM peptide.
(PDF)

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Appendix 2

Publication II

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Toxicity of Amyloid-β Peptides Varies Depending on Differentiation Route of SH-SY5Y Cells

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Abstract. Alzheimer's disease (AD) is a currently incurable neurodegenerative disorder being the major form of dementia worldwide. AD pathology is initiated by cerebral aggregation of amyloid-β (Aβ) peptides in the form of amyloid plaques; however, the mechanism how Aβ peptide aggregates participate in the disease progression and neurodegeneration is still under debate. Human neuroblastoma cell line SH-SY5Y is a convenient cellular model, which is widely used in biochemical and toxicological studies of neurodegenerative diseases. This model can be further improved by differentiation of the cells toward more neuron-like culture using different protocols. In the current study, dbcAMP, retinoic acid with TPA, or BDNF were used for differentiation of SH-SY5Y cells, and the resulting cultures were tested for the toxicity toward the $A\beta_{42}$ peptide. The toxicity of $A\beta_{42}$ peptide depended on the type of differentiated cells: RA and TPA- differentiated cells were most resistant, whereas dbcAMP and RA/BDNF- differentiated cells were more sensitive to Aβ toxicity as compared with non-differentiated cells. The differentiated cultures provide more appropriate cellular models of human origin that can be used for studies of the mechanism of Aβ pathogenesis and for a screening of compounds antagonistic to the toxicity of Aβ peptides.

Keywords: Alzheimer's disease, amyloid-β, differentiation, SH-SY5Y, toxicity

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disease that is responsible for up to 75% of all dementia cases [1]. Brains of AD patients are characterized by the presence of amyloid plaques, consisting of amyloid-β (Aβ) peptides, and neurofibrillary tangles consisting of hyperphosphorylated tau proteins. Aβ peptide aggregates accumulate in the brain long before the onset of neurological symptoms and play a significant role in the neurodegeneration. Toxicity of Aβ is intensively studied in cellular models, which can shed light on the mechanisms of disease progression at cellular level. In these studies, a variety of *in vitro* cellular models, which include primary rodent neuronal cultures, neuroblastoma cell lines (mouse Neuro-2A, rat PC12, human SH-SY5Y cells), and human induced pluripotent stem cells (iPSCs), are widely used [2, 3]. Each model has its own advantages and disadvantages; however, human origin, price advantage, and accessibility make human neuroblastoma cell line SH-SY5Y the most popular cellular model in toxicological and biochemical studies of AD. Unfortunately, SH-SY5Y cells in the non-differentiated state lack several essential features of neurons specifically affected in AD. Most importantly, they expose several morphological subtypes with rounded cell bodies and only few short neurites [4] and moreover, they proliferate rapidly. Cancerous cell line-based models can be further improved by application of specific agents, which induce differentiation toward neuron-like

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morphology, expression of neuron-specific proteins, and inhibition of proliferation.

It is known that differentiation of SH-SY5Y cells can be induced by various agents including retinoic acid (RA), brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), tetradecanoylphorbol acetate (TPA), 17 beta-Estradiol (E_2), 3β-hydroxy-5-cholestene (cholesterol), N(6),2′-O-dibutyryladenosine 3′:5′ cyclic monophosphate (dbcAMP), insulin-like growth factor 1 (IGF-1), and several others [5–9].

RA treatment enhances the outgrowth of neurites [10] and leads to increase of the survival of SH-SY5Y cells through the upregulation of BCL-2 protein [11] and changes in cellular sodium homeostasis [12]. Treatment with RA makes SH-SY5Y cells responsive to neurotrophins including BDNF and sensitive to further differentiation. Sequential treatment of SH-SY5Y cells with RA and BDNF leads to outgrowth of neurites, synaptogenesis, and modulates the cellular survival. At the molecular level, treatment leads to increased expression of VAChT and ChAT, and to increased activity of AChE, suggesting differentiation toward the cholinergic neuronal phenotype [13, 14].

Differentiation of SH-SY5Y cells with dbcAMP also induces morphological changes in cells toward a neuron-like phenotype with neurite outgrowth and branching, exposing noradrenergic phenotype [9]. DbcAMP is degraded by intracellular esterases to butyrate and monobutyryl cAMP (mbcAMP), which activates protein kinase A (PKA). It was shown that both the butyrate and the activation of PKA play an important role in dbcAMP mediated differentiation of SH-SY5Y cells [9]. Several studies have demonstrated that dbcAMP increases the expression of amyloid-β protein precursor (AβPP), the precursor molecule for Aβ peptides [15, 16].

12-O-tetradecanoylphorbol-13-acetate (TPA) is a biologically active phorbol ester affecting cell growth and differentiation via protein kinase C (PKC) [13]. During differentiation by TPA, SH-SY5Y cells undergo morphological changes, discontinue replication, and reach a stable cell population. It has been previously shown that sequential exposure of SH-SY5Y cells to RA and TPA induces 3-fold increase in TH, 4-fold increase in DAT, 3-fold increase in D2 and 6-fold increase in D3 receptor levels as compared to undifferentiated cells, characteristic for a dopaminergic cellular phenotype [7, 17].

We have differentiated SH-SY5Y cells by using three protocols: RA/BDNF, RA/TPA, and dbcAMP, which all induced differentiation toward more neuron-like phenotype characterized by dense neurite network. The cultures were tested against toxicity of $A\beta_{42}$ peptide by using different viability tests as well as microscopy and immunocytochemistry. After 48 h exposure to $A\beta_{42}$, all differentiated cells and neurites were covered with Aβ layer and neurite fragmentation was observed. However, cells exhibited different sensitivity toward $A\beta_{42}$ peptide in viability tests: dbcAMP treatment and RA/BDNF treatment increased the sensitivity whereas RA/TPA- differentiated cells were the most resistant toward Aβ toxicity.

MATERIALS AND METHODS

Cell culture

SH-SY5Y human neuroblastoma cells (ATCC, Europe) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 50 U/ml penicillin, 50 μg/ml streptomycin solution (Gibco) in an incubator at 37°C and 5% CO_2. The medium was changed every 2-3 days and cells were split using Trypsin-EDTA solution (Gibco). Cells were seeded in poly-L-lysine (PLL) (Sigma) coated (dbcAMP) or uncoated (RA/TPA and RA/BDNF) white clear bottom 96-well plates (Greiner Bio-One) for toxicity experiments or 24-well plates (Greiner Bio-One) for microscopy experiments. Prior to the application of differentiating agents, cells were allowed to adhere for 24 h.

Obtained cells were treated according to the following differentiation protocols: cells were pre-differentiated with RA (10 μM; Sigma Aldrich) in full media for 4 days and then treated with BDNF (50 ng/mL; Alomone Labs) in serum free media for 2 days or with 80 nM TPA (Sigma Aldrich) in serum free media for 3 days; dbcAMP (Sigma Aldrich) treatment was conducted with 2 mM dbcAMP in full media for 2 days and subsequently in serum-free media containing 2 mM dbcAMP for an additional day.

Cells were visualized with Zeiss Axiovert 200 M, Axiocam MRc5 with 20x A-Plan with Ph2 and 20x Plan NeoFluo objectives.

Cell viability measured by WST-1

The effects of $A\beta_{42}$ on the viability of cells were determined using the cell viability assay WST-1 (Roche). WST-1 allows colorimetric measurement of

cell viability due to reduction of tetrazolium salts to water-soluble formazan by viable cells. The amount of formed formazan dye correlates with the number of viable cells. The measurements were completed 48 h after cells treatment with $A\beta_{42}$. 1 μM staurosporine (Santa Cruz) added to serum-free control group was used as a positive control to induce apoptosis during 48 h. The experiments with HEPES buffer were used as a negative control. 5 μl/well of WST-1 reagent was added to 100 μl cell culture medium, incubated at 37°C for 2 h and absorbance was measured at 450 nm using TECAN Genios Pro microplate reader.

Propidium iodide assay

Propidium iodide (PI) is a red-fluorescent DNA-binding dye used to detect nonviable cells with disrupted cell membranes as it cannot cross intact cell membranes. 0.5 mM PI in phosphate buffered saline (PBS, Sigma) was added to 100 μl cell culture 0.5 μl/well and incubated for 10 min at 37°C. Fluorescence was measured using TECAN Genios Pro microplate reader (excitation 540 nm, emission 612 nm). Results are presented as the fold increase from control islets.

Peptide preparation

Lyophilized amyloid-β 1–42 peptide (Aβ42) (rPeptide) was dissolved in 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) (Sigma) to disaggregate the peptide oligomeric and fibrillar assemblies, vortexed and incubated for 1 h at room temperature, divided into aliquots and dried overnight in a vacuum desiccator. The aliquots were stored at –80°C. One day before experiments, the aliquots were treated with HFIP again as described before. Defibrillized $A\beta_{42}$ aliquot was dissolved in 10 mM NaOH and incubated on ice for 10 min. Next, equal amount of 40 mM HEPES buffer containing 200 mM NaCl (pH 7.3) was added to a final peptide concentration of 400 μM. Prepared peptide solution was immediately applied to the serum-free differentiated cell culture at final concentration of 10 μM and 20 μM.

Immunocytochemistry

Cells were seeded on glass coverslips in 24-well plates. After differentiation, culture media was removed and cells were washed twice with PBS. Cells were fixed using 4% PFA (for TUJ antibody - methanol for 15 min at 4°C) for 20 min at room temperature, washed twice with PBS and blocked with 3% goat serum diluted in PBS for 20 min at room temperature to avoid non-specific staining. Cells were washed twice with PBS and primary antibodies diluted in 0.2% Tween solution were applied for incubation overnight at 4°C. Primary antibodies - anti-Synaptophysin 1 (1:2000) (Synaptic Systems), anti-βIII tubulin (1:2000), anti-AβPP/β-Amyloid (1:2000) NAB228 (antigen is 1–11aa from Aβ) were used (Cell Signaling Technology).

Samples were washed three times for 5 min with PBS and incubated with secondary antibodies diluted in PBS for 1 h at room temperature in dark. Secondary antibodies, Alexa Fluor 488 goat anti-mouse and Alexa Fluor 568 goat anti guinea pig (1:2000) (Thermo Fisher Scientific), were used.

Samples were washed three times for 5 min each with PBS, and nuclei were counterstained with DAPI for 5 min. Coverslips were rinsed once with PBS and once with water and applied onto the microscope slides mounted with a drop of ProLong® Diamond antifade reagent (Life Technologies). Cells were visualized using a confocal microscope Zeiss Duo 510 META with 63X oil immersion objective.

Statistical analysis

Statistical analysis was performed by using one-way analysis of variance (ANOVA) with the *post hoc* Dunnett's multiple comparison test. The graphs represent data from at least three independent experiments, all performed in triplicates as mean ± standard error of the mean (SEM). In the cell viability assay, positive cells were normalized to 100% in negative control (HEPES buffer). In the PI assay, results are presented as the fold increase from control islets. Statistical significance of $p < 0.05$ is represented as *, $p < 0.002$ as **, $p < 0.0002$ as ***, $p < 0.0001$ as ****. Statistical analyses were performed with GraphPad Prism 7.

RESULTS

Morphology of differentiated SH-SY5Y cells

Treatment of SH-SY5Y cells with differentiating agents used induces neurite outgrowth with elongated neurites that form connections with surrounding

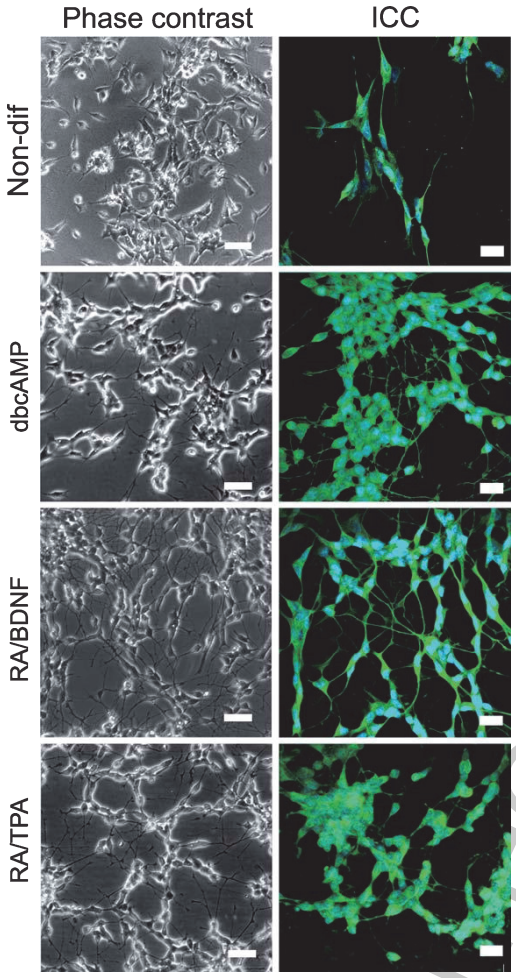

Fig. 1. Visualization of the differentiated SH-SY5Y cells. Phase contrast (left column) images obtained with Zeiss Axiovert 200 M, Axiocam MRc5 with 20x A-Plan with Ph2. Scale bar 50 μm. Immunohistochemistry for TUJ (right column) with counterstained nuclei (DAPI) were visualized with the confocal microscope Zeiss Duo 510 META and 63x oil immersion objective. Stack layers with z-interval of 320 nm are presented in sum intensity projection. Brightness and contrast were increased for clarity. Images were edited by using Fiji software. Scale bar 20 μm.

cells comparing to non-differentiated cells (Fig. 1). RA/BDNF resulted in almost homogeneous cell population whereas small populations of undifferentiated cells were present in dbcAMP and RA/TPA differentiated cell cultures. The success of differentiation was also visualized using immunostaining with an early neuronal marker beta III tubulin (Fig. 1, right column).

Toxicity of $A\beta_{42}$ on non-differentiated SH-SY5Y cells

The toxicity of Aβ on non-differentiated SH-SY5Y cells was determined, which served as a reference for comparison of the effects of differentiation (Fig. 2A, B). The results showed that after 48 h, 10 μM $A\beta_{42}$ was not toxic in viability (WST-1) test ($100.2 \pm 3.4\%$ compared with negative control) as well as in cell permeability (PI) tests (1.1 ± 0.1 fold increase over control). In contrast, 20 μM $A\beta_{42}$ exposed significant toxicity by reducing viability to $57.4 \pm 8.7\%$, and increasing PI uptake was 1.8 ± 0.2 fold over control.

Toxicity of $A\beta_{42}$ on dbcAMP differentiated cells

Incubation with $A\beta_{42}$ had a statistically significant effect on the viability of dbcAMP differentiated cells after 48 h incubation period as measured with WST-1 assay (Fig. 2C). 10 μM and 20 μM $A\beta_{42}$ induced respectively a $51.3 \pm 4.2\%$ and $37.2 \pm 1.7\%$ decrease in the cell viability, which was in both cases statistically significant ($p < 0.0001$).

PI assay revealed that 20 μM $A\beta_{42}$ induced a significant ($p < 0.0002$), almost 2-fold increase in number of permeabilized cells (1.94 ± 0.1 fold increase over control) (Fig. 2D), incubation with 10 μM $A\beta_{42}$ caused 1.37 ± 0.1 fold increase, which was also statistically significant ($p < 0.04$).

Toxicity of $A\beta_{42}$ on RA/BDNF differentiated cells

RA/BDNF differentiated cells were also susceptible to Aβ toxicity; however, 10 μM $A\beta_{42}$ led to a slightly lower relative viability (59.4 ± 5.3 %, $p < 0.0002$) as compared to the effect of 20 μM $A\beta_{42}$ ($77.2 \pm 6.8\%$, $p < 0.0021$) after 48 h incubation (Fig. 2E).

Similarly, treated cells exhibited also increase in amount of permeabilized cells, measured by PI test (Fig. 2F). In case of 10 μM $A\beta_{42}$, there was a 1.8 ± 0.2 fold increase over control, whereas 20 μM $A\beta_{42}$ displayed lower 1.6 ± 0.2 fold increase over control.

Toxicity of $A\beta_{42}$ on RA/TPA differentiated cells

RA/TPA differentiated cells were not sensitive to $A\beta_{42}$ toxicity. In the WST-1 assay, both peptide concentrations induced only minor changes in relative viability of the cells (Fig. 2G), being $85.7 \pm 3.7\%$ in

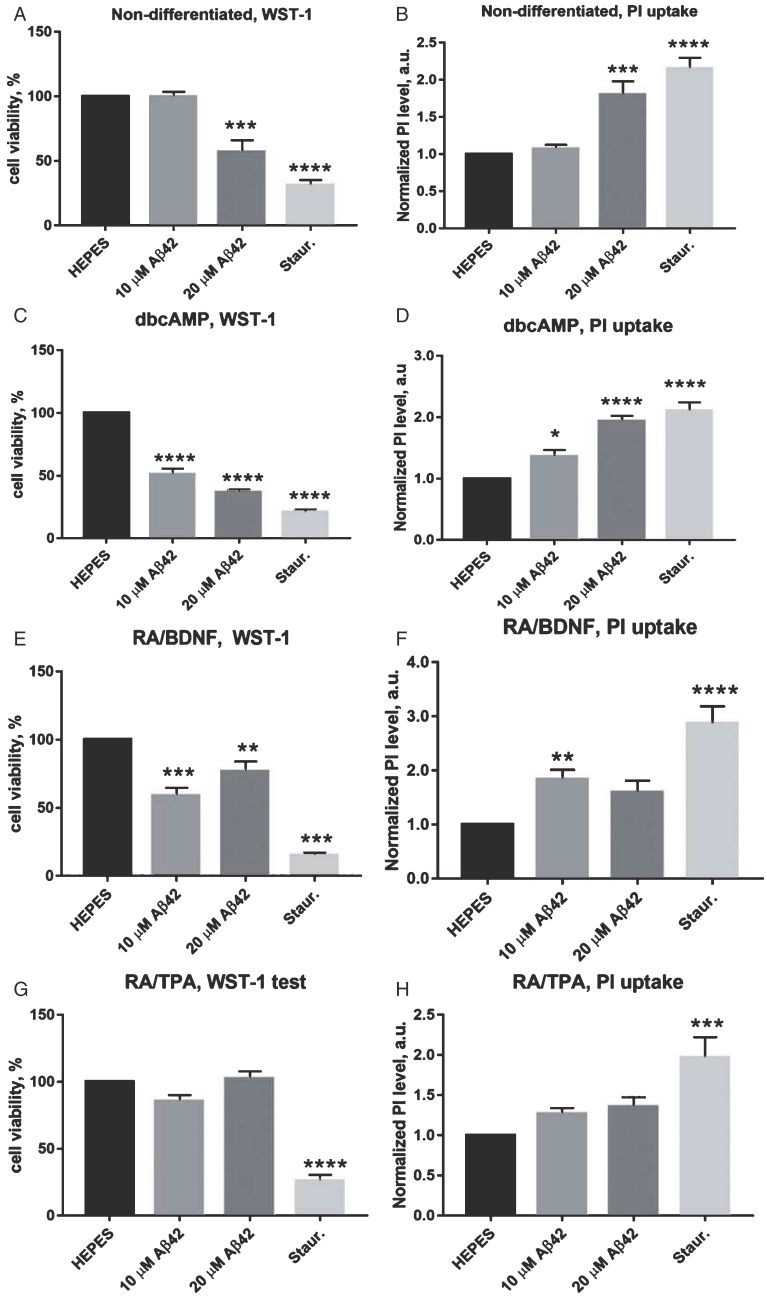

Fig. 2. Quantitation of viable and dead cells after 48 h incubation with $A\beta_{42}$. WST-1 viability test results are on panels A, C, E and G. PI uptake estimation results are on panels B, D, F and H. Negative control: HEPES buffer; positive control: staurosporine. The figure displays the mean ± SEM; $n = 4$ for non-differentiated cells; $n = 4$ for dbcAMP cells; $n = 10$ for RA/BDNF cells; $n = 6$ for RA/TPA cells. $^{****}p < 0.0001$; $^{***}p < 0.0002$; $^{**}p < 0.0021$; $^{*}p < 0.0489$. One-way ANOVA followed by a Dunnett's multiple comparisons test at the 0.05 level was used for statistical analysis.

the case of 10 μM $A\beta_{42}$ and 102.6 ± 5.3% in the case of 20 μM $A\beta_{42}$. Viability of staurosporine-treated cells as a positive control was decreased significantly to 26.4 ± 4.1% ($p < 0.0001$) from control.

Quantification of PI-positive cells also did not expose statistically significant toxic effects of $A\beta_{42}$ on RA/TPA differentiated cells after 48 h incubation (Fig. 2H). In case of 10 μM $A\beta_{42}$, the number of permeabilized cells was increased 1.3 ± 0.1 fold over control, and in the case of 20 μM $A\beta_{42}$, a 1.4 ± 0.1 fold increase was observed. The positive control, staurosporine, induced nearly two-fold increase in PI-positive cell count (2.0 ± 0.2).

Pathological changes in cell culture and coverage with Aβ peptide

Pathological changes were detected in all differentiated cultures after 48 h incubation with 10 μM $A\beta_{42}$ peptide; however, the signs of pathology were completely different from the effects of staurosporine (Supplementary Figure 1). DbcAMP differentiated cells lost all neurites after 48 h incubation even with 10 μM $A\beta_{42}$ (Supplementary Figure 1F, J). In the case of RA/TPA differentiated cells, which were most resistant to amyloid, we observed a relatively small increase in the amount of dead cells after 48 h incubation even with 20 μM $A\beta_{42}$; regardless, neurite fragmentation was seen in the case of both 10 and 20 μM $A\beta_{42}$ as compared with the negative control (Supplementary Figure 1H, L). Both effects were substantially larger in the case of RA/BDNF differentiated cells (Supplementary Figure 1G, K). It is known that Aβ interacts with the plasma membrane and there is also evidence for localization of $A\beta_{42}$ on the cell membranes in the brain of AD patients [18]. As distinct differentiation methods revealed different sensitivities to $A\beta_{42}$, the next step was to evaluate the differences in the distribution of the $A\beta_{42}$ peptide in the cell cultures. For these experiments, cells were stained for AβPP/Aβ and synaptophysin 1 after the treatment with 10 μM $A\beta_{42}$ for 24 h. Synaptophysin 1 is a major synaptic vesicle membrane protein, whose expression is a broad-range marker of neural and neuroendocrine cells including neuroblastoma cells [19]. By means of ICC, we detected that in case of all cell types studied (non-differentiated cells and three differentiated cell cultures), the cell bodies and neurites were too large extent covered with the $A\beta_{42}$ peptide aggregates (Fig. 3), whereas Aβ aggregates were also present in the intracellular space (Fig. 3B, G, L, Q, white arrows).

DISCUSSION

To expand our understanding of the neurotoxicity of amyloid peptides in AD, we compared the effects of $A\beta_{42}$ on human neuroblastoma cell line SH-SY5Y differentiated toward more neuron-like morphology, exposing different biochemical phenotypes [20]. Three different treatments included: 1) dbcAMP, 2) RA followed by BDNF, and 3) RA followed by TPA, which supposedly induce noradrenergic [9], cholinergic [14], and dopaminergic [7] phenotypes, respectively. In all cases, differentiation induced the outgrowth of βIII-tubulin positive networked neurites.

One of the most important factors in Aβ toxicity studies is peptide pre-treatment and preparation for cell culture experiments. It has been shown that the origin of the Aβ peptide [21] as well as the protocol of the preparation of the peptide before applying it to the cells can affect the results [22, 23]. Therefore, comparative studies of Aβ toxicity should be performed using strictly standardized protocols of peptide preparation. In the current study, we used freshly HFIP-treated and alkali-solubilized recombinant $A\beta_{42}$, which minimizes the amount of preformed fibrillary seeds present in the initial solution. In our previous study with RA/BDNF differentiated SH-SY5Y cells, we used a slightly different Aβ dissolving protocol solubilizing the HFIP-pretreated peptide at neutral pH and observed a lower toxic effect, especially in PI assay [24]. In many studies, amyloid peptide is pre-treated with organic solvents before toxicity experiments in order to get oligomers [25, 26] or protofibrils [27, 28] with enhanced toxicity. However, since an increasing amount of evidence points to the decisive role of intermediate fibrillization species in the cellular toxicity [22], we suggest that toxicity of the peptide in the state of active fibrillization can be better model for the *in vivo* toxicity of the peptide than artificially generated formulations.

By comparing the toxicity of similarly pre-treated $A\beta_{42}$ peptide toward differently prepared SH-SY5Y cells, we established that dbcAMP treatment toward noradrenergic phenotype and RA/BDNF treatment toward cholinergic phenotype increase the susceptibility of cells to the toxicity of $A\beta_{42}$ (Tables 1 and 2). In the study with rat primary neuronal cell cultures, it has been demonstrated that Aβ is more toxic to noradrenergic neurons in comparison with cholinergic neurons [29], similarly to our results with human derived cell line. It can also be speculated that the higher susceptibility to $A\beta_{42}$ peptide might be caused

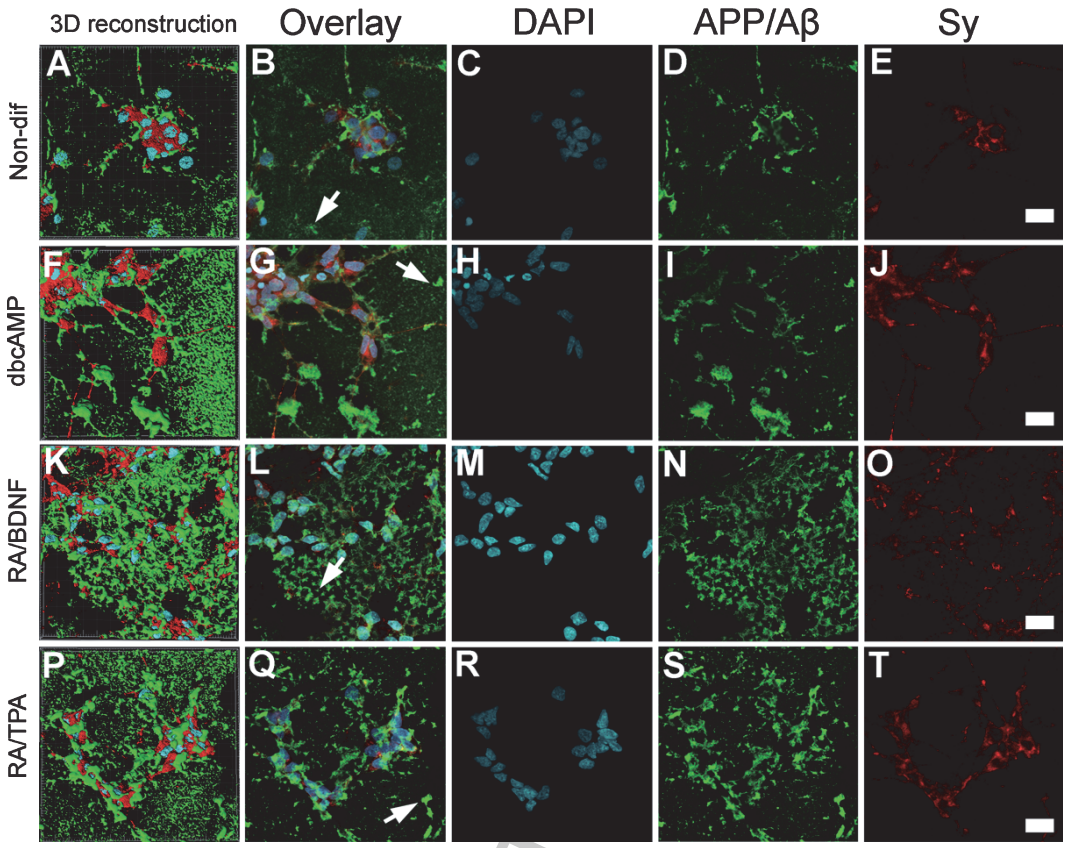

Fig. 3. Distribution of Aβ in cell cultures after 24 h incubation. Immunocytochemistry of 10 μM $A\beta_{42}$-treated SH-SY5Y cells for APP/Aβ (panels D, I, N, and S) and Sy (panels E, J, O, and T) with DAPI counterstained nuclei (panels C, H, M, and R) visualized with the confocal microscope Zeiss Duo 510 META and 63x oil immersion objective. White arrows (panels B, G, L, and Q) indicate the aggregates in the intracellular space. Stack layers with z-interval of 320 nm presented in sum intensity projection. Three-dimensional reconstruction of stacks (panels A, F, K, and P) generated with Imaris software. Brightness and contrast were increased for clarity. Images were edited by using Fiji software. Scale bar 20 μm.

by the dbcAMP-induced overexpression of AβPP [15], which proteolytic processing yields Aβ peptides and may enhance the toxic effects endogenously. Surprisingly, in the case of RA/BDNF differentiated cells the $A\beta_{42}$ peptide at 10 μM concentration was more toxic than at 20 μM level. The reason for this unordinary concentration dependence is not clear; however, it can be hypothesized that as 20 μM peptide fibrillizes faster, then the exposure time of cells to the toxic intermediate species crucial for Aβ-induced neurotoxicity [22, 24] is shorter. Significant sensitivity of RA/BDNF cells toward amyloid can also be enhanced by increased expression of AβPP, showed by Holback et al [30], similarly to dbcAMP- differentiated cells; however, the irregular concentration effect on this type of cells need further investigation. RA/TPA differentiated dopaminergic SH-SY5Y cells were almost resistant to $A\beta_{42}$ toxicity. Such cells are also more resistant to other toxic compounds like 6-OHDA [17], which is used to destroy dopaminergic neurons by different mechanisms as compared to Aβ. Moreover, TPA treatment had protective effect against Aβ toxicity also in case of hippocampal primary neurons, which is assumingly mediated by activation of protein kinase C [31], known to support cell survival [32]. Thus, RA/TPA differentiated SH-SY5Y culture is characterized by high resistance against Aβ-mediated toxicity applicable for studies of the underlying mechanisms of tolerance.

In parallel with toxicological studies, we also monitored distribution of extracellularly applied $A\beta_{42}$ in cell cultures by ICC. The visualized pattern of peptide

Table 1
Comparative table of cell viability WST-1 test results

Differentiation type	10 μM $A\beta_{42}$	20 μM $A\beta_{42}$	Staurosporine
Non-differentiated	100.20 ± 3.45	57.36 ± 8.71	31.58 ± 3.60
dbcAMP	51.34 ± 4.23	37.19 ± 1.73	21.36 ± 1.74
RA/BDNF	59.44 ± 5.30	77.20 ± 6.83	15.59 ± 1.31
RA/TPA	85.75 ± 4.37	102.60 ± 5.26	26.45 ± 4.15

The table displays the mean ± SEM; $n = 4$ for non-differentiated cells; $n = 4$ for dbcAMP cells; $n = 10$ for RA/BDNF cells; $n = 6$ for RA/TPA cells.

Table 2
Comparative table of PI test results

Differentiation type	10 μM $A\beta_{42}$	20 μM $A\beta_{42}$	Staurosporine
Non-differentiated	1.08 ± 0.05	1.81 ± 0.17	2.16 ± 0.14
dbcAMP	1.37 ± 0.10	1.94 ± 0.08	2.11 ± 0.13
RA/BDNF	1.84 ± 0.17	1.61 ± 0.20	2.88 ± 0.31
RA/TPA	1.28 ± 0.06	1.36 ± 0.11	1.98 ± 0.25

The table displays the mean ± SEM; $n = 4$ for non-differentiated cells; $n = 4$ for dbcAMP cells; $n = 10$ for RA/BDNF cells; $n = 6$ for RA/TPA cells.

distribution on cell bodies and neurites in different cultures was generally similar. We expected that the increased resistance of RA/TPA-differentiated cells toward Aβ might be associated with decreased peptide association with cell membranes, which composition might differ from other cells studied. It is known that the composition of cell membranes is crucial for seeding of Aβ peptide aggregation [33, 34] and involved also in cell death signaling [35]. However, our assumption was not confirmed, as we did not find any correlations between the extent of Aβ distribution in the studied cell cultures and the peptide toxicity, which indicates that the reasons of RA/TPA-differentiated cell resistance toward Aβ toxicity need further clarification.

To conclude, we have generated three neuron-like cellular models of human origin by differentiation of SH-SY5Y cells according to different prescribed protocols, which possess several advantages over non-differentiated cell line model, and compared the toxic effects of $A\beta_{42}$ peptide on these systems. We have found that RA/TPA differentiated cells SH-SY5Y cells were almost resistant against toxicity of Aβ. DbcAMP-differentiated and RA-BDNF-differentiated SH-SH5Y cells might be useful for further studies of the mechanisms of Aβ toxicity as well as in the screening of compounds protecting the neuronal cells from the toxic effects of Aβ. DbcAMP differentiation should be preferred because of the normal concentration dependence; RA/TPA differentiated cells can be used for unravelling the mechanisms of Aβ tolerance, which might contribute to better understanding of the neurodegeneration and neuroprotection in case of AD.

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Authors' disclosures available online (https://www.j-alz.com/manuscript-disclosures/19-0705).

SUPPLEMENTARY MATERIAL

The supplementary material is available in the electronic version of this article: http://dx.doi.org/10.3233/JAD-190705.

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Appendix 3

Publication III

Metsla, K.*, Kirss, S.*, Laks, K., Sildnik, G., Palgi, M., Palumaa, T., Tõugu, V., & Palumaa, P. (2022). α-Lipoic Acid Has the Potential to Normalize Copper Metabolism, Which Is Dysregulated in Alzheimer's Disease. *Journal of Alzheimer's disease: JAD*, *85*(2), 715–728. https://doi.org/10.3233/JAD-215026

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α-Lipoic Acid Has the Potential to Normalize Copper Metabolism, Which Is Dysregulated in Alzheimer's Disease

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Abstract.
Background: Alzheimer's disease (AD) is an age-dependent progressive neurodegenerative disorder and the most common cause of dementia. The treatment and prevention of AD present immense yet unmet needs. One of the hallmarks of AD is the formation of extracellular amyloid plaques in the brain, composed of amyloid-β (Aβ) peptides. Besides major amyloid-targeting approach there is the necessity to focus also on alternative therapeutic strategies. One factor contributing to the development of AD is dysregulated copper metabolism, reflected in the intracellular copper deficit and excess of extracellular copper.
Objective: In the current study, we follow the widely accepted hypothesis that the normalization of copper metabolism leads to the prevention or slowing of the disease and search for new copper-regulating ligands.
Methods: We used cell culture, ICP MS, and *Drosophila melanogaster* models of AD.
Results: We demonstrate that the natural intracellular copper chelator, α-lipoic acid (LA) translocates copper from extracellular to intracellular space in an SH-SY5Y-based neuronal cell model and is thus suitable to alleviate the intracellular copper deficit characteristic of AD neurons. Furthermore, we show that supplementation with LA protects the *Drosophila melanogaster* models of AD from developing AD phenotype by improving locomotor activity of fruit fly with overexpression of human Aβ with Iowa mutation in the fly brain. In addition, LA slightly weakens copper-induced smooth eye phenotype when amyloid-β protein precursor (AβPP) and beta-site AβPP cleaving enzyme 1 (BACE1) are overexpressed in eye photoreceptor cells.
Conclusion: Collectively, these results provide evidence that LA has the potential to normalize copper metabolism in AD.

Keywords: Alzheimer's disease, copper metabolism, metalloneurochemistry, α-lipoic acid

INTRODUCTION

Alzheimer's disease (AD) is characterized by the occurrence of amyloid plaques and neurofibrillary tangles in the brain, which results in neurodegeneration and clinical diagnosis of dementia [1]. AD is an age-dependent disease affecting approximately 50 million people worldwide, and this number is expected to increase dramatically because of the population aging [2]. AD is the costliest disease for the developed countries as there is no cure and the patients require long-term support [3, 4]. It is universally accepted that the prevention and treatment of

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AD is one of the major current medical problems and the development of effective treatment and prevention strategies will considerably decrease the global healthcare burden [2].

According to the prevalent amyloid cascade hypothesis [5, 6], the formation of amyloid plaques, consisting of amyloid-β (Aβ) peptides, and the consequent appearance of neurofibrillary tangles, composed of aggregated hyperphosphorylated tau proteins ultimately lead to neurodegeneration in AD. Aβ peptides are produced through proteolytic cleavage of amyloid-β protein precursor (AβPP) by beta-site AβPP cleaving enzyme 1 (BACE1) and gamma secretase. Most therapeutic approaches to AD have focused on targeting Aβ and tau, but unfortunately, many of them have failed in clinical trials [7–9]. However, one Aβ-targeting antibody, Aducanumab, was recently approved by FDA [10]. Currently there is conflicting evidence about the clinical benefit of the costly Aducanumab treatment [11]. Therefore, there is still the need to investigate alternative treatment options.

It has been confirmed that copper metabolism is dysregulated in AD [12, 13], which may trigger the development of AD. Copper is an essential redox cofactor for more than twenty enzymes with crucial roles in cellular energy production (cytochrome c oxidase), antioxidative defense (Cu,Zn-superoxide dismutase-1), oxidative metabolism (lysyl oxidase, tyrosinase, dopamine β-monooxygenase, peptidylglycine α-amidating monooxygenase, etc.), and metabolism of iron (ceruloplasmin). However, "free" or weakly complexed copper ions interact with oxygen metabolites, generating reactive oxygen species, including highly toxic hydroxyl radicals [14]. This double-faceted nature of copper ions dictates the requirement for their tight control [15, 16]. Dysregulation of copper metabolism, such as deficiency, misdistribution, or excessive accumulation is detrimental and leads to various diseases [17]. Classical examples of excessive accumulation and deficiency of copper are Wilson's disease (WD) and Menkes disease, caused by loss-of-function mutations in copper transporters ATP7B [18] and ATP7A [19], respectively. Importantly WD and Menkes disease can be treated by correcting the abnormal copper metabolism by using copper chelators [20] or copper supplements [21], accordingly.

Disturbance of copper metabolism in AD is characterized by copper misdistribution. AD is accompanied by substantially elevated levels of copper in extracellular space, e.g., blood serum and cerebrospinal fluid (CSF) [22–26] and simultaneous copper deficiency in brain tissue [22, 27, 28]. These changes result in decoppering of cellular copper proteins and can hypothetically be detrimental to neurons. Similar changes on a smaller scale also occur during the normal aging process [29, 30]. Recently, a new CuAD hypothesis has been proposed [22]: it theorizes a copper imbalance in AD among the contributing early drivers of this multifactorial condition. This hypothesis posits an age aggravated gradual shift from copper from the pool bound to proteins, with both loss of energy production and antioxidant function, to pools of loosely bound copper ions that increase in the extracellular space and in general circulation and facilitate oxidative stress. According to such a scenario, dysregulation of copper metabolism is an early event in AD pathology and its normalization may be an effective strategy for the prevention and/or treatment of AD.

Attempts to regulate copper metabolism in AD have thus far been unsuccessful. In our opinion, the failures have been caused by several reasons. Generally, so far, the attempts have been largely trial and error cases and were not based on a comprehensive understanding of the copper-binding properties of the ligands in comparison with organismal copper proteins and peculiarities of copper metabolism in healthy and AD brains. Second, synthetic Cu(II) chelators [31–33] have been used, which are known to induce undesirable decoppering of the organism [34–36]. Third, application of synthetic copper ionophores such as clioquinol leads in contrary to an abnormal increase of cellular copper [37, 38] which becomes toxic [39]. A more detailed analysis of copper links with AD and copper-focused therapeutic strategies is presented in the Discussion.

In the current study, we propose molecular tools to normalize copper metabolism in AD based on systematic knowledge about the metal-binding properties of potential AD drug candidates, which have the potential to normalize distorted copper metabolism in AD. Our lead compound is α-lipoic acid (LA). Earlier work from our group has shown that the reduced dihydro-LA (DHLA) form has a substantial Cu(I)-binding affinity [40]. Its affinity for Cu(I) is higher than glutathione but lower than intracellular copper chaperones and enzymes (Fig. 1) [40, 41]. LA acts as Cu(I) chelator only in the intracellular space, where it exists in reduced DHLA form and may shift the copper equilibrium from extracellular to intracellular.

LA is an extensively studied natural ligand that is synthesized enzymatically in the mitochondrion

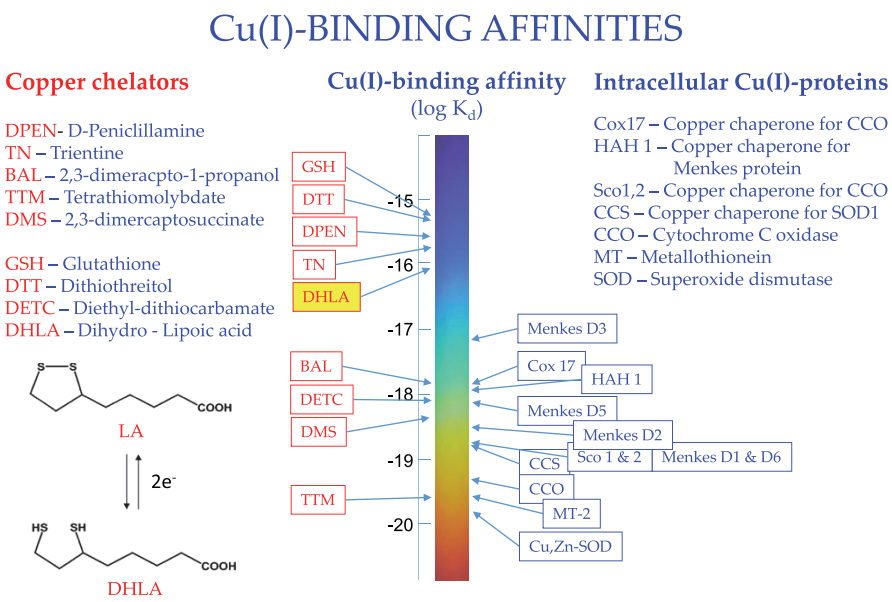

Fig. 1. Copper(I)-binding affinities of intracellular Cu(I) proteins and copper chelators according to [41] and [40].

from octanoic acid [42] and is functioning primarily as a cofactor covalently linked to mitochondrial α-ketoacid dehydrogenases [43, 44]. LA is also absorbed from dietary sources and elicits a unique set of biochemical activities [42]. Thus far, the biological effects of LA have been explained mainly by its antioxidant action, however, its potential in detoxification of heavy metals has also been recognized [45].

LA has been also studied in the context of AD and aging. For example, LA improves the memory of aged nontransgenic (NMRI) mice [46] as well as transgenic AD (Tg2576) mice [47]. Moreover, supplementation with LA extends the lifespan of immunosuppressed mice [48] and also of *Drosophila melanogaster* [49]. LA has a clinically proven therapeutic value in the treatment of diabetic polyneuropathy [50]. Most importantly, LA has been tested in AD clinical trials. A daily dose of 600 mg showed a positive effect by slowing the progression of cognitive impairment in patients with mild AD (43 patients, trial duration 48 months) [51, 52] and in patients with mild to moderate AD with and without insulin resistance (126 patients, trial duration 16 months) [53]. Despite these promising results, clinical trials with LA have not been taken forward. The therapeutic effect of LA in these trials has mainly been attributed to its antioxidative and anti-inflammatory effects [43], whereas its copper regulating properties have not been considered or studied.

In this study, we tested the potential of LA to regulate the cellular copper metabolism in a way necessary for the treatment of AD. We demonstrate that supplementation with LA promotes the influx of copper into SH-SY5Y cells in a dose-dependent manner. In addition, by using *Drosophila melanogaster* models of AD, we show that LA can alleviate the disease phenotype reflected in a negative geotaxis experiment of fruit flies overexpressing Aβ Iowa mutation in the fly brain and weakened the development of copper-induced smooth eye phenotype when BACE1 and AβPP were overexpressed in photoreceptor cells. These results, together with surplus data from literature support the hypothesis that LA may be suitable for the normalization of copper distribution, which is disrupted in AD.

MATERIALS AND METHODS

Cell culture experiments

Human SH-SY5Y neuroblastoma cells (ATCC) were grown in Dulbecco's Modified Eagle Medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin and streptomycin (Gibco) at 37°C in a humidified atmosphere containing 5%

CO_2. The culture medium was changed every 2 to 3 days and the cells were split every 5 to 7 days using 0.25% Trypsin-EDTA (Gibco), up to 20 times. Cells were plated with a density of 2×10^5 cells/ml into white clear-bottom 6-well plates (Greiner Bio-One) 2 ml per well for inductively coupled plasma mass spectrometry (ICP-MS) experiments. SH-SY5Y cells were differentiated in cell culture plates, using the following differentiation protocol: cells were pre-differentiated with 10 μM retinoic acid in full medium for 4 days, followed by differentiation with 50 ng/mL brain-derived neurotrophic factor (Alomone Labs) in serum-free medium for 2 days [54]. Phase-contrast images of cells were taken using Zeiss Axiovert 200M microscope with 20x objective. For cell count measurement, 10 μl of cell suspension was mixed with an equal volume of trypan blue stain, pipetted into Countless cell counting chamber slide, and inserted into the Countess Automated Cell Counter (Invitrogen). To measure copper content, non-differentiated and differentiated SH-SY5Y cells were treated for 24 h with 5 or 10 μM $CuCl_2$ (Sigma-Aldrich) in the presence of 5–50 μM LA (Sigma-Aldrich), 5–200 μM D-penicillamine (D-PA) (Sigma-Aldrich), and 5–200 μM triethylenetetramine hydrochloride (TETA) (Sigma-Aldrich). For toxicity experiments, cells were grown and differentiated in white clear bottom 96-well plates. Differentiated SH-SY5Y cells were treated for 24 h with different concentrations of D-PA, TETA, and LA in the presence of 10 μM $CuCl_2$ (Sigma-Aldrich). For the assessment of cell viability with propidium iodide (PI), 0.5 mM PI in phosphate buffered saline (PBS, Sigma) was added to 100 μl cell culture 0.5 μl/well and incubated for 10 min at 37°C. TECAN Genios Pro microplate reader was used to measure fluorescence (excitation 540 nm, emission 612 nm) [54]. Cells on the plate were imaged with Zeiss Axiovert 200 M microscope.

ICP-MS analysis

Differentiated SH-SY5Y cells were collected from 6-well plate wells into acid-washed 2 ml tubes and centrifuged for 1 min at 10,000 x g to separate the medium from cells. Cells were washed twice in 500 μl PBS (Sigma Aldrich), and a sample of 10 μl from cell suspension sample was taken to measure cell count with Countess Automated Cell Counter as previously described. Cells in PBS were centrifuged for 3 min at 10 000 x g to separate cells from PBS. PBS was discarded and the obtained cell samples were stored at –20°C until the ICP-MS analysis. One day before ICP-MS analysis, 100 μl of 68% HNO_3 was added to the cell samples. Acidified samples were incubated for 24 h at room temperature. For ICP-MS analysis, samples were diluted to 2% HNO_3. Ultrapure Milli-Q water with a resistivity of 18.2 MΩ/cm, produced by a Merck Millipore Direct-Q & Direct–Q UV water purification system (Merck KGaA, Darmstadt, Germany), was used for all sample preparations.

ICP-MS analyses for Cu-63 were performed on an Agilent 7800 series ICP-MS instrument (Agilent Technologies, Santa Clara, USA) and Agilent SPS-4 autosampler was used for sample introduction. For instrument control and data acquisition, ICP-MS MassHunter 4.4 software Version C.01.04 from Agilent was used. ICP-MS analysis was performed in peak-hopping mode, 6 points per peak, 100 scans per replicate, 3 replicates per sample, and the instrument was operated under general matrix working mode under the following conditions: RF power 1550 W, nebulizer gas flow 1.05 l/min, the plasma gas flow 15 l/min, nebulizer type: MicroMist. Elements monitored: Sc-45 (internal standard) and Cu-63. The ICP-MS apparatus was calibrated using multielement calibration standard 2A in 2% HNO_3 (Agilent Technologies, USA) containing Ag, Al, As, Ba, Be, Ca, Cd, Co, Cr, Cs, Cu, Fe, Ga, K, Li, Mg, Mn, Na, Ni, Pb, Rb, Se, Sr, Tl, U, V, and Zn at levels 0.5, 1, 5, 10, and 25 ppb with Sc-45 (ICP-MS internal standard mix 1 μg/mL in 2% HNO_3, Agilent Technologies) as the internal standard for Cu-63.

Drosophila melanogaster experiments

Used stocks, maintenance, and husbandry of Drosophila melanogaster

The following stocks were used for the experiments: driver lines *30Y-Gal4* (a gift from Mark Fortini) [55] and *ninaE*^{GMR}*-Gal4* [56] (Bloomington Drosophila Stock Center (BDSC) #1104), responder lines UAS-APP.Aβ42.D694N.VTR (Iowa flies) (Vitruvian; BDSC #33779) and UAS-APP695-N-myc, UAS-BACE1/ TM6B (AβPP/BACE1 flies), (Vitruvian; BDSC #33797). Fly stocks were maintained at 25°C (12 h:12 h light: dark cycle; 60–70% humidity) on malt-semolina based food prepared from 6.5 g agar, 38 g semolina, 70.5 g malt flour, 17.5 g dry yeast, 5.9 ml nipagin (Tegosept 30%; 30 g/100 ml 94% EtOH; Dutcher Scientific), and 6.8 ml propionic acid (Sigma) per 1000 ml water. Drosophila crosses were performed at 29°C to obtain a higher

activity of *Gal4-UAS* system. By crossing *30Y-Gal4* and Iowa flies, the offspring (AD Iowa flies) have $A\beta_{42}$ Iowa mutant D23N overexpression driven by *30Y-Gal4* pattern in the fruit fly brain. For controls we used the pure UAS-APP.Aβ42.D694N.VTR line inactive in the absence of Gal4. For overexpression in the eye photoreceptors (AD BACE1/AβPP flies), *ninaE*^{GMR}*- Gal4* flies were crossed to AβPP/BACE1 flies, where *ninaE*^{GMR}*- Gal4/+; TM6B/+*flies were serving as a control.

LA feeding regimen

To study the effect of LA on the locomotor activity of AD flies, dietary supplementation with LA was performed. For negative geotaxis assay, LA was dissolved in 96% ethanol to obtain a 200 mM stock solution and added into the previously described food at a final concentration of 2 mM (LA-food). Control food (food) contained the same amount of ethanol that was used to produce LA-food. Adult male and female flies were separated and added to LA-food and food within 24 h after eclosion and aged at 29°C for 7 days before the experiments. To study the effect of LA on the developed AD phenotype, separated male and female flies were maintained on regular food for 7 days and transferred to LA-food and food for further 7 days.

For the eye phenotype experiment, $CuCl_2$ was dissolved in 0.5% HCl for a 1 M stock solution and added to the food at a final concentration of 500 μM (Cu-food). In addition, to measure the effect of LA, food was supplemented with both 500 μM $CuCl_2$ and 2 mM LA (Cu/LA-food). Transgenic flies were kept on food and Cu-food from the egg-laying stage. Adult female and male flies were separated and added to food, LA-food, Cu-food, and Cu/LA-food within 24 h after eclosion and aged at 29°C for 5 days before the experiments. Images from eyes were obtained with the scanning electron microscope Carl Zeiss EVO LS15 (SEM).

In all experiments, flies were transferred to fresh food every 2 days.

Negative geotaxis assay for Drosophila melanogaster

A negative geotaxis assay for *Drosophila melanogaster* was performed as described earlier in [57]. Before the test, 7–10 flies of each group were transferred into empty 15 ml vials, covered with another upside-down vial, and vials were sealed together with transparent tape. Obtained vials were placed in front of a 20 cm high background that was divided into 10 equal spaces. For the measurement, flies were knocked to the bottom of the vial three times, and photos were taken after 10 s. The climbing height of each fly was registered, and the average climbing distance score for each vial of flies was calculated.

Statistical analyses

Statistical analyses were performed using GraphPad Prism 8. Data of PI assay and ICP-MS were analyzed using a one-way analysis of variance (ANOVA) with the *posthoc* Dunnett's multiple comparison test. Negative geotaxis scores were averaged per vial (5–20 flies per vial) and the mean score of each vial was treated as an individual replicate for further analysis. Two-way ANOVA with Sidak's multiple comparison correction was used to compare the scores of different experimental groups. Data on graphs are presented as mean ± standard error of the mean (SEM) and a p value of ≤ 0.05 was considered as statistically significant. Statistical significance is represented as $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, and $^{****}p \leq 0.0001$.

RESULTS

Toxicity of metal chelating agents in the presence of copper ions on differentiated SH-SY5Y cells

As *in vitro* cellular model, we used human neuroblastoma cell line SH-SY5Y, which is a widely used cellular model in neuroscience in the non-differentiated and differentiated form [58]. In the current study, we differentiated SH-SY5Y cells with retinoic acid and brain-derived neurotrophic factor, which induces neuronal phenotype of cells reflected in the outgrowth of long neurites and formation of neuronal network [59] (Fig. 2A, B). To exclude the toxic effect of LA in the presence of copper ions PI assay was performed on differentiated SH-SY5Y cells. Two copper chelators, used in the treatment of WD, were also included in the test. After a 24 h incubation period, all agents and Cu(II) ions were individually nontoxic (Supplementary Figure 1A, C, E). LA and TETA were also nontoxic in a presence of 10 μM $CuCl_2$ (Supplementary Figure 1B, D); however, D-PA at higher concentrations was toxic in a presence of 10 μM $CuCl_2$ as PI uptake doubled at 100 μM D-PA concentration (Supplementary Figure 1F). The phase-contrast image and fluorescent microscopy confirmed the presence of a substantial number of dead cells (Supplementary Figure 2D).

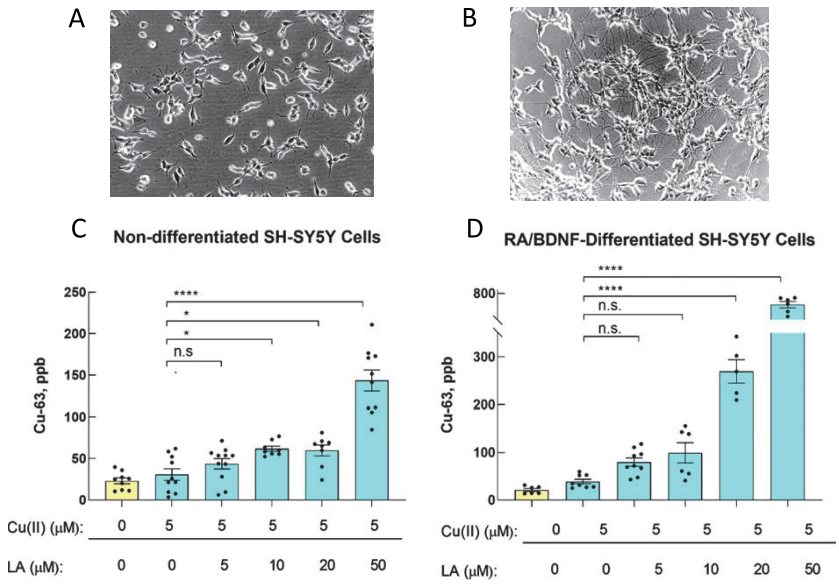

Fig. 2. Effect of LA on the distribution of copper between medium and cells. Phase contrast images showing the morphology of non-differentiated (A) and differentiated SH-SY5Y cells (B). Non-differentiated (C) and differentiated (D) SHSY-5Y cells were treated with 5–50 μM LA in the presence of 5 μM $CuCl_2$; The columns display the mean ± SEM; $n = 8–11$. One-way ANOVA followed by a Dunnett's multiple comparisons test at the 0.05 level was used for statistical analysis. Main effect of treatment $^{****}p < 0.0001$; $^{*}p < 0.05$; n.s., not significant.

The effect of LA, DPA, and TETA on the distribution of copper ions in cell culture

To study whether LA and other copper chelators DPA and TETA can redistribute copper from extracellular to intracellular environment, we used differentiated and non-differentiated SH-SY5Y cells. Both types of cells were treated with 5–50 μM LA in the presence of 5 μM $CuCl_2$. The results demonstrate that LA promotes the translocation of copper ions from the extracellular environment into cells in a concentration-dependent manner (Fig. 2C, D). The effect was evident already at a low micromolar concentration of LA and was more pronounced in the case of differentiated SH-SY5Y cells (Fig. 2C, D). DPA did not increase intracellular copper concentration and in the presence of TETA the intracellular copper concentration decreased (Supplementary Figure 3).

The effect of LA on the phenotype of Drosophila melanogaster AD model

To model AD in fruit flies, we used two fly responder lines: Iowa flies and AβPP/BACE1 flies, overexpressing Aβ peptide with an Iowa mutation D23N and human AβPP/BACE1 respectively (reviewed by [60]). For the ectopic overexpression in Drosophila, the two-component Gal4-upstream activating sequence (UAS) system is widely exploited [61]. We used also two driver lines - *30Y-Gal4* and *ninaE*^{GMR}-*Gal4* with specific expression in the Drosophila mushroom body previously shown to affect also the negative geotaxis [57] and in the eye photoreceptor cells, which models neurodegeneration.

The offspring of AD Iowa flies and control flies were provided with standard food (food) and food with LA added (LA-food) after hatching. After 7 days of incubation, the control flies kept on food and LA-food did not display any difference in the climbing score determined by the negative geotaxis experiment (Fig. 3B). The climbing score of AD Iowa flies incubated on food declined compared to control flies (Fig. 3B), whereas AD flies kept on LA-food showed a lower decline, indicating that LA protects AD Iowa flies from developing AD phenotype. Analysis of male and female flies separately demonstrated that the effect of LA was statistically significant in both sexes (Fig. 3C).

We also studied the effect of LA on flies that had already developed the AD phenotype. In this experiment, AD flies were grown on food for 7 days and half of the flies were thereafter transferred to food+LA

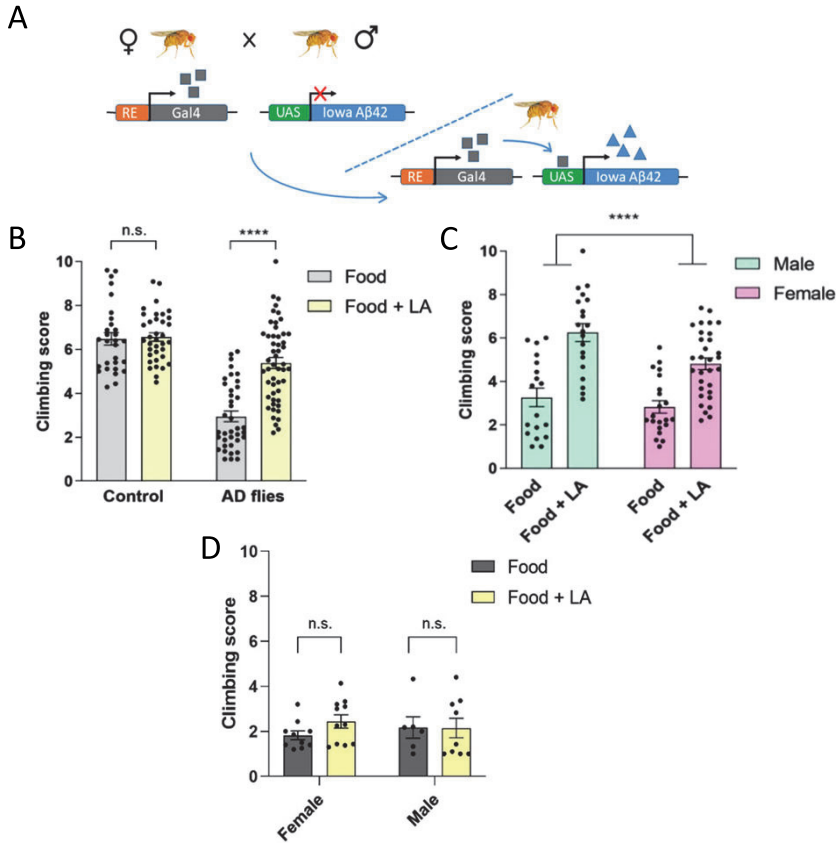

Fig. 3. Effect of LA on the climbing ability of Control versus AD flies. The crossing of RE (regulatory element 30Y) driven Gal4 flies with flies containing UAS in tandem with $A\beta_{42}$ gene containing Iowa mutation (D23N) (A). AD flies were incubated for 7 days on food or food+LA before a negative geotaxis assay (B, C). AD flies were incubated on food for 7 days and food or food+LA for the next 7 days (D). The climbing score displays the mean ± SEM of climbing distance for $n = 32–57$ in groups, each consisting of 7–10 flies. Two-way ANOVA with Sidak's multiple comparison correction was used to compare the scores of different experimental groups. Main effect of treatment $^{****}p < 0.0001$; n.s., not significant.

for the subsequent 7 days. We discovered that the supplementation of LA did not affect the results of the negative geotaxis assay in male and female flies (Fig. 3D) These results suggest that in fly model LA is not able to rescue the AD phenotype that has already been developed.

The offspring of AD AβPP/BACE1 flies and control flies were kept on food and Cu-food starting from the egg laying stage. After hatching male and female flies were separated onto food, LA-food, Cu-food, and Cu/LA-food. After 5 days of incubation, the fly eyes were examined using SEM. AD AβPP/BACE1 flies on the food and LA-food had visible neurodegenerative phenotype with degenerated eye size and smooth external eye surface (Fig. 4E, F). Cu-food had a slightly more severe effect on the eye phenotype, where dark deposits were observed (marked with asterisks in Figure 4G) and had a smoother external eye surface, compared to control flies (Fig. 4E). Eyes of flies on Cu/LA-food exposed slightly more visible ommatidia (marked with asterisks in Fig. 4H), which indicates that LA weakened the effect of copper.

DISCUSSION

In this study, we investigated the molecular tools to normalize copper metabolism, which is dysregulated in AD. Quantitative meta-analyses of numerous independent studies conducted on AD patients have revealed that AD is accompanied by substantially

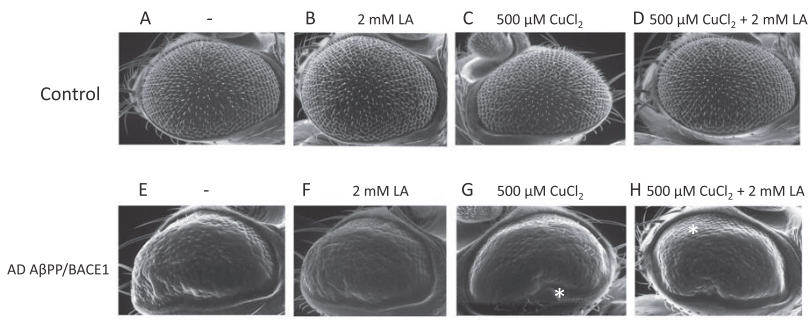

Fig. 4. Effect of LA on the eye phenotype of AD AβPP/BACE1 flies. SEM images showing the eye morphology of *ninaE*^{GMR}*- Gal4/+; TM6B/+*flies as control and AD AβPP/BACE1 5-day old flies grown on food (A and E), food supplemented with 2 mM LA (B and F), food supplemented with 500 μM $CuCl_2$ (C and G), or food supplemented with 500 μM $CuCl_2$ and 2 mM LA (D, H). Asterisks show the presence of dark precipitate on (G) or photoreceptor cells on (H).

elevated copper levels in serum [22–25] as well as in the CSF [26] and simultaneous copper deficiency in the brain tissue [23, 27, 28]. The more precise analysis shows that in the serum and CSF of AD patients mainly non-ceruloplasmin (CP)-bound fraction of copper is increased [23, 25, 62, 63] and in AD brain copper deficiency affects the hippocampus (average decrease 35%), amygdala (average decrease 41%), frontal cortex (average decrease 20%) and several other areas of the cerebral cortex, which are the most damaged in the AD pathology, whereas the average copper deficiency in AD brain is 24% [22].

The molecular and genetic background of disturbed copper metabolism has also been extensively studied in the context of AD. Genetically, a higher probability of having AD has been associated with certain variants of *ATP7B*, the copper transporter defective in WD [64]. Furthermore, copper affects several cellular aspects of AD pathology. First, AβPP, a central molecule in AD pathology, the proteolysis of which produces Aβ peptides [65], is a copper-binding protein [66, 67] and its expression [68], oligomeric state [69], cellular localization [70, 71], and proteolysis [72, 73] are copper-regulated. Therefore, the misbalance of copper metabolism may shift the proteolysis of AβPP towards the amyloidogenic pathway, leading to increased production of Aβ peptides or more amyloidogenic peptide $Aβ_{42}$, which plays a crucial role in initiating and perpetuating the AD pathologic cascade [6]. Second, copper ions bind with relatively high affinity to full-length and truncated Aβ peptides [74–76], accelerating their aggregation [77, 78]. Third, copper ions are enriched in Aβ fibrils *in vivo* in AD brains [79] as well as *in vitro* [80] and cause oxidative stress, which is neurotoxic and can cause neurodegeneration [81, 82]. Listed evidence indicates that normalizing copper metabolism provides an attractive avenue for the treatment and prevention of AD.

Because of the connection between copper metabolism and AD, several attempts have been made to treat AD by modifying copper metabolism. Three different strategies: copper supplementation, chelation, and redistribution have all been tested in laboratory experiments as well as in clinical trials. Copper supplementation was attempted with copper orotate [83, 84], chelation with D-PA [36] and copper redistribution with clioquinol (CQ, 5-Chloro-8-hydroxy-7-iodoquinoline) and its derivative PBT2 (5,7-dichloro-8-hydroxy-2-[(dimethylamino)methyl]quinoline) [85, 86]. In clinical trials, copper supplementation showed no effect on the progression of AD phenotype over a 12-month treatment period [83]. D-PA promoted decoppering and reduced serum oxidative stress, but did not affect the clinical progression of the disease in a 6-month trial [36]. CQ was also tested in a phase II clinical trial with 36 patients [87]. There was no statistically significant difference in cognition between the treatment and placebo groups at 36 weeks [87, 88]. The drug, however, has been withdrawn from development due to safety concerns as a mutagenic ingredient "di-iodo" CQ that could not be reduced to an acceptable level [88]. PBT2, an improved version of CQ, was tested in two clinical trials [89]. BPT2 showed a favorable safety profile [87] and did reduce $Aβ_{42}$ concentration in CSF compared with those treated with placebo [90]. In the initial publication, it was concluded that BTP2 did not improve the cognitive function of AD patients [90]; however, in later

analysis of the results, some improvement of cognition was detected [89, 91]. The second trial of PBT2 was more rigorously conducted and showed that after 12 weeks this compound appeared to be safe and well-tolerated in people with mild AD; however, larger trials are required for reliable demonstration of cognitive efficacy [87].

In addition to clinically tested compounds, numerous other synthetic Cu(II)-binding ligands [31–33, 92–95], including TETA [96], an FDA-approved WD drug, as well as zinc treatment [97] have been proposed for the treatment of AD. TETA and all other this type of drugs as well as zinc treatment result in a decoppering of an organism [98, 99], which may further decrease the copper levels in the brain.

Therefore, in our opinion the treatments, which lead to decoppering of the organism are not suitable for the normalization of copper dysmetabolism in AD. Rather, substances with the ability to translocate excessive extracellular copper to the intracellular space in the brain are needed. We aimed to normalize copper metabolism by using Cu(I)-binding ligand, which acts only in intracellular space and can shift the equilibrium of copper distribution from extracellular to intracellular location. We established that dihydro-LA has substantial Cu(I)-binding affinity owing to its two closely located SH groups [40]. SH groups of LA are reduced inside the cell and oxidized to a disulfide bond in the extracellular environment thus enabling selective intracellular Cu(I) binding and shifting of equilibrium of copper distribution towards intracellular space through a direct or indirect mode of action.

There are numerous benefits of LA over other synthetic compounds in drug development as well as in further therapeutic use. LA has been approved for the treatment of diabetic polyneuropathy [50] and could be repurposed for therapeutic application in AD [100]. The known toxicology and pharmacodynamic profiles of repurposed drugs significantly accelerate the drug development process, decrease the related costs, and increase the probability of success. By using the PI test, we demonstrated that LA and synthetic compounds, such as D-PA and TETA are individually non-toxic in differentiated SH-SY5Y cell culture. However, when cells were treated with chelating agents in the presence of 10 μM Cu(II) ions, LA and TETA were non-toxic, whereas D-PA in the presence of copper ions was toxic. It is known, that D-PA treatment in WD worsens neurological symptoms in the early phase of treatment [101], which may be caused by neurotoxicity of D-PA in the presence of copper, demonstrated in our study.

We demonstrated that supplementation with LA significantly increases the intracellular copper level of SH-SY5Y cells in a dose-dependent manner. An increase of intracellular copper occurred already at 5 μM concentration of LA and the increase was moderate, which is similar to the decrease of copper level in AD brain tissue [23]. The mechanism of LA action in promoting copper redistribution remains to be established. The effect may involve a direct interaction of DHLA with copper ions in the intracellular environment, which shifts the equilibrium of copper distribution towards the intracellular compartment. Alternatively, LA may affect the regulation of copper metabolism indirectly by enhancing or suppressing the synthesis of some proteins. In direct action, LA has to compete with extracellular human serum albumin (HSA), which has a high (picomolar) affinity Cu(II) binding site [102] and is present in CSF at 3 μM concentration [103]. HSA did not hinder copper translocation by LA in experiments with non-differentiated SH-SY5Y cells where the culture media contained 75 μM of HSA, which demonstrates that intracellular LA outcompetes extracellular HSA. In addition, indirect or even pleiotropic action of LA is not excluded [43]. It is known that LA increases cellular glutathione levels [104] and functions as an antioxidant by promoting mitochondrial functioning [105]. The mechanism of LA action in promoting copper intake needs further elucidation.

Furthermore, we tested the effect of LA in two transgenic AD *Drosophila melanogaster* models. The first model was AD Iowa flies overexpressing human Aβ with an Iowa mutation D23N in mushroom bodies. We showed that early supplementation with LA prevents the development of AD phenotype in AD Iowa flies in a negative geotaxis experiment; however, LA cannot alleviate the already developed phenotype in AD flies. Although the expression of Aβ peptides is not directly connected with copper metabolism, there is evidence that the phenotype of these flies is affected by copper metabolism. For example, the phenotype of $A\beta_{42}$ expressing flies is ameliorated through the inhibition of high-affinity copper influx transporter Ctr1 orthologues in the fly nervous system [106]. The second model was AD AβPP/BACE1 flies overexpressing human AβPP and BACE1 in the eye photoreceptors. In this model, the strongest smooth eye phenotype with dark deposits came up after the addition of 500 μM $CuCl_2$ to the food. This effect was slightly weakened by the addition of 2 mM LA to the food, which indicates that LA may have a protective effect on the neurotoxicity of $A\beta_{42}$ in the presence of

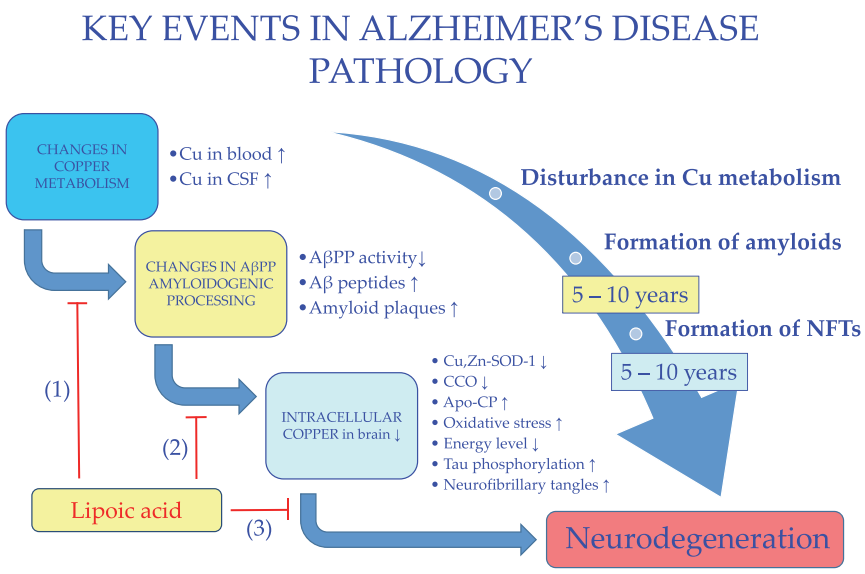

Fig. 5. Key events in Alzheimer's disease pathology. Disturbance of copper metabolism is an early event before initiation of amyloid cascade composed from formation of amyloid plaques, formation of NFTs and following neurodegeneration. According to our hypothesis LA can correct extra- and intracellular copper distribution (1), suppress amyloidogenic processing of AβPP (2), and reverse decoppering of cellular copper proteins leading to neurodegeneration (3) characteristic of AD. Aβ, amyloid-β; AβPP, amyloid-β protein precursor; Apo-CP, apoceruloplasmin; CCO, cytochrome c oxidase; CSF, cerebrospinal fluid; Cu,Zn-SOC-1, Cu,Zn-superoxide dismutase-1; NFTs, neurofibrillary tangles

Cu(II) ions. Our data agree with the previous report showing that the phenotype of flies expressing $A\beta_{42}$ in their eyes is changed by adding Cu(II) ions to the food and these changes could be reversed by copper chelators [107]. In addition, the phenotype of flies expressing $A\beta_{42}$ specifically in their eyes is changed after mutations in copper transporter ATP7 [108], which highlights the link between Aβ toxicity and copper metabolism in AD model flies. Therefore, the beneficial effect of LA on AD Iowa flies may also be connected with the normalization of copper metabolism; however, it has to be confirmed in follow-up studies. Moreover, LA might act through an anti-amyloid mechanism different from the regulation of copper since LA protected cultured rat primary neurons against Aβ toxicity [109].

In conclusion, we propose a hypothetical role of LA in key events of AD, which is presented in Fig. 5. According to our hypothesis, LA can avoid or inhibit the following processes in AD pathology: 1) an increase of extracellular copper levels during aging; 2) misfunctioning of AβPP and its amyloidogenic proteolytic cleavage into amyloid peptides; 3) demetallation of essential copper proteins (Fig. 5).

Disturbed copper metabolism may lead to further downstream processes such as increase of oxidative stress, depletion of cellular energy reserves, phosphorylation of tau proteins and formation of neurofibrillary tangles leading ultimately to neurodegeneration. Disturbance of copper metabolism is an early event before initiation of amyloid cascade where there are 5–10 years periods between the formation of amyloid plaques and formation of neurofibrillary tangles as well as between formation of neurofibrillary tangles and neurodegeneration/appearance of symptoms (Fig. 5). The proposed mechanism of LA ion waits for verification in further animal experiments and human clinical trials.

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SUPPLEMENTARY MATERIAL

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Lisanna Laanela, bachelor's degree, 2018, (sup) Kristel Metsla; Peep Palumaa, The toxicity of copper ions on differentiated SH-SY5Y cells. The effect of metal chelators. Tallinn University of Technology, School of Science, Department of Chemistry and Biotechnology

Sigrid Kirss, master's degree, 2020, (sup) Kristel Metsla; Peep Palumaa, Investigation of copper metabolism and its regulation in cell culture and Drosophila models of Alzheimer's disease, Tallinn University of Technology, School of Science, Department of Chemistry and Biotechnology

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Lisanna Laanela, bakalaureusekraad, 2018, (juh) Kristel Metsla; Peep Palumaa, The toxicity of copper ions on differentiated SH-SY5Y cells. The effect of metal chelators. Tallinna Tehnikaülikool, Loodusteaduskond, Keemia ja biotehnoloogia instituut

Sigrid Kirss, magistrikraad, 2020, (juh) Kristel Metsla; Peep Palumaa, Investigation of copper metabolism and its regulation in cell culture and Drosophila models of Alzheimer's disease (Vase metabolismi ja selle regulatsiooni uurimine Alzheimeri tõve rakukultuuri ja äädikakärbse mudelis), Tallinna Tehnikaülikool, Loodusteaduskond, Keemia ja biotehnoloogia instituut

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