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# Application of Differentiated SH-SY5Y Cells for Toxicological Studies of Alzheimer's Amyloid Beta Peptide

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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 any academic degree.

Jekaterina Krištal

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# Diferentseeritud SH-SY5Y rakkude kasutamine Alzheimeri amüloid beeta peptiidi toksilisuse uurimiseks

JEKATERINA KRIŠTAL

TAL TECH
KIRJASTUS

# Contents

## List of publications

Current thesis is based on the following publications, which will be referred to in the text by their Roman numerals.

I Tiiman, Ann; Krishtal, Jekaterina; Palumaa, Peep; Tõugu, Vello (2015). In vitro fibrillization of Alzheimer's amyloid-β peptide (1-42). AIP Advances, 5, 092401.10.1063/1.4921071

II Krishtal, J.; Bragina, O.; Metsla, K.; Palumaa, P.; Tõugu, V. (2017). In situ fibrillizing Amyloid-beta 1-42 Induce Neurite Degeneration and Apoptosis of Differentiated SH-SY5Y Cells. PLoS ONE.10.1371/journal.pone.0186636. DOI: 10.1371/journal.pone.0186636

III Krishtal, J.; Metsla, K.; Bragina, O.; Tõugu, V.; Palumaa, P. (2019). Toxicity of amyloid beta peptides varies depending on differentiation route of SH-SY5Y cells. J Alzheimers Dis. 2019;71(3):879-887; DOI 10.3233/JAD-190705

## Author's contribution to the publications

I Amyloid beta defibrillization procedures, fibrillization experiments with mixtures of amyloid beta 40 and 42, preparation of fibrils probes for transmission electron microscopy

II Cell culture and differentiation, peptide preparation, toxicity experiments, immunocytochemistry and microscopy, image analysis, preparation of the peptide probes for transmission electron microscopy, preparation of the manuscript

III Cell culture and differentiation, peptide preparation, toxicity experiments, immunocytochemistry and microscopy, preparation of the manuscript

# Introduction

Alzheimer's disease (AD) is the most common form of dementia affecting approximately 50 million people worldwide, and this number is expected to increase up to 152 million by 2050. There is still no effective treatment and no cure in sight, which might be caused by the fact that the mechanisms of neurodegeneration in AD are still not completely understood. Many efforts have been made to create appropriate models for studying AD. As opposed to rodent primary cultures, immortalized cell cultures of human origin are simpler to use for evaluating the neuropathological mechanisms of AD since, obviously, no genetic modifications are required for „humanization" of such cells. To date, the human neuroblastoma cell line SH-SY5Y has been one of the most widely used cellular models for AD studies. The untreated SH-SY5Y cells lack many features of mature neurons such as expression of various neuronal markers and outgrowth of neurites with synaptic connections and consequently cannot form neuronal networks. At the same time, there are also advantages of using SH-SY5Y cells as AD model e.g. the cell line has been comprehensively described in literature and can be differentiated into a neuron-like culture with several neuronal properties resembling the diversity of neurons found in the brain. The two main hallmarks of AD are the accumulation of extracellular amyloid plaques and intracellular neurofibrillary tau tangles. Amyloid beta peptide (Aβ), the main component of the plaques, has been shown to form toxic species in the brain that may trigger neurodegeneration or contribute to disease progression. In the current study, SH-SY5Y cells were differentiated into neuron-like cultures using three different protocols to obtain cells with distinct neuronal properties for subsequent use in testing Aβ toxicity by using of different assays. The protocol for Aβ preparation for toxicity studies was also optimized. The results show that differentiated SH-SY5Y cells have different sensitivity toward *in situ* fibrillizing Aβ as compared to non-differentiated cells. Moreover, noradrenergic and cholinergic cultures were more sensitive to Aβ toxicity as compared to dopaminergic culture. These findings are consistent with the cholinergic and noradrenergic hypothesis of AD, since these types of neurons are among the first to die in the course of the disease. Elaborated models based on differentiated SH-SY5Y cells can be used to reveal the mechanisms of neurodegeneration as well as for preliminary studies of new drug candidates.

## Abbreviations

| | |
|---|---|
| 2D | two dimensional |
| 3D | three dimensional |
| ACh | acetylcholine |
| AChE | acetylcholine esterase |
| AD | Alzheimer's disease |
| ApoE | apolipoprotein E |
| APP | amyloid precursor protein |
| ATCC | American Type Culture Collection |
| Aβ | amyloid beta |
| Aβ3pE-42 | pyroglutamated amyloid beta 3-42 |
| BCL-2 | B-cell lymphoma 2 |
| BDNF | brain derived neurotrophic factor |
| BMP4 | bone morphogenetic protein 4 |
| C83 | APP carboxy-terminal fragment α |
| C99 | APP intracellular domain |
| ChAT | choline acetyltransferase |
| ChT1 | choline transporter 1 |
| CSF | cerebrospinal fluid |
| dbcAMP | dibutyryl cyclic adenosine monophosphate |
| DMEM | Dulbecco's Modified Eagle Medium |
| DMSO | dimethylsulfoxide |
| fAD | familial Alzheimer's disease |
| FBS | fetal bovine serum |
| HFIP | 1,1,1,3,3,3-Hexafluoroisopropanol |
| HSV | herpes simplex virus |
| IGF | insulin-like growth factor |
| iPSC | induced pluripotent stem cells |
| LC | locus coeruleus |
| MAP2 | microtubule associated protein 2 |
| mbcAMP | monobutyryl cyclic adenosine monophosphate cAMP |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| NA | noradrenaline |
| NGF | neuronal growth factor |
| NMDA | N-methyl-D-aspartate |
| NMR | nuclear magnetic resonance |
| PBS | phosphate buffered saline |
| PI | propidium iodide |
| PKA | protein kinase A |
| PKC | protein kinase C |
| PS1 | presenilin 1 |

| | |
|---|---|
| PS2 | presenilin 2 |
| RA | retinoic acid |
| RPMI | Roswell Park Memorial Institute |
| sAD | sporadic Alzheimer's disease |
| sAPPα | secreted ectodomain APP alpha |
| sAPPβ | secreted ectodomain APP beta |
| TEM | transmission electron microscopy |
| TH | tyrosine hydroxylase |
| ThT | Thioflavin T |
| TPA | 12-O-tetradecanoylphorbol-13-acetate |
| TREM2 | triggering receptor expressed on myeloid cells-2 |
| TUJ | neuron-specific class III beta-tubulin |
| VAChT | vesicular acetylcholine transporter |
| VTA | ventral tegmental area |
| WST-1 | water soluble tetrazolium (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium) |

# Review of the literature

## 1. Alzheimer's disease

In 1906 German psychiatrist, Alois Alzheimer described a demented patient with a postmortem histopathological finding of amyloid plaques and neurofibrillary tangles in her brain for the first time. This type of the disease now bears his name: Alzheimer's disease (AD). According to the Alzheimer's Association's annual report for 2019, 5.8 million people in USA have AD and this number is expected to grow in the future due to aging of the baby boom generation (Gaugler, James et al. 2019). It was also estimated in the report that AD is the sixth leading cause of death in the United States. In general, AD is an age-dependent disease, characterized by neuronal cell loss that is accompanied by memory impairment and cognitive decline. Importantly, the first symptoms of AD appear many years after the formation of amyloid plaques (Jack, Knopman et al. 2010). Approximately 95% of AD patients have the sporadic disease form (sAD) with onset after 65 years. Less than 5% of AD patients have familial AD (fAD), with the average age of onset being 45 years (Masters, Bateman et al. 2015). AD has been intensively studied by multidisciplinary approaches and state-of-the-art techniques for many decades (Cline, Bicca et al. 2018, Molinuevo, Ayton et al. 2018). It has been known since the mid-1980s that extracellular amyloid plaques and intracellular neurofibrillary tangles, the hallmarks of AD, mainly consist of fibrillized amyloid beta (Aβ) peptides and hyperphosphorylated tau proteins, respectively (Hardy and Selkoe 2002). However, there is still no clear understanding of the mechanisms of neurodegeneration.

## 2. Amyloid beta origin and physiological role

Aβ is a product of proteolytic cleavage of amyloid precursor protein (APP), a transmembrane protein expressed in several isoforms mainly in neuronal tissue (Nalivaeva and Turner 2013). To date, APP has been shown to be involved in a number of physiological processes such as nervous system development, function and formation of synapses, modulation of neural circuits (Muller, Deller et al. 2017). APP is typically processed in two ways: a non-amyloidogenic pathway and an amyloidogenic pathway (Figure 1); however, there are also non-canonical pathways such as the C-terminal cleavage by caspases that are involved in neuronal apoptosis (Muller, Deller et al. 2017). APP processing usually happens in the endosomal compartment originating from trans-Golgi Network, or on the cell membrane (Chow, Mattson et al. 2010). In the case of the non-amyloidogenic pathway, α-secretase cleaves APP to produce a soluble derivate, sAPPα, and APP carboxy-terminal fragment α (C83) that is membrane anchored. This fragment is subsequently cleaved by γ-secretase into the p3 fragment and the APP intracellular domain (Chow, Mattson et al. 2010). In the amyloidogenic pathway, APP is cleaved by β-secretase into sAPPβ and APP carboxyl-terminal fragment β. The latter fragment is processed by γ-secretase, resulting in formation of APP intracellular domain (C99) and Aβ (Chow, Mattson et al. 2010). γ-secretase enzymatic complex consists of 4 proteins: nicastrin, presenilin enchancer-2 (PEN-2), anterior pharynx defective-1 (Aph-1), presenilin 1 (PS1) or presenilin 2 (PS2) that are all required for the γ-secretase activity (Zhang, Li et al. 2014). γ-secretase participates not only in APP processing, but also in the Notch signaling pathway, which controls various developmental and physiological processes (Bray 2016). PS1 and PS2 are aspartyl proteases that play a major role in determining the length of the Aβ produced. The released Aβ peptide

contains 37-43 amino acid residues, since γ-secretase complex performs intramembrane step-by-step carboxyterminal cleavage of the polypeptide chain (Steiner, Fukumori et al. 2018). The approximate proportions of the various Aβ peptides produced are: Aβ40 - 80%, Aβ42 - 10%, Aβ38- 10%, Aβ43 and Aβ37 - less than 1% (Steiner, Fukumori et al. 2018). Aβ has a hydrophilic N-terminal and hydrophobic C-terminal parts. The N-terminal part of the peptide is involved in binding of transition metal ions. His6, His13 and His14 residues can bind $Zn^{2+}$ and His13, His14 and Asp1 of Aβ coordinate $Cu^{2+}$ (Tiiman, Palumaa et al. 2013, Srivastava, Pittman et al. 2019). The C-terminal part of Aβ (25-35aa) is involved in formation of intermolecular β-sheets and Aβ peptide fibrillization (Pike, Walencewicz-Wasserman et al. 1995).

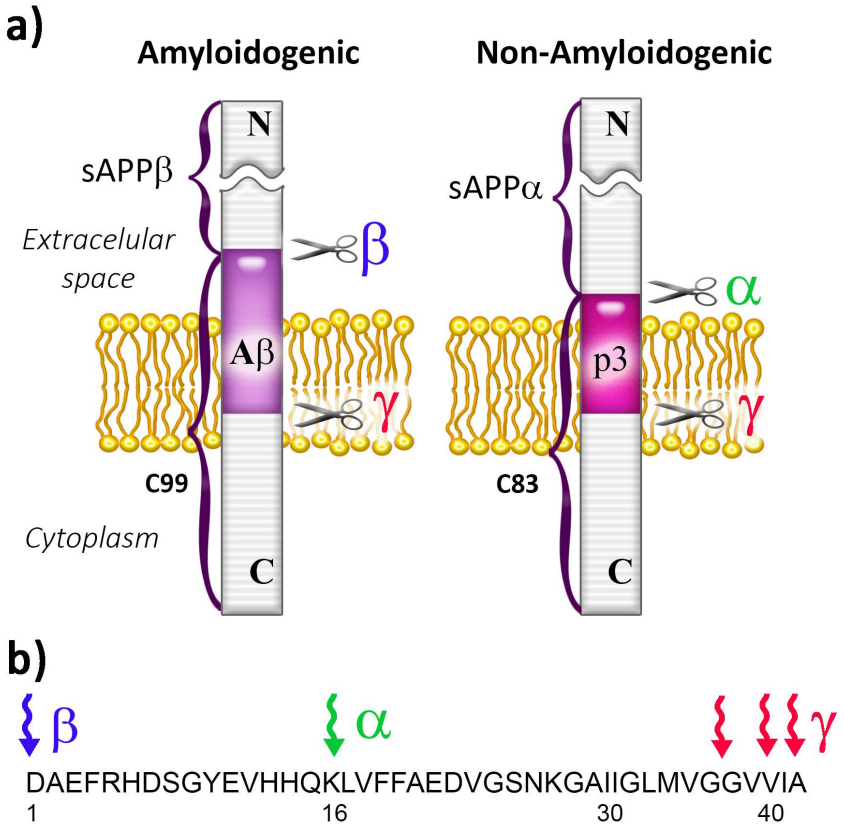


***Figure 1 a) Amyloidogenic and non-amyloidogenic processing of amyloid precursor protein,*** *α-secretase cleavage site is indicated in green; β-secretase cleavage site is indicated in blue; γ-secretase cleavage site is indicated in red;* ***b) Amino acid sequence of Aβ42***

Following amyloidogenic cleavage of APP, Aβ is released into the extracellular space with several possible outcomes. The peptide monomer may interact with other monomers and form a nucleus that gives rise to fibrillary structures, or it may form soluble oligomers that do not grow into fibrils (Srivastava, Pittman et al. 2019). Importantly, various Aβ aggregates can be removed by microglia (Lee and Landreth 2010) or degraded with neprilysin, a peptidase expressed by the presynaptic terminals of all neurons (Saido 2013). Aβ can also be transported across the blood-brain barrier to the blood plasma for further degradation (Deane, Bell et al. 2009).

In spite of vast amount of information regarding Aβ neurotoxicity, there are some proponents of the beneficial physiological roles of Aβ in the brain. These opinions are based on the following observations: Aβ has antimicrobial activity, plays a role in tumor suppression, general brain recovery after a head trauma and may protect the brain by intercepting viruses, toxic compounds etc. (Brothers, Gosztyla et al. 2018). These roles of Aβ were mainly suggested by analyzing mice where β-secretase had been genetically eliminated or suppressed - it was concluded that the absence of β-secretase was not beneficial for nervous system performance (Brothers, Gosztyla et al. 2018). Recovery after the spinal cord injury was slower in β-secretase deficient mice than in the control group (Pajoohesh-Ganji, Burns et al. 2014), or synaptic plasticity during memory formation was abolished when β-secretase had been partially suppressed (Lombardo, Chiacchiaretta et al. 2019). Consistently with the phenotype of β-secretase knock-out mice (Munro, Nash et al. 2016), a number of serious side effects, including worsening of cognitive capabilities were documented in clinical trials of β-secretase inhibitors, which were therefore terminated (Liu, Xie et al. 2019).

To conclude, the mechanisms of Aβ production are known in considerable detail; however, the role of this peptide in the brain has not been unequivocally established. Obviously, Aβ's physiological and non-physiological roles need further investigation.

## 3. Amyloid cascade hypothesis

In 1992 Hardy and Higgins proposed the „Amyloid cascade hypothesis" of AD (Hardy and Higgins 1992). The proposed cascade begins with Aβ accumulation in the brain and continues with the formation of tau tangles, accompanied with widespread neurodegeneration in the brain (Figure 2). This hypothesis was based on genetic studies of APP and the first published reports of Aβ toxicity. Several years later, discovery of mutations in presenilin and APP genes in the fAD patients supported the hypothesis (Hardy 1996). Several years later an extended version of the „amyloid cascade" was published, where Aβ oligomers rather than fibrils or monomers were regarded as the toxic species that cause neuronal death; however, no explanation was proposed for their origin in the brain (Hardy and Selkoe 2002).

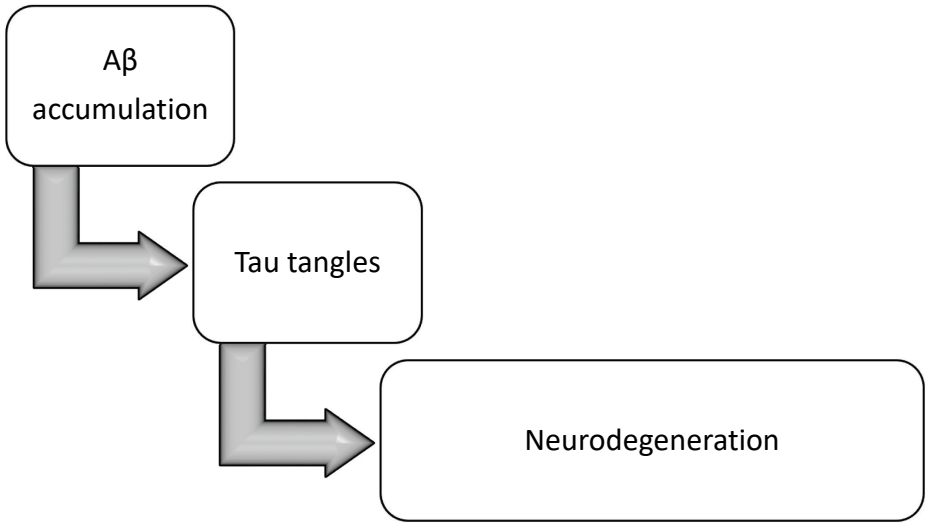


***Figure 2. Amyloid cascade hypothesis of AD.***

These days, the amyloid hypothesis is not universally accepted and its critics like to point out a number of inconsistencies. First, the amount of amyloid deposition does not correlate with the severity of the disease including memory loss (Kametani and Hasegawa 2018). Second, amyloid plaques have been also found in the brains of patients without a dementia (Higashi, Nishii et al. 2018). Third, rodent AD models with only fAD mutations in APP or presenilin genes do not develop tau tangles (Oakley, Cole et al. 2006). However, neurofibrillary tangles are formed in primary cultures following the expression of human tau isoforms (Jin, Shepardson et al. 2011) or in the induced pluripotent stem cell-based models following introduction of only fAD mutations (Muratore, Rice et al. 2014). Rapoport et al. showed that primary hippocampal neurons become more sensitive towards Aβ in the presence of hyperphosphorylated tau (Rapoport, Dawson et al. 2002). While the amyloid hypothesis is consistent with the data obtained from fAD patients, sAD, i.e. the prevalent from of the disease, does not fit well with the amyloid cascade hypothesis. For example, sAD is not accompanied by life-long overproduction of Aβ. The authors based their hypothesis regarding sAD on many independent studies, proposing that impaired Aβ clearance mechanisms induce accumulation of amyloid peptide into plaques with further enhancement of the cascade (Selkoe and Hardy 2016).

Analysis of AD biomarkers indicates that a decrease of Aβ levels in the CSF points to the peptide accumulation in the brain; and those are the first events occurring in the disease pathogenesis (Jack and Holtzman 2013). The cascade of events triggered by Aβ includes many pathological processes such as synaptic dysfunction, microglia activation and inflammation, oxidative stress and tau hyperphosphorylation (Selkoe and Hardy 2016).

Nonetheless, the amyloid cascade hypothesis is supported by many observations and studies; however, the ultimate cause of neurodegeneration as well as the exact role of Aβ in AD pathology remain unclear.

## 4. Neurodegeneration in AD

### 4.1. Amyloid fibrils and plaques

It has been long known that amyloid plaques consist of fibrillary amyloid peptides of different lengths with non-truncated and truncated N- or C-termini, e.g. Aβ3pE-42 with pyroglutamate at N terminus (Iwatsubo, Saido et al. 1996). Plaques can be divided into two types: cored plaques and diffuse plaques with various compositions and surroundings, e.g. diffuse plaques containing ApoE or cored APP positive plaques that are surrounded by APP-positive neurites (Thal, Capetillo-Zarate et al. 2006). The diffuse plaques do not stain with amyloid specific fluorescent dyes Thioflavin S or Congo red and do not co-localize with activated microglia or astrocytes. Cored neuritic plaques, in contrast, are Thioflavin S-positive and usually surrounded by dystrophic neurites and are therefore termed neuritic plaques (Thal, Capetillo-Zarate et al. 2006). In a recent study, Michno et al. showed that the core of AD patient plaques mostly consisted of Aβ40, but not Aβ42. N-terminally truncated pyroglutamated Aβ3pE-42 and Aβ11-42 peptides were present in the diffuse plaques (Michno, Nystrom et al. 2019). These findings suggest that N-terminal modifications of Aβ may induce the formation of new plaques since pyroglutamated peptide is more hydrophobic and aggregates faster than Aβ1-42 (D'Arrigo, Tabaton et al. 2009).

AD is similar to a prion disease, as it can spread and induce pathological transformations of Aβ (Jucker and Walker 2018), e.g. injection of AD brain extracts into the hippocampus of APP transgenic mouse induces amyloid deposition in the brain (Kane, Lipinski et al. 2000, Langer, Eisele et al. 2011).

An intriguing study was performed with „seeding" of Aβ fibrillization with the extracts from AD brain cortex (Qiang, Yau et al. 2017). „Seeding" means the ability of the preformed small fibrillary aggregates to initiate growth of new fibrils. Qiang et al. showed that the amyloid seeds extracted from different AD brain parts are highly heterogeneous and initiate fibrils with different morphology as revealed by TEM experiments and structure as identified with NMR, especially in the case of Aβ42 when compared to Aβ40. This observation demonstrates the diversity of amyloid intermediates even within one brain extract (Qiang, Yau et al. 2017). Thus, these data indicate that the appearance of pathological seeds might be a factor defining the onset of AD.

Another hypothesis associates amyloid aggregation into plaques with a head trauma, since traumatic brain injury has been identified as a risk factor for accelerating the age of AD onset by two or more years (Li, Risacher et al. 2016). After a head trauma, pro-inflammatory mediators are released, e.g. S100A9, that modulate Aβ aggregation (Wang, Klechikov et al. 2014, Wang, Iashchishyn et al. 2018).

There is growing evidence that already formed amyloid fibrils can serve as templates for Aβ monomers for increasing the number of new nucleation centers in a process termed secondary nucleation (Linse 2017, Linse 2019). Cohen et al. showed that toxic Aβ oligomers can be produced via secondary nucleation. Moreover, selective inhibition of the secondary nucleation reduced Aβ42 toxicity in cell lines and mouse brain slices (Cohen, Arosio et al. 2015). The idea of secondary nucleation is also consistent with fAD onset, since Yang et al. showed that some familial mutations of Aβ increase the rate of secondary nucleation or generation of oligomers (Yang, Meisl et al. 2018).

Metal ions are enriched in amyloid plaques: the concentrations of copper, iron and zinc in AD plaques exceed those in healthy brain tissue by more than twofold (Lovell, Robertson et al. 1998). The role of fibril-associated metal ions is still not known (Liu, Nguyen et al. 2019). Metal ions bound by plaques can be involved in generation of hydroxyl radicals; and accumulation of metals in plaques may cause an imbalance of metal ions in brain, e.g. reduce superoxide dismutase activity (Bayer, Schafer et al. 2003).

To conclude this section, both amyloid plaques and fibrils most likely have important roles in generation of Aβ toxic species and seeds and can cause changes in the metal ion homeostasis.

### 4.2. Aβ Oligomers

As an outcome from many studies with AD models and AD patient samples, the amyloid oligomers were identified as the main toxic species of amyloid peptide, although their origin is still not fully understood (Selkoe and Hardy 2016, Cline, Bicca et al. 2018).

To date, Aβ oligomers have been shown to induce synaptic dysfunction and reduce synaptic density, cause tau hyperphosphorylation and oxidative stress, disrupt $Ca^{2+}$ homeostasis, induce microglia activation, endoplasmic reticulum stress, inhibition of axonal transport, insulin resistance etc. (Viola and Klein 2015, Cline, Bicca et al. 2018). These effects of Aβ oligomers were studied using oligomers extracted from patient brain or CSF samples and *in vitro* made oligomers. There was strong criticism regarding the use of detergents to induce Aβ oligomerization and artificial production of oligomers for *in vitro* and *in vivo* studies (Benilova, Karran et al. 2012), thus the effects of Aβ oligomers described in many studies can be overestimated.

Cline and co-authors divided Aβ oligomers into two types in a recently published review: 1) oligomers derived from Aβ monomers immediately after APP processing and associated with memory impairment and 2) oligomers derived from amyloid plaques or plaque seeds and not affecting memory (Cline, Bicca et al. 2018). However, such division

of oligomers is based on data from various AD mouse models and there is no clear evidence that the same types of oligomers are present in AD brain. Importantly, it was shown that the plaque to oligomer ratio in the brain allows to distinguish between demented and non-demented patients (Esparza, Zhao et al. 2013). Thus, Hardy and Selkoe proposed that diffuse plaques can „hold" oligomers until the disease onset, when oligomers diffuse from the plaques into neighboring brain compartments, thereby causing neurodegeration (Selkoe and Hardy 2016).

Importantly, the toxic properties of monomeric, oligomeric and fibrillar forms of Aβ are quite variable when comparing results from separate studies (Cavallucci, D'Amelio et al. 2012). Therefore, it remains to be determined whether the toxic oligomeric species are generated by soluble monomeric Aβ, by amyloid fibrils, or by a combination of monomers with fibrils.

### 4.3. Tau protein in AD pathology

One of the main hallmarks of AD is the presence of hyperphosphorylated tau protein that forms intracellular, insoluble neurofibrillary tangles. Tau is expressed mainly in neurons, where it associates with microtubules (Guo, Noble et al. 2017). In mature neurons, tau is located mainly in axons and stabilizes microtubules (Guo, Noble et al. 2017). The most important posttranslational modification of tau is phosphorylation, because it causes aggregation of tau and subsequent destabilization of microtubules. While tau has 85 phosphorylation sites, it can also be acetylated, glycosylated etc. (Tapia-Rojas, Cabezas-Opazo et al. 2019). Tau glycosylation has been proposed to be protective against hyperphosphorylation of tau and subsequent tangle formation (Liu, Iqbal et al. 2004). Except for frontotemporal dementia caused by certain mutations of microtubule-associated protein, tau pathology is usually accompanied by accumulation of amyloidogenic protein such as Aβ, α-synuclein or huntingtin (Guo, Noble et al. 2017). Recent studies have shown that tau is not only localized intracellularly, but it can also be secreted into extracellular space, and thereby tau pathology is also propagated throughout the brain (Yang and Wang 2018, Pernegre, Duquette et al. 2019). The consequent cascade of events in AD indicates that formation of tau tangles is a secondary event after amyloid deposition suggesting that Aβ can induce tau hyperphosphorylation (Selkoe and Hardy 2016). Importantly, the presence of tau tangles correlates with the disease progression (Braak and Braak 1991, Jack and Holtzman 2013). Taking together, tau hyperphosphorylation contributes to AD progression.

### 4.4. Autophagy impairment in AD

A considerable amount of literature has been published regarding impairment of autophagy as a potential pathological mechanism underlying AD (Funderburk, Marcellino et al. 2010, Uddin, Stachowiak et al. 2018). Autophagy is a physiological process responsible for removal of impaired organelles and other long-lived cytoplasmic structures. Autophagosome is a vesicle formed around an organelle or a large supramolecular structure. Autophagosomes are subsequently delivered to lysosomes for degradation (Hara, Nakamura et al. 2006). The transport of structures destined for degradation is highly important for postmitotic cells e.g. neurons, where autophagosomes reside mostly in dendrites or axons and lysosomes locate mainly in the cell body. Thus, an efficient transport of autophagosomes from the periphery to the cell body is vital for neuronal health (Nixon 2007). Fusion of autophagosomes with lysosomes is a microtubule-dependent process. Consequently, hyperphophorylated tau might impair axonal traffic and lead to the accumulation of autophagic vesicles containing Aβ (Nixon, Wegiel et al. 2005).

In case of AD, several authors have proposed that endosomal-lysosomal pathway acts as a regulator of APP processing (Grbovic, Mathews et al. 2003, Pasternak, Bagshaw et al. 2003). Studies in APP/PS1 mouse model showed that autophagic vacuoles include γ-secretase complex and APP (Yu, Cuervo et al. 2005). Also, it was shown that failure to deliver autophagic vacuoles to the lysosomes in cell bodies for the degradation results in abnormal accumulation of amyloid in neurites (Funderburk, Marcellino et al. 2010). Pickford et al. found a relationship between Aβ overproduction and beclin 1, a component of a lipid kinase complex that is essential for autophagy. The APP transgenic mice crossed with beclin 1 deficient mice are characterized by reduced autophagy and accumulation of abnormal lysosomal structures. In contrast, when beclin 1 was overexpressed to enhance autophagy, extracellular and intracellular levels of Aβ were reduced. Pickford et al. also found that levels of beclin 1 were decreased in the brain samples of AD patients (Pickford, Masliah et al. 2008). It is also thought that hyperphosphorylated tau accumulation and autophagy are also connected in AD.

**4.5. Mitochondrial cascade (primary vs secondary)**

Mitochondrion is an organelle responsible for cellular energetics. In neurons mitochondria supply synaptic processes with energy (Flannery and Trushina 2019). In recently published review on the role of mitochondria in AD (Swerdlow 2018), author proposed that there is a primary mitochondrial cascade in AD: failure of the respiratory chain functioning causes changes in neurotransmitter release, overproduction of ROS, cell stress responses that lead to altered APP homeostasis and Aβ accumulation. Alternatively, the author also suggested the possibility of a secondary mitochondrial cascade, which is consistent with the amyloid cascade hypothesis and changes in mitochondria occurring downstream of Aβ accumulation (Swerdlow 2018). The primary mitochondrial cascade hypothesis is strongly supported by the observation that aerobic glycolysis is increased in the same brain regions where senile plaques are located (Vlassenko, Vaishnavi et al. 2010); suggesting that changes in cellular energy metabolism may in fact induce Aβ production and accumulation. Nevertheless, there is also a possibility that Aβ accumulation causes failure of mitochondrial bioenergetics: decreased aerobic glycolysis in the brain, which usually accompanies with aging, resulting from amyloid burden correlates with increased deposition of tau (Vlassenko, Gordon et al. 2018). However, changes in glucose metabolism have been identified in not just sAD patients but also elderly people without dementia (Goyal, Vlassenko et al. 2017). This data may support both primary and secondary mitochondrial cascades as mechanisms underlying AD pathogenesis.

To conclude, there are still many gaps in the explanation of what might trigger AD.

**4.6. Which type of neurons degenerate in AD?**

Since mid-1970s it has been known that significant degeneration of cholinergic neurons occurs in AD patients (Davies and Maloney 1976). The authors studied activity of enzymes, which participate in neurotransmitter synthesis in AD patients and healthy controls. The finding of reduced activity of choline acetyltransferase (ChAT) in AD brain leaded to cholinergic hypothesis. Cholinergic transmission regulates associative learning, memory, sensory functions etc. (Ahmed, Knowles et al. 2019). It was found that the extent of failure in the cholinergic transmission correlates with the severity of symptoms, similarly to the presence of neurofibrillary tangles (Braak and Braak 1991, Vana, Kanaan et al. 2011). A center of cholinergic innervation, the nucleus basalis of Meynert (the part of the basal forebrain that innervates the cortex) undergoes neurodegeneration in AD

(Hampel, Mesulam et al. 2018). Amyloid burden correlates with neuronal loss in the basal forebrain and decreased ChAT activity correlates with increased abundance of Aβ as revealed by postmortem studies (Hampel, Mesulam et al. 2019). Treatment of AD patients with cholinesterase inhibitors improves cognitive functioning and memory by increasing the availability of acetylcholine (ACh) in synapses (Schneider, Mangialasche et al. 2014). However, the reason why cholinergic system deteriorates first is not explained by the cholinergic hypothesis of AD.

There is evidence that noradrenergic neurons located mainly in the locus coeruleus (LC) also degenerate in AD and may have a role in the disease onset (Gannon, Che et al. 2015). Increased availability of noradrenalin was shown to improve cognitive functions in APP transgenic mice whereas noradrenalin depletion caused functional impairment of microglia and an increase in the Aβ load (Heneka, Nadrigny et al. 2010). As reviewed by Gannon et al., degeneration of noradrenergic neurons can exaggerate certain neuropathological changes characteristic of AD, such as amyloid burden or abnormalities associated with the tau protein, at least in mouse models of AD (Gannon, Che et al. 2015). Noradrenergic neurons of LC degenerate in AD patients even to a greater extent than cholinergic neurons in the nucleus basalis (Zarow, Lyness et al. 2003).

Moreover, authors of a recent review proposed that the loss of dopaminergic neurons of the ventral tegmental area (VTA) and LC is an important event causing memory loss in AD patients (Krashia, Nobili et al. 2019). VTA consists mainly of tyrosine hydroxylase (TH) positive neurons that innervate the prefrontal cortex, hippocampus etc. (Gasbarri, Verney et al. 1994). VTA was identified as the first region to degenerate in the APP transgenic mice Tg2576, thus explaining the memory impairment found in this model (Nobili, Latagliata et al. 2017). Therefore, Krashia et al. hypothesized that dopaminergic dysfunction leads to cognitive dysfunction and other symptoms of AD such as depression, apathy and disturbances in circadian rhythms (Krashia, Nobili et al. 2019); however, the vulnerability of VTA in AD patients has still not been thoroughly studied.

Although there is a clearly defined temporal sequence of the degeneration of different brain parts in AD (Braak and Braak 1991, Serrano-Pozo, Frosch et al. 2011), we still do not know why certain types of neurons are more vulnerable than other ones.

### 4.7. Risk factors for AD

The first genetic risk factors identified for development of sAD was the presence of the apolipoprotein E (ApoE) ε4 allele. ApoE regulates lipid metabolism in the brain and may have a significant role in deposition and clearance of Aβ (Karch and Goate 2015). Another genetic risk factors for developing AD are certain variants of the triggering receptor expressed on myeloid cells-2 (TREM2)-, that are expressed mainly in microglia and are responsible for normal immune functions in the brain (Gratuze, Leyns et al. 2018). Interestingly, TREM2 is the putative receptor for ApoE4 in microglia, and their interactions may have a significant role in the onset of AD (Wolfe, Fitz et al. 2018). It is suggested that the ApoE and TREM-2 interaction might initiate microglia to switch from homeostatic to neurodegenerative microglia on the transcriptional level (Gratuze, Leyns et al. 2018).

The onset of AD eventually depends on a combination of a number of risk factors including aging, high blood pressure, traumatic brain injury, type II diabetes, physical inactivity (Xia, Jiang et al. 2018). Recently, the impaired gut microbiome and its metabolites were shown to trigger inflammation in the central nervous system, leading to alterations in the levels of neurotransmitters through the vagal afferent fibers (Bostanciklioglu 2019). There is also evidence that infections, such as herpes simplex virus (HSV) may play a significant role in the disease onset: reactivation of HSV with

subsequent secretion of type M anti-HSV immunoglobulins was found to increase the risk for AD (Lovheim, Gilthorpe et al. 2015). However, in the latter study no possible mechanism connecting HSV infection with neurodegeneration was proposed.

In summary, AD is a multifactorial disease where a number of different pathological events, genetic and environmental factors have been implicated as likely causes. However, there is a lack of the unequivocal understanding: how these factors cause neurodegeneration in AD and in which temporal sequence.

### 4.8. Potential protective factors against AD

Epidemiological studies have identified the following factors, which can make elderly people less vulnerable to AD: social activity and intellectual stimulation, regular physical activity, vitamin-rich and omega-3 fatty acid containing diet (Stozicka, Zilka et al. 2007). As an example, physical activity has been shown to increase the levels of brain derived neurotrophic factor (BDNF) that supports neuronal cell survival and promotes non-amyloidogenic APP processing in the AD mouse model (Nigam, Xu et al. 2017). There was also evidence that non-steroidal anti-inflammatory drugs and cholesterol-lowering drugs or statins may lower the risk to get AD, however, estimated effects on AD patients in clinical trials were mostly symptoms relieving (Stozicka, Zilka et al. 2007).

### 4.9. Treatment strategies for AD

Symptoms of AD, such as dementia and impairment of cognitive functions, appear many years after the beginning of amyloid accumulation and are accompanied by other neuropathological features, such as the formation of tau tangles, decreased volume of hippocampus, changes in glucose metabolism of the brain etc. (Masters, Bateman et al. 2015). To date, the only AD drugs available are the NMDA receptor antagonist (Memantine), or cholinesterase inhibitors (Donepezil, Rivastigmine, Galantamine), which only relieve the symptoms of AD by targeting the neurotransmitter systems affected by the disease (Khoury, Patel et al. 2017, Liu, Xie et al. 2019). In addition, norepinephrine transporter inhibitors are also used to improve behavioral and mood disorders in AD patients (Gannon, Che et al. 2015).

There are several drug-development strategies for AD proposed: disease-modifying and system-reducing or symptomatic (Cummings, Lee et al. 2019). Disease-modifying drug candidates are based on the amyloid cascade hypothesis: a number of anti- Aβ monoclonal antibodies-based drugs such as aducanumab and crenezumab have been designed to enhance Aβ clearance (Liu, Xie et al. 2019). Unfortunately, clinical trials of nearly all anti-amyloid-based strategies have failed so far (Panza, Lozupone et al. 2019). There are also other disease-modifying drugs candidates, such as tau aggregation inhibitors in clinical trials (Cummings, Lee et al. 2019). System-reducing strategies are targeted to reduce AD symptoms and enhance cognition, where cholesterol-lowering, anti-inflammation, neuroprotective drugs are used (Cummings, Lee et al. 2019). Since mechanisms of AD pathogenesis have turned out to be more complicated than expected, several combined therapies have also been proposed (Cummings, Tong et al. 2019). Therefore, combinations of disease-modifying drugs, e.g. amyloid aggregation inhibitors with ibuprofen or other anti-inflammatory drugs are currently in clinical trials. Combinations of symptom-relieving drugs are also being tested (Cummings, Tong et al. 2019).

Overall, it is difficult to cure the disease with unknown cause of onset; however, there is still a hope for drugs or combinational therapies that might prevent neurodegeneration.

## 5. *In vitro* studies of Alzheimer's disease

AD is a slowly progressing neurodegenerative disease where changes in the brain appear years before the onset of symptoms and the actual diagnosis. Therefore, studying AD mechanisms in animal models and creating appropriate *in vitro* models has been challenging. *In vitro* and *in vivo* studies of AD are often problematic since even small differences between the various models used complicate interpretation of experimental data and drawing of meaningful conclusions. As an example, the APP/PS double transgenic mouse, does not developed tau tangles unless a mutated tau gene has been introduced into the genome (3xTg mouse) (Mullane and Williams 2019). Importantly, the simplest and well controlled *in vitro* experiments have made small but incremental contributions to our understanding of the mechanisms of neurodegeneration in AD.

### 5.1 Amyloid beta fibrillization *in vitro*

Fibrillization of peptides is a process where monomers form intermolecular β-sheet structures that grow further into long stable fibrils of certain morphology (Tycko 2014). Creating a model of Aβ fibrillization *in vitro* allows performing this process in controlled conditions. Fibrillization *in vitro* follows a sigmoidal curve (Figure3). Initial phase is a lag-phase or primary nucleation, which can be either homogeneous or heterogeneous (Srivastava, Pittman et al. 2019). In the case homogeneous nucleation, Aβ is aggregating spontaneously in the solution containing monomers at appropriate concentration. Primary nucleation is heterogeneous if the formation of Aβ aggregates occurs on surfaces, e.g. cell membranes or phase boundaries (Srivastava, Pittman et al. 2019). The middle part of fibrillization is dominated by the elongation phase when propagation of nucleation centers continues. This phase is induced via fragmentation of the fibrils in the solution, creating new elongation centers (Knowles, Waudby et al. 2009), and via secondary nucleation, whereby fibrils act as templates for the creation of new nucleation centers or seeds (Linse 2017, Srivastava, Pittman et al. 2019). Finally, a plateau or stationary phase is achieved when most of the monomeric peptides have aggregated.

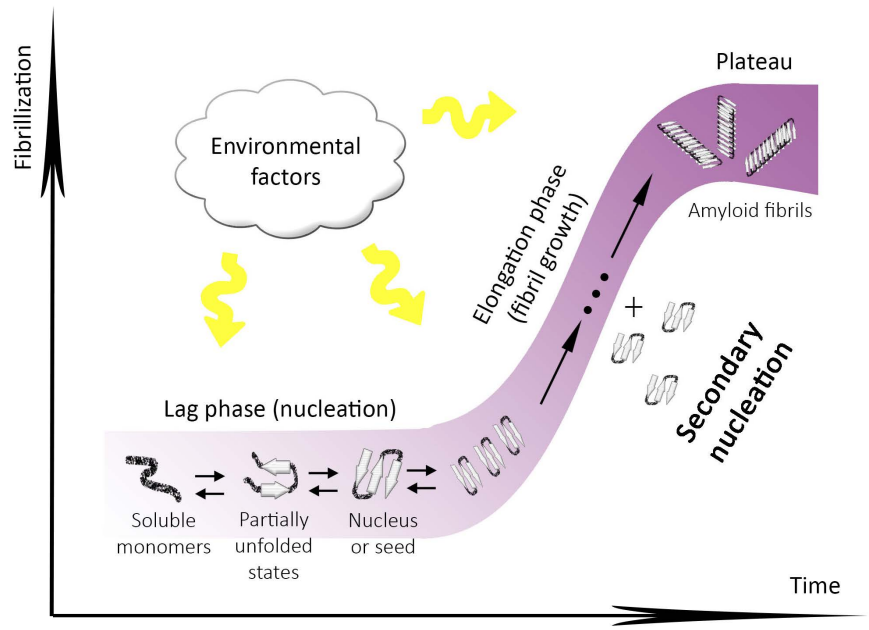


***Figure 3. Schematic representation of amyloid β fibrillization.***

The simplest experiment for studying Aβ aggregation and its fibrillization is measuring aggregation kinetics in a solution by using amyloid specific dyes like Thioflavin T. To optimize the conditions and accelerate the process of fibrillization, experiments can be performed with the agitation using a magnetic stirrer (Karafin, Palumaa et al. 2009). Alternatively, Aβ fibrillization can be studied under quiescent conditions, i.e. without a constant agitation; however, under such conditions the process can take days (Tiiman, Noormagi et al. 2013, Cukalevski, Yang et al. 2015). Fibrillization *in vitro* can be influenced by various environmental factors such as temperature, pH of the solution; or presence of inhibitors, such as polyphenols, sterols, peptides, antibodies (Giorgetti, Greco et al. 2018). The generation of fibrillary seeds is crucial for initiation of fibrillization *in vitro*, as previously described; however, what promotes this process *in vivo* is still not well understood. Matsuzaki et al. studied heterogeneous nucleation on cell membranes and effects of the membrane composition on the initiation of Aβ fibrillization. They showed that ganglioside clusters, components of the cellular membrane, form centers for fibril growth (Matsuzaki 2014). In addition to this, other amyloidogenic proteins can also modulate Aβ fibril formation, such as S100A9, a pro-inflammatory peptide secreted by neurons and microglia (Wang, Klechikov et al. 2014). It was shown that metal ions $Cu^{2+}$ and $Zn^{2+}$ could promote Aβ aggregate formation; however, those aggregates are mainly amorphous, which undergo further transformation into fibrils (Tougu, Tiiman et al. 2011, Srivastava, Pittman et al. 2019).

Since the prevalent form of Aβ is Aβ40, it was hypothesized that this shorter peptide may have a protective effect by interfering with aggregation of the more amyloidogenic and toxic Aβ42 peptide (Murray, Bernstein et al. 2009, Terrill-Usery, Colvin et al. 2016). Kuperstein et al. showed that small changes in the peptide ratios affect kinetic and toxic properties of mixed Aβ aggregates when tested on primary cultures (Kuperstein, Broersen et al. 2010). However, a more recent and detailed study showed that Aβ40 and Aβ42 monomers could promote the formation of fibrillary seeds and the formed fibrils were homomolecular (Cukalevski, Yang et al. 2015). Alternatively, there is an evidence that peptides form interlaced fibrils, which means that Aβ40 and Aβ42 β-strands alternate with each other (Gu and Guo 2013). It is difficult to determine whether the processes described can occur in the brain since the amyloid species extracted from plaques are diverse and Aβ40 can influence fibrillization or oligomerisation of Aβ42 and vice versa (Cabrera, Mathews et al. 2018).

*In vitro* studies of Aβ fibrillization can help to understand the amyloidogenic properties of the peptides and propose new hypotheses to suppress amyloid cascade.

### 5.2 Primary neuronal and organotypic slice cultures in AD research

Rodent-derived primary neuronal cultures are widely used in AD studies as well as neuroscience in general (Gordon, Amini et al. 2013). Primary neurons are advantageous since they constitute a brain-derived cell type, which, unlike non-primary cells, do not require further differentiation and overexpression of the main neuronal markers such as TUJ, MAP2, synapsin. There are also a number of disadvantages: firstly, primary cultures are not immortal, and thus the cell number obtained is limited; secondly, it is always important to prove the absence of non-neuronal cells if mixed cultures should be avoided (Gordon, Amini et al. 2013).

Rodent *ex vivo* models termed organotypic brain slice cultures are also widely used in AD studies (Humpel 2015). On the one hand, this *in vitro* model is more complex than the 2D models and can be prepared from aged animals, a feature that is particularly important for AD studies. On the other hand, since it is a rodent-based model, organotypic

brain slices could be derived only from appropriate transgenic rodent models of AD (Jang, Kim et al. 2018).

### 5.3 Stem cell-derived AD models

Since the arrival of reprogramming of somatic cells into pluripotent stem cells in 2006, a number of stem cell-based AD models have been developed by using of various strategies (de Leeuw and Tackenberg 2019). Patient-specific approach entails creation of induced pluripotent stem cells (iPSC) from somatic cells of the AD patient with subsequent differentiation toward mature neurons of the type affected in AD disease (e.g. basal forebrain cholinergic neurons) and comparing these to the neurons derived from the cells of healthy controls. Monogenic or isogenic approach means that somatic cells from AD patient or healthy individual are reprogrammed to stem cells and the iPSC cells obtained are edited in accordance with the aims of an experiment, e.g. by introduction of sAD risk gene variants or editing of fAD gene variants etc. On the last stage, genetically edited stem cells are differentiated and compared to healthy control or non-edited AD control cells (de Leeuw and Tackenberg 2019). iPSC cells derived from fAD patients were used to create a 3D model to study the mechanisms of AD pathology involving amyloid overproduction, oxidative stress, ER-stress and tau-hyperphosphorylation (Choi, Kim et al. 2014, de Leeuw and Tackenberg 2019).

To conclude, the involvement of stem cell-based models in AD research is a great advance that has a potential in further studies to reveal mechanisms of neurodegeneration.

### 5.4 Immortalized cell lines in AD studies

The first choice for preliminary studies of drug candidates are usually immortalized cancer cell lines because the cells can be easily cultured at an amount needed for experiments. Immortalized cells are easy to use and have many characteristics of the tissue where they have been derived from. These affordable models enable to carry out toxicity experiments and elucidate pathological mechanisms on cellular level.

The most popular rodent immortalized cell lines in AD studies are Neuro2A and PC12. Neuro2A is a mouse neuroblastoma cell line, which is widely used in toxicity studies (Hong, Maezawa et al. 2007, Manzoni, Colombo et al. 2011). Neuro2A cells can be differentiated into neuron-like cells using various agents such as retinoic acid (RA), dibutyryl cyclic adenosine monophosphate (dbcAMP), bone morphogenetic protein 4 (BMP4), transforming growth factor-β 1 (TGFβ 1) (Tremblay, Sikorska et al. 2010). PC12 is rat pheochromocytoma cell line that is usually used after differentiation with neuronal growth factor (NGF) toward a noradrenergic phenotype (Greene and Tischler 1976, Wang, Jiang et al. 2018). However, there is also evidence that undifferentiated PC12 can be used if treated with autophagy inhibitors that can serve as a model for AD since inhibition of autophagy leads to impaired amyloid clearance (Tian, Lin et al. 2019).

Immortalized cell lines of human origin are also used in AD studies. The most common are human neuroblastoma cell lines e.g. SK-N-SH cells, which are used in Aβ toxicity studies (Pinkaew, Changtam et al. 2016) or SH-SY5Y cells. The main disadvantage of the above mentioned cell lines is the lack of many characteristics for mature neurons, i.e. the cell type that degenerates in AD. To resolve this problem, the cultures can be differentiated with various agents, which inhibit cell proliferation and induce a more neuron-like phenotype. The SK-N-SH cell line can be differentiated with RA and 12-O-tetradecanoylphorbol-13-acetate (TPA) toward a more neuron-like phenotype (Niewiarowska-Sendo, Patrzalek et al. 2015). Another possibility for creating more relevant cellular model of AD is to induce overexpression of a protein of interest, e.g. to use

SH-SY5Y cells stably expressing mutated PS1 as a model for fAD (Tong, Lee et al. 2016). There is also a possibility to introduce tau hyperphosphorylation promoting mutations (Loffler, Flunkert et al. 2012).

The toxic effects of Aβ on the neurons can be elucidated when comparing toxicity on cell cultures of different origins. For example, sensitivity toward Aβ has been estimated in neuroblastoma cells *vs* embryonic kidney cells 293 or human IMR-32 neuroblastoma cells (Saikia, Pandey et al. 2019) or SH-SY5Y vs SK-N-BE(2)-C human neuronal cell lines or C6 mouse glial cell line (Pacifico, Piccolella et al. 2014).

A recent and quite sophisticated approach for studying AD was developed by the biotechnology company ReNeuron's, who derived and immortalized neural progenitor cells from human fetal mesencephalon (midbrain). This cell line, termed ReNcell VM, can be differentiated into neurons and glial cells and was developed to the 3D culture-based model exhibiting features of AD pathology (Choi, Kim et al. 2014). To this end, the authors introduced APP and/or PS1 fAD mutations to the ReN cells' genome, differentiated the cells into mature neurons for several weeks to be able to observe Aβ formation and tau hyperphosphorylation. Compared to mouse-based models, this model is advantageous for having both hallmarks of AD present without requiring the introduction of tau mutations (Choi, Kim et al. 2016).

To conclude, immortalized cell lines are widely used in toxicological studies for drugs candidates or for revealing pathological AD-associated changes on the cellular level. Working with human cell lines is of crucial importance since AD is a human neurodegenerative disease (De Felice and Munoz 2016).

### 5.5 Differentiation routes and neuronal properties of SH-SY5Y

SH-SY5Y was obtained by sub-cloning of the SK-N-SH cell line, a human cell line derived from a bone marrow aspirate of a 4-year-old girl with metastatic neuroblastoma, which was heterogeneous since it included cells of neuronal, glial and intermediate phenotypes (Ross, Spengler et al. 1983, Macleod, Allsopp et al. 2001). The original SH-SY5Y cell line differs significantly from mature neurons: it lacks long neurites, a formation of neuronal networks, and has a low expression of neuron-specific markers e.g. β-III Tubulin (Agholme, Lindstrom et al. 2010). Non-differentiated SH-SY5Y cells express neuronal marker enzymes (tyrosine hydroxylase and dopamine-β-hydroxylase), several neurofilament proteins, opioid, muscarinic, and nerve growth factor receptors; they specifically take up norepinephrine and are therefore proposed to possess the properties of catecholaminergic and dopaminergic neurons (Biedler, Roffler-Tarlov et al. 1978, Xie, Hu et al. 2010). Due to these characteristics, this cell line was considered for deployment as a cellular Parkinson's disease model, where mainly dopaminergic neurons degenerate; however, this cell line is not exclusively dopaminergic (Xicoy, Wieringa et al. 2017). Importantly, non-differentiated SH-SY5Y cells express the shortest isoform of tau only, which is not involved in the formation of neurofibrillar tangles (Agholme, Lindstrom et al. 2010).

Treatment with a range of differentiating agents can change the SH-SY5Y cells' morphology and physiological properties to make them more neuron-like as well as to acquire more neuronal properties. There are a number of protocols for differentiation of SH-SY5Y neuroblastoma cell line and the most relevant ones are briefly reviewed below.

Treatment of SH-SY5Y cells with RA leads to enhanced outgrowth of neurites (Cheung, Lau et al. 2009), increases their survival by upregulating the antiapoptotic B-cell lymphoma 2 (BCL-2) protein (Itano, Ito et al. 1996), and also brings about changes in cellular $Na^+$ homeostasis (Toselli, Tosetti et al. 1996). Also, RA treatment increases tau

levels with transposition of phosphorylation sites involved in the stabilization of microtubules (Haque, Tanaka et al. 1999). The RA-based differentiation protocol can be used to create a model for studying Parkinson's disease due to the many features of dopaminergic neurons mentioned above (e.g. TH expression) (Xie, Hu et al. 2010, Xicoy, Wieringa et al. 2017). Importantly, some non-differentiated cells are still present in the culture, because they do not response to RA treatment (Nishida, Adati et al. 2008).

Treatment with RA reprograms SH-SY5Y cells to become responsive to neurotrophins including BDNF (Encinas, Iglesias et al. 2000). Sequential treatment of SH-SY5Y cells with RA and BDNF leads to the outgrowth of neurites, synaptogenesis and modulates positively their survival. There is also evidence that sequential treatment of RA and BDNF not only increases tau production, but also promotes its relocalizaton from the cell soma to the neurites, thereby strengthening the microtubules (Chen, Zhou et al. 2014). Additionally, the RA/BDNF treatment leads to the increased expression of vesicular acetylcholine transporter (VAChT), choline acetyltransferase (ChAT) and microtubule associated protein 2 (MAP2). Encinas et al. hypothesized that this protocol might induce cholinergic phenotype in SH-SY5Y; however, ChAT activity measurements did not reveal any significant difference between only RA treatment or combined RA/BDNF treatments (Encinas, Iglesias et al. 2000). Increased activity of acetylcholine esterase (AChE) and increased expression of cholinergic receptor was reported by Goldie et al., suggesting differentiation toward the cholinergic phenotype (Goldie, Barnett et al. 2014). A recently published study confirmed the acquisition of cholinergic phenotype following RA and BDNF treatment of SH-SY5Y cells since a microarray-based analysis revealed an increase in the expression of ChAT, AChE and important cholinergic receptors; however, the authors have applied RA and BDNF simultaneously (for 3 days) rather than sequentially (de Medeiros, De Bastiani et al. 2019). Another group described a SH-SY5Y-based 3D model for Parkinson's disease after differentiation of the cells with RA and BDNF, which led supposedly to a dopaminergic phenotype (Taylor-Whiteley, Le Maitre et al. 2019). Interestingly, treatment of SH-SY5Y cells with BDNF was shown to increase the expression of APP (Holback, Adlerz et al. 2005) and initiate non-amyloidogenic APP processing by α-secretase (Nigam, Xu et al. 2017).

Differentiation of SH-SY5Y cells with dbcAMP also induces morphological changes in cells, including neurite outgrowth and branching concomitant with an increased release of NA, consistent with the acquisition of a noradrenergic phenotype (Kume, Kawato et al. 2008). Intracellular esterases degrade dbcAMP to butyrate and monobutyryl cAMP (mbcAMP) that activate protein kinase A (PKA). Unlike RA/BDNF differentiation, dbcAMP treatment does not induce expression of MAP2 (Sanchez, Jimenez et al. 2004). It was subsequently shown that both butyrate and activation of PKA have important roles in dbcAMP-mediated differentiation of SH-SY5Y cells (Kume, Kawato et al. 2008). Several studies have demonstrated that dbcAMP increases the expression of APP also in NG108-15 cells and in rat primary cultures (Shekarabi, Bourbonniere et al. 1997, Sagy-Bross, Kasianov et al. 2015), implying that the effects of dbcAMP on the expression of APP apply across several different cellular contexts.

TPA is a biologically active phorbol ester affecting cell growth and differentiation via protein kinase C (PKC) activation (Encinas, Iglesias et al. 2000). During differentiation by TPA, SH-SY5Y cells undergo morphological changes, discontinue replication and enter quiescence. Sequential exposure of SH-SY5Y cells to RA and TPA induces a 3-fold increase in TH, a 4-fold increase in dopamine transporter (DAT), a 3-fold increase in dopamine receptor 2 and a 6-fold increase in dopamine receptor 3 levels compared to the untreated

cells (Presgraves, Ahmed et al. 2004, Looyenga, Resau et al. 2013). All these facts imply that SH-SY5Y cells adopt a dopaminergic cellular phenotype in response to RA and TPA treatment. At the same time, Pahlman et al. showed that TPA drives differentiation toward noradrenergic phenotype (Pahlman, Ruusala et al. 1984).

Staurosporine, a non-specific protein kinase inhibitor, is able to induce differentiation of SH-SY5Y cells in a serum-free medium (Prince and Oreland 1997). Differentiation with staurosporine was shown to induce catecholaminergic phenotype with increased production of noradrenalin and upregulation of the expression levels of related genes (Filograna, Civiero et al. 2015). Another protocol used silica-ε-polycaprolactone-nanoparticles (designed to modulate neuronal differentiation) to guide the differentiation of SH-SY5Y cells into dopaminergic neurons, as evidenced from increased TH expression and decreased expression of choline transporter 1 (ChT1) (Wiedmer, Ducray et al. 2019).

Treatment of SH-SY5Y cells with insulin-like growth factor (IGF) also induces neurite outgrowth with more neuron-like phenotype (Kim, Leventhal et al. 1997, Dwane, Durack et al. 2013). In addition, 17 beta-Estradiol ($E_2$), 3β-hydroxy-5-cholestene (cholesterol) accompanied with RA treatment were also shown to direct differentiation the cells toward neuron-like phenotype (Teppola, Sarkanen et al. 2016).

Coating of the cell plates to induce or support differentiation of the cells is also widely used (Nishida, Adati et al. 2008, Dwane, Durack et al. 2013, Korecka, van Kesteren et al. 2013). Algholme et al. tested several cell substrate-coating variants for SH-SY5Y cells for their capacity to induce neurite outgrowth, such as extracellular matrix, collagen as well as various agents including vitamin $D_3$, NGF, BDNF (Agholme, Lindstrom et al. 2010). There are also protocols to induce differentiation of neuroblastoma using the growth medium normally used for human neural stem cells with the addition of RA (Yang, Wang et al. 2016).

To conclude, the protocols reviewed in current section promote more neuron-like phenotypes of SH-SY5Y cells with the changes in the relevant neuronal marker expression and the synthesis of different neurotransmitters; however, a consensus about the neuronal properties of differentiated cells has not been reached.

## 6. Amyloid beta toxicity studies in SH-SY5Y cell line

Human neuroblastoma SH-SY5Y cell line is the most widely used *in vitro* neuronal model for studies of Aβ toxicity. A number of seminal publications using SH-SY5Y cell line for this purpose are listed in Table 1.

***Table 1 Summary of published Aβ toxicity studies using SH-SY5Y cells***

| Differentiation | Aβ origin and solvent | Aβ aggregation state | Test for toxicity | Aβ concentration | Incubation time | Main result | Author, year |
|---|---|---|---|---|---|---|---|
| **RA with serum composi-tion changes** | Aβ25-35, NA | fibrils | CellTiter GLO | 25µM | 24h | viability 50% | Seyb et al. 2008 |
| **none** | Aβ25–35, Sigma | oligomers | MTT | 30µM | 24h | viability 50% | Xiao et al. 2013 |
| **none** | Aβ25 – 35, NA | fibrils | MTT | 20µM | 24h | viability 60% | Chi et al. 2013 |
| **none** | Aβ25 – 35, NA | fibrils | MTT | 10µM | 48h | viability 50% | Zeng et al. 2014 |
| **none** | Aβ25-35, Bachem | NA | MTT | 20µM | 24h | viability 40% | Kim et al. 2014 |
| **N-12 neuro-blastoma growth factor** | Aβ25–35, (American Peptide Company) | pre-aggregated | CellTiter Glo (ATP) | 50µM | 48h | viability 80% | Soumyanath et al. 2014 |
| **RA** | Aβ25–35, Sigma | pre-aggregated | MTT | 20µM | 24h | viability 80% | Zhang et al. 2017 |
| **none** | Aβ25–35, Sigma | pre-aggregated | MTT | 20µM | 16h | viability 80% | Liu et al. 2017 |
| **none** | Aβ1-42*, Aβpy3-42; AnaSpec | oligomers | MTT | 100nM | 72h | viability 54% for Aβpy3-42; 74% for Aβ1-42 | Galante et al. 2012 |
| **none** | Aβ42, Bachem | NA | MTT | 5µM | 24h | viability 45% | Milton et al. 2012 |

**Table 1 continued I**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **none** | Aβ42*, American Peptide Company | oligomers | MTT | 10µM 20µM | 24h | viability 75%/55% | More et al. 2013 |
| **none, fibronectin coated plates** | Aβ42, Funakoshi | NA | MTT | 15µM | 24h | 55-60% viability | Villareal et al. 2016 |
| **1% serum deprivation** | Aβ42, Biochem | oligomers | MTT | 1-1.5µM | 24h | viability 50% | Lin et al. 2017 |
| **RA** | Aβ42**, Aβ40**, Anaspec | pre-aggregated | MTT | 30µM | 72h | viability 60% | De Lorenzi et al. 2017 |
| **RA** | Aβ42, Peptide Institute Inc. | oligomers | MTT | 2.5µM | 20h | viability 50-60% | Oguchi et al. 2017 |
| **none** | Aβ42*, Biopeptide | oligomers | MTT | 10µM | 24h | 80% viability | Zatsepina et al. 2018 |
| **none** | Aβ42*, APExBIO | pre-aggregated fibrils | MTT | 20µM | 24h | viability 50% | Haris Omar et al. 2019 |
| **RA/BDNF** | Aβ42, Sigma-Aldrich Chemical Co | pre-aggregated, oligomers | MTT | 2.5µM | 24h | viability 50% | Singh et al. 2017 |
| **RA/BDNF** | Aβ42* Abcam | oligomers | MTT | 0.1nM | 24h | 55% viability | Medeiros et al. 2019 |

| Table 1 continued II | | | | | | | |
|---|---|---|---|---|---|---|---|
| **RA** | Aβ42*, rPeptide | oligomers, fibrils | LDH release | 5µM | 48h | viability 90% for fibrills, 82% for oligomers | Garzon et al. 2007 |
| **none** | Aβ1-42, Bachem | fibrils | WST-8 | 20µM | 48h | viability 80% | Chonpathom-pikunlert et al. 2011 |
| **none** | Aβ1-42, rPeptide | prefibrillar | CytC release | 10µM | 1h | cytC release 60% | Camilleri et al. 2013 |
| **none** | Aβ40**, Aβ42**, AnaSpec | monomer | peptide uptake | 1µM | 24h | Aβ42 uptake is twice as high for Aβ40 | Wesén et al. 2017 |
| **none** | Aβ42, Sigma | pre-aggregated | Trypan blue | 20µM | 24h | viability 50% | Hwang et al. 2017 |
| **none** | Aβ42, Anaspec | fibrils | Cell-Counting Kit, WST-8 | 10µM | 48h | viability 80% | Ni et al. 2017 |
| **RA** | Aβ40*, Aβ42*, rPeptide | oligomers | Plaque assay, p-tau | 0.5, 1 or 10µM | 24h | no p-tau increase | Mokhtar et al. 2018 |
| **RA/BDNF** | MetAβ42** recombi-nant | monomers | Presto Blue | 7µM | 72h | viability 50% | Cristóvão et al. 2018 |

*„NA" - not available; Aβ42 is Aβ1-42; Aβ40 is Aβ1-40; p-tau – hyperphosphorylated tau; asterix*- DMSO used as solvent; **-alkaline solvent used; absence of an asterix means neutral solvent e.g. water or PBS; yellow shadowing indicates articles, where only MTT test was used to estimate toxicity.*

Most authors optimize cell-culturing conditions, differentiation protocols and peptide preparation techniques before implementing them for the actual experiments. SH-SY5Y cells are mainly used without prior differentiation (Chonpathompikunlert, Yoshitomi et al. 2011, Galante, Corsaro et al. 2012, Camilleri, Zarb et al. 2013, Chi, Wang et al. 2013, Wesen, Jeffries et al. 2017). The composition of the medium used in the mentioned

studies varies. American Type Culture Collection (ATCC) recommends growing the cells in a mixture comprising 50% DMEM and 50% Ham's F12. The studies listed in Table 1 used either DMEM, DMEM replaced with neurobasal medium or RPMI. Xicoy et al. reviewed the use of SH-SY5Y cells as a cell culture model for Parkinson's disease studies (Xicoy, Wieringa et al. 2017) and also indicated a wide range of cell medium that had been used in a number of independent studies.

The simplest differentiation protocol entails treatment of SH-SY5Y cells with RA to render this cellular model system more neuron-like (Zhang, Jiao et al. 2017, Mokhtar, Kim et al. 2018). Interestingly, RA-differentiated SH-SY5Y cells turned out to be relatively resistant towards Aβ toxicity: De Lorenzi et al. showed that a 3-day treatment with 30 µM Aβ42 reduced these cells' viability to 60% as compared to non-treated control (De Lorenzi, Chiari et al. 2017). However, De Lorenzi et al. tested the effects of Aβ in the presence of 5% FBS that might have interfered with the formation of amyloid aggregates. Retrieval or elimination of serum is important when studying toxicity of Aβ, since albumin and other serum components can interact with the Aβ peptide (Reyes Barcelo, Gonzalez-Velasquez et al. 2009). Another group showed that Aβ42 oligomers are highly toxic to RA-differentiated cells (45% of viable cells remained after 20 h incubation with 2.5 µM oligomers) (Oguchi, Ono et al. 2017).

SH-SY5Y cells differentiated with RA followed with BDNF treatment are used to a lesser extent in studies of Aβ toxicity (Table 1). Again, it is difficult to compare the results in the mentioned studies, since the Aβ concentrations used ranged from nanomolar to micromolar. In addition, the origin of the Aβ peptide used was different, since recombinant or synthetic peptides were used (Table 1). Singh et al. and de Medeiros et al. showed that the synthetic peptides appeared to be highly toxic as revealed by the MTT assay (after 24 h incubation, 2.5µM and 0.1 nM Aβ reduced viability by 50% and 45%, respectively) (Singh, Bissoyi et al. 2017, de Medeiros, De Bastiani et al. 2019). Recombinant MetAβ peptide was less toxic since 7µM of the peptide reduced viability by 50% after a 72-hour treatment, as shown by the PrestoBlue test (Cristovao, Morris et al. 2018). One possible reason why BDNF is not used after RA for differentiation of the SH-SY5Y cells is its potential protective effect on the neuronal cells' survival (Kim 2014, Nigam, Xu et al. 2017), thereby masking the toxic effects of the peptide.

The origin of Aβ, i.e. whether it is recombinant or synthetic, has a significant effect on toxicity and amyloidogenic properties of the peptide (Finder, Vodopivec et al. 2010). On the one hand, the recombinant form of Aβ peptide is more preferable in toxicity studies due to the high fidelity of ribosomes compared to those of organic synthesis systems. On the other hand, purification procedures after overexpression of the amyloid peptides may co-purify the additives that could influence the peptide properties (Finder, Vodopivec et al. 2010, Suvorina, Selivanova et al. 2015, Adams, Nemkov et al. 2017). The presence of additives in different peptide batches could also explain the heterogeneity of the results obtained even when using the same peptide concentration, time of exposure, toxicity assay etc. (Table1).

Importantly, in many *in vitro* studies not only Aβ40 and Aβ42, but also the Aβ fragment from residues 25 to 35 have been used (Yu, Suen et al. 2006, Chi, Wang et al. 2013, Xian, Lin et al. 2013, Xiao, Huang et al. 2013, Chen, Chen et al. 2014). This region of Aβ is highly hydrophobic and forms stable aggregates whose toxicity is higher than that of Aβ42 (Pike, Walencewicz-Wasserman et al. 1995). Since the Aβ25-35 peptide can be produced

only synthetically (Pike, Burdick et al. 1993), then the results obtained with this peptide are only poorly related to AD pathogenesis *in vivo*.

It has been also found that the N-terminally truncated Aβ3pE-42 peptide is present in plaques of AD patients (Iwatsubo, Saido et al. 1996). Studies of its fibrillization kinetics revealed that it is as amyloidogenic, as Aβ42 (Dammers, Schwarten et al. 2017). However, Aβ3pE-42 toxicity to SH-SY5Y cells was shown to be significantly lower than that of full-length Aβ42 (Galante, Corsaro et al. 2012).

The choice of a proper solvent for dissolving of Aβ peptides plays a crucial role in their toxic properties. Although the use of dimethylsulfoxide (DMSO) precludes formation of β-sheet structures and therefore peptide fibrillization (Shen and Murphy 1995), it still induces non-fibrillar oligomerization of the peptides (Novo, Freire et al. 2018). Importantly, the Aβ oligomers, obtained in the presence of DMSO, were shown to be highly toxic at micromolar and nanomolar concentrations (Oguchi, Ono et al. 2017, de Medeiros, De Bastiani et al. 2019). Despite of that, such artificial production of Aβ oligomers is not well suited for revealing the neurotoxic mechanism of the peptide *in vivo*.

Aβ can also interfere with the method used for testing of cellular viability. For example, the widely used MTT test, based on tetrazolium salt reduction in viable cells, was shown to negate the toxic effect of Aβ42 on SH-SY5Y cells, which have been damaged with the exocytosis of formazan (Isobe, Michikawa et al. 1999, Lu, Zhang et al. 2012). MTT test was also inaccurate when estimating the toxicity of glycolysis inhibitors, as compared to other assays for enumerating non-viable cells such as neutral red or resazurin (van Tonder, Joubert et al. 2015). Nevertheless, over 60% of the studies listed in Table 1 used MTT tests and seven of them (yellow shading) did not apply any additional toxicity assays e.g. AnnexinV/PI. It can be seen from the Table 1, that the results obtained using MTT tests showed that approx. 50% of cells remained viable after 24h incubation with Aβ regardless of the concentration applied, which obviously complicates the comparison of the results obtained from different studies of Aβ toxicity.

To sum it up, it can be seen that the most established cellular model based on SH-SY5Y cells is mainly used without any additional differentiation steps. Moreover, the conclusions about Aβ toxicity reached on the basis of this model are contradictory due to the differences in the source of the peptide, various peptide preparation protocols and the choice of a toxicity test.

## Aims of the study

The aims of current thesis were as follows:

- To investigate systematically the effects of environmental factors on Aβ40 and Aβ42 fibrillization *in vitro* and elaborate a protocol for the peptides preparation for toxicological studies
- To validate differentiated SH-SY5Y cells as improved cellular model for the studies of neurodegeneration and Aβ toxicity
- To compare the sensitivity of three SH-SY5Y cell cultures, which were differentiated to various neuronal phenotypes, toward Aβ42 toxicity

## Materials and methods

Preparation of Aβ peptides for experiments (Publications I, II, III)

Fibrillization of Aβ peptides studied with Thioflavin T and fluorescence spectroscopy (Publication I)

TEM experiments (Publication I and II)

Cell culture and differentiation (Publications II and III)

Cellular toxicity studies with WST-1 and PI uptake assays (Publications II and III)

Microscopy and Immunocytochemistry (Publications II and III)

Image analysis to evaluate neurite abnormalities (Publication II)

# Results

**Publication I**

- Concentration of Aβ42 had a small effect on the peptide fibrillization: 3 μM and higher concentrations showed constant fibril elongation rate values.
- Aβ42 fibril elongation rate constant increased with the temperature.
- Aβ42 fibrillization processes were not affected within the pH range of 7-9; however, lower pH significantly decreased the fibrillization rate.
- Fibrillization rate constant for Aβ42 was 2.5 times higher than that for Aβ40 under agitated conditions.
- Addition of 10% of Aβ42 to Aβ40 did not significantly influence fibrillization rate constant.
- Transmission electron microscopy images showed that fibrils of Aβ42 and Aβ40 peptides have a similar morphology.

**Publication II**

- WST-1 viability test showed that 20 μM Aβ40 was non-toxic to the non-differentiated SH-SY5Y cells, whereas 20 μM Aβ42 decreased cell viability by 20% after 48h incubation; PI test showed a 20% increase of dead cells only after 72h incubation with 20 μM Aβ42.
- RA/BDNF-differentiated cells were more susceptible to Aβ toxicity than non-differentiated SH-SY5Y cells after 72h incubation with 20 μM Aβ40 and Aβ42 peptides. For Aβ40, viability decreased by 30% and cell death increased by 29%; Aβ42 decreased viability by 43% and increased cell death by 50%, determined by WST-1 and PI tests, respectively.
- Caspase 3/7 activation test showed that Aβ42, but not Aβ40, induces apoptotic cell death in RA/BDNF-differentiated SH-SY5Y cells.
- Both peptides Aβ40 and Aβ42 peptides induced beading and fragmentation of neurites in RA/BDNF- differentiated culture; however, the Aβ42 had a stronger effect than Aβ40.
- Immunocytochemistry revealed that Aβ42 aggregates covered cell bodies and neurites with the peptide coating, which was less dense in case of Aβ40 as compared to Aβ42.
- TEM confirmed the presence of Aβ42 fibrils, but not Aβ40 fibrils, in the cell culture medium after 24 h incubation.

**Publication III**

- Treatment of SH-SY5Y cells with RA followed by BDNF or TPA for 7 days, or dbcAMP alone for 3 days induced neurite outgrowth and the formation of neuronal network, visualized with light microscopy. RA/BDNF-differentiated cells were morphologically the most homogeneous ones when compared to the other two differentiated cultures, which also contained non-differentiated cells.
- Cell viability assay WST-1 results after 48h incubation with 10 and 20 μM Aβ42 were as follows:

- non-differentiated SH-SY5Y cells' viability decreased only with 20 μM peptide;
- dbcAMP-treated culture was more sensitive toward 20 μM peptide as compared to 10 μM peptide;
- RA/BDNF-treated culture was more sensitive toward 10 μM peptide as compared to 20 μM peptide;
- RA/TPA-treated culture did not show any statistically significant decrease of viability at both peptide concentrations.

- PI assay of cell permeability after 48h incubation with 10 and 20 μM Aβ42 revealed the following:
  - Membrane permeability of non-differentiated SH-SY5Y cells increased significantly only after incubation with 20 μM peptide;
  - dbcAMP-treated culture showed regular dose dependence since the effect of 20 μM peptide exceeded that of 10 μM peptide by nearly two-fold;
  - RA/BDNF-treated culture was 20% more susceptible toward 10 μM peptide than to 20 μM peptide;
  - RA/TPA-treated cells showed approx. 30% increase in membrane permeability in response to both 10 and 20 μM Aβ42.
- Immunocytochemistry revealed that a pattern of Aβ42 distribution in all three SH-SY5Y cultures, differentiated to different neuronal phenotypes, was common for all cultures. Visual inspection of morphological changes revealed beading and fragmentation of neurites in all differentiated cultures, including the most resistant RA/TPA-treated culture.

# Discussion

*In vitro* amyloid studies can characterize Aβ fibrillization and elucidate the mechanism for generation of Aβ toxic assemblies. The process of fibrillization includes nucleation and propagation of new fibrillization centers, elongation and maturation of fibrils. Each process can be influenced by environmental factors and by the substances that can interfere with fibrillization. The determination of the effects of peptide concentration, pH, temperature and several common substances on Aβ aggregation is important not only for studying the mechanism of fibrillization, but also for planning cell culture experiments (Publication I and II). We showed that concentration had a relatively small effect on the fibrillization of Aβ42 since the elongation rate of fibrils remained constant at peptide concentrations above 3 μM (Publication I). It is proposed that the fibril elongation rate constant can become concentration independent at the high peptide concentration, when the first step of monomer binding to the fibril is reversible (Massi and Straub 2001). Importantly, the fibrillization of the peptide that has an additional Met residue at its N-terminus (MetAβ42) shows a different concentration dependence (Hellstrand, Boland et al. 2010).

Fibrillization of Aβ42 can be initiated by using preformed fibrillary seeds or stirring. While the mechanism of a primary nucleation is not known, it has been suggested that it can occur on various interfaces (Srivastava, Pittman et al. 2019). Increasing the temperature increased the rate of Aβ42 fibrillization, this effect was characterized by a linear Arrhenius plot (Publication I, F3). Relatively high activation energy (12kcal/mole) of this process suggested that the rate limiting step of the process is not diffusion. The rate of fibril elongation may be limited by a conformational rearrangement of the peptide molecules during fibrillization (Massi and Straub 2001).

We found that at pH values below 6.5 reduced the fibrillization rate of Aβ42, due to the simultaneous protonation of His13 and His14 (Publication I). We propose that positively charged His residues repel each other, thereby inhibiting fibril formation. Recently, Brännström et al. showed by using a surface plasmon resonance method that lower pH stabilize Aβ fibrils (Brannstrom, Islam et al. 2017), however, they determined the thermodynamic stability, but not the fibrillization rate.

We have studied fibrillization of Aβ40 and Aβ42 in agitated conditions, which accelerate the process. Previously, we have found that agitation is essential during the initial phase of fibrillization (Tiiman, Noormagi et al. 2013). Importantly, in case of Aβ42, elongation phase can continue with the same rate without agitation, whereas Aβ40 elongation phase slowed down remarkably once agitation had been terminated (Tiiman, Noormagi et al. 2013). We compared fibrillization of Aβ40 and Aβ42 in near-physiological conditions, calculated fibrillization rate constants and the duration of lag phases for the peptides alone and their mixtures (Publication I). The lag phases of the peptide mixtures were different from the lag phases of the peptides alone; however, this fibrillization parameter is highly variable (around 60%) even under agitated conditions (Publication I). The fibrillization rate constant for 9:1 mixture of Aβ40 and Aβ42 was similar to that of Aβ40. Therefore, the peptide mixtures were not used in subsequent toxicological studies with SH-SY5Y cells.

We identified massive fibril formation in the SH-SY5Y cell culture in case of Aβ42, but not in case of Aβ40 (Publication II). Aβ42 fibrillization within 24 h under quiescent conditions was also revealed by ThT test in the cell medium without FBS (Publication II, S7). Immunocytochemistry showed that Aβ40 aggregates were also present on cells and in

the intracellular space (Publication II), It should be noted, that antibody-based detection of Aβ in cultures does not reveal whether the aggregates have a fibrillar or non-fibrillar structure. It is known that the initiation of the fibrillization in the cell medium without agitation can be influenced by many components present in the solution, including metal ions (Tougu, Tiiman et al. 2011). Cell membranes, plastic or glass surfaces can promote generation of nucleation centers for fibril growth (Srivastava, Pittman et al. 2019). There is also evidence revealed by fluorescence correlation spectroscopy that HFIP-treated Aβ peptide, which is dissolved in an alkaline solution, forms immediately some ThT-positive aggregates (Tiiman, Jarvet et al. 2015).

Importantly, Aβ40 was almost nontoxic for non-differentiated SH-SY5Y cells and only had a minor, but statistically insignificant toxic effect on RA/BDNF-differentiated cells after 72 h of incubation (Publication II, F2). The presence of other factors which can initiate Aβ fibrillization can influence toxicity, .e.g. increased amounts of ganglioside clusters in the cell membrane (Matsuzaki 2014) and the shift in a proportion of other cell membrane components (Drolle, Negoda et al. 2017). It is also not excluded that Aβ40 may need prolonged incubation to induce toxicity via cell membrane deformation by growing fibrils or the formation of pores (Matsuzaki 2014).

Our study showed that Aβ42 treatment induced caspase3/7 activation in RA/BDNF-differentiated SH-SY5Y cells, whereas Aβ40 treatment did not (Publication II). Similar results have been obtained with primary cultures, where Aβ42 treatment induced apoptosis (Han, Hu et al. 2017). Recently, Aβ-induced apoptosis was also described in RA/BDNF-differentiated SH-SY5Y cells treated with recombinant MetAβ42 (Cristovao, Morris et al. 2018). In addition, there is evidence that Aβ40 induces apoptosis in primary cultures (Boland and Campbell 2003); however, in this study, the peptide was incubated in DMSO solution to induce Aβ oligomerization and prevent fibrillization (Shen and Murphy 1995).

In AD research, divergent results have been published for Aβ toxicity most likely due to the differences in the sources and handling protocols of the peptides, and it is rarely analyzed whether monomers, oligomers or fibrils of Aβ are actually applied to the cell culture (Table 1). For the Aβ toxicity studies (Publication III), we have slightly modified the Aβ preparation protocol reported in Publication II. Freshly HFIP-defibrillized peptide was first dissolved in 10mM NaOH to minimize the presence of prefibrillar aggregates in the initial solution. We found that the Aβ42 peptide prepared according to the modified protocol had a higher toxicity than observed previously (Table 2). We presume that addition of freshly dissolved Aβ to the cells without pre-aggregation is important, since it precludes stochastic appearance of the aggregates, which can alter their toxicity.

Our study showed that fibrillization of Aβ *in situ* was crucial for Aβ toxicity, because preformed matured fibrils were not toxic to the cells (Publication II, S8). This observation supports the hypothesis that secondary nucleation during fibrillization might be responsible for generation of toxic Aβ species (Cohen, Linse et al. 2013).

Another important aspect to consider in Aβ toxicity studies is the choice of an appropriate cellular model. It is reasonable to choose a cell culture of human origin, since AD is a human disease. In addition to this, the choice of neuronal cells or cells differentiated toward neuron-like phenotypes is a more relevant model to study mechanisms of neurodegeneration. We have differentiated human neuroblastoma SH-SY5Y cells using three different protocols to compare their sensitivity toward Aβ. Importantly, RA/TPA-differentiated cells, that have been previously described as dopaminergic (Presgraves, Ahmed et al. 2004), were almost resistant to Aβ42. This

phenomenon could be explained by the activation of protein kinase C by TPA, which inhibits apoptotic pathways in neurons (Pastore, Pacifici et al. 2019). In addition, TPA treatment alone has also a protective effect against Aβ toxicity (Han, Zheng et al. 2004). Neurite abnormalities were seen in all differentiated SH-SY5Y cultures (Publication III, S1), pointing to the ability of Aβ to damage the cell membranes (Arbor, LaFontaine et al. 2016).

***Table2 Comparison of toxicity results between Publication II and Publication III.***

| Differentiation | PI test | | WST-1 test | |
|---|---|---|---|---|
| | **10 µM Aβ42** | **20 µM Aβ42** | **10 µM Aβ42** | **20 µM Aβ42** |
| *None* | 1.1 ± 0.1 | 1.8 ± 0.2 | 100.2 ± 3.5 | 57.4 ± 8.7 |
| *dbcAMP* | 1.4 ± 0.1 | 1.9 ± 0.1 | 51.3 ± 4.2 | 37.2 ± 1.7 |
| *RA/BDNF* | 1.8 ± 0.2 | 1.6 ± 0.2 | 59.4 ± 5.3 | 77.2 ± 6.8 |
| *RA/TPA* | 1.3 ± 0.1 | 1.4 ± 0.1 | 85.8 ± 4.4 | 102.6 ± 5.3 |
| *None** | | 1.1 ± 0.5* | | 83.6 ± 4.6* |
| *RA/BDNF** | | 1.3 ± 0.2* | | 77.6 ± 9.5* |

*Asterix * indicates results obtained in Publication II.*

Surprisingly, RA/BDNF-differentiated cholinergic cell culture was more sensitive toward 10 µM Aβ42 than 20 µM Aβ42. We can only speculate about the reason for this concentration dependence. It is possible that the peptide fibrillized faster at higher concentration in the cell culture; however, it is not clear why this effect was observed only in the RA/BDNF-treated culture. There might be other factors affecting Aβ42 toxicity such as differences in cell membrane composition (Drolle, Negoda et al. 2017). Nevertheless, noradrenergic and cholinergic SH-SY5Y cell cultures had a higher sensitivity toward Aβ42 than the non-differentiated or dopaminergic cultures. There is evidence that dbcAMP and BDNF treatments increase the expression of APP (Shekarabi, Bourbonniere et al. 1997, Holback, Adlerz et al. 2005) that can potentially lead to the increase of endogenous Aβ production. We can hypothesize that extracellularly applied rapidly fibrillized Aβ can interact with endogenously produced Aβ peptide monomers to produce more toxic oligomers.

Analyzing toxicity, we raised a working hypothesis that increased Aβ aggregate load on the cells may induce the higher toxic effect. We thus compared the extent of amyloid association with the cells in the three differentiated cell cultures with the toxicity results. Surprisingly, we found that the Aβ distribution was almost identical for each obtained culture despite the fact that the toxicity was different (Publication III).

In conclusion, the cholinergic and noradrenergic SH-SY5Y cell cultures obtained had a higher susceptibility to Aβ toxicity than the dopaminergic culture, consistent with the observation that the same types of neurons are most vulnerable during AD onset and progression. It can be also concluded that the models of differentiated human neuroblastoma SH-SY5Y cells, used in the current study, can have a potential to reveal the mechanisms of Aβ toxicity on cellular level.

## Conclusions

- The effects of the main environmental factors on Aβ42 fibrillization *in vitro* were determined and suitable Aβ preparation protocols for toxicity studies were elaborated. It was shown that different protocols for Aβ preparation yield peptide samples with different toxicity.
- RA/BDNF-differentiated SH-SY5Y cells were more susceptible to Aβ40 and Aβ42 than non-differentiated SH-SY5Y cells. Aβ42 fibrillizing in cell culture, but not Aβ40 or prefibrillized Aβ42, activated apoptosis, caused neurite's beading and fragmentation.
- It was shown that the SH-SY5Y cells differentiated toward three different phenotypes vary in their sensitivity to Aβ42. DbcAMP-based differentiation toward noradrenergic phenotype and RA/BDNF treatment toward cholinergic phenotype increased the susceptibility of cells to Aβ42, whereas RA/TPA treatment toward dopaminergic phenotype decreased the sensitivity to Aβ42.
- As compared to non-differentiated SH-SY5Y cells, differentiated neuron-like SH-SY5Y cells represent more relevant models to study the cellular mechanisms of neurodegeneration and to screen new potential drugs.

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## Abstract

## Application of differentiated SH-SY5Y cells for toxicological studies of Alzheimer's amyloid beta peptide

The most prevalent form of dementia is Alzheimer's disease (AD), which affects more than 50 million people worldwide. AD neuropathological mechanisms are complex and still not fully understood. The main hallmarks of the disease are amyloid plaques and tau tangles in the brain. The principal components of the plaques are amyloid beta (Aβ) peptides, which are prone to aggregation, generation of toxic species and seeding of fibrillary structures throughout the brain. The toxicity of Aβ peptide has been intensively studied, however, no clear mechanism for neurotoxicity has emerged. During recent decades, many models, including cell line-based ones, have been created to study mechanisms of AD. Differentiation of human neuroblastoma SH-SY5Y cells toward neuron-like cultures affords the possibility to improve the initial cellular model based on non-differentiated SH-SY5Y cells for Aβ toxicity studies. In current thesis, the effects of the environmental factors on Aβ40 and Aβ42 *in vitro* fibrillization were studied, and the results obtained were taken into account in the peptide preparation for subsequent cell toxicity studies. Next, we differentiated cells toward noradrenergic, cholinergic and dopaminergic neuronal phenotypes and used these neuronal cultures to study their sensitivity to the toxicity of Aβ by knowing the fact that cholinergic and noradrenergic neurons are the first to die in case of AD. By using both cell viability and membrane permeability tests, we established that the obtained cultures show different susceptibility to Aβ42: noradrenergic and cholinergic cultures were most sensitive, whereas dopaminergic culture had the lowest sensitivity. A more detailed study of the cholinergic culture revealed that Aβ42 fibrillized in the cell culture, induced apoptosis and neurite abnormalities as compared to Aβ40 or preformed Aβ42 fibrils. Our results show that differentiated neuron-like SH-SY5Y cells represent more relevant models to study mechanisms of Aβ-induced neurodegeneration and to screen new potential AD drug candidates.

# Lühikokkuvõte

## Diferentseeritud SH-SY5Y rakkude kasutamine Alzheimeri amüloid beeta peptiidi toksilisuse uurimiseks

Alzheimeri tõbe (AT) põeb maailmas ligikaudu 50 miljonid inimest ning see on kõige levinum ja kõige kulukam neurodegeneratiivne haigus arenenud riikides. AT ei ole ravitav ning vaatamata väga intensiivsele ja põhjalikule uurimisele on selle käigus toimuva neurodegeneratsiooni põhjused ja mehhanismid on ebaselged. AT iseloomustavad ajurakkude vahel tekkinud amüloidsed naastud ning rakusisesed neurofibrillaarsed kämbud. Naastud on ajaliselt kõige varem ilmuvaks AT tunnuseks ning nende põhikomponendiks on amüloid beeta (Aβ) peptiid, mis on võimeline tekitama toksilisi agregaate ajus ning käivitama neurodegeneratsiooni. Tänapäeval on välja töötatud palju AT mudeleid, et uurida haiguse tekkemehhanismi ning pakkuda välja uusi ravimkandidaate. Rakukultuuride kasutamine on kõige lihtsam viis uurida neurodegeneratsiooni mehhanisme. Inimese neuroblastoomi SH-SY5Y rakuliin on kõige populaarsem AT uuringuteks kasutatav rakuliin, seda hoolimata asjaolust, et originaalne rakuliin ei oma enamikku küpsete neuronite tunnuseid, st rakud ei ekspresseeri vastavaid neuronaalseid markereid ja ei moodusta sünapsitega seotud neuraalset võrgustikku. Rakuliini eelisteks on siiski võimalus seda diferentseerimise abil neuronilaadseteks muuta ning tekitada erinevate neuronaalsete omadustega rakukultuurid, mis suurendab antud rakuliini rakendamisalasid ning sobivust AT neurodegeneratsiooni uurimiseks. Samuti on SH-SY5Y rakukultuuri põhjalikult iseloomustatud. Käesoleva doktoritöö eesmärgiks oli uurida Aβ *in vitro* fibrillisatsiooni ning keskkonna faktorite mõju sellele protsessile, mis võimaldab teostada tema toksilise mõju uurimist kindlalt kontrollitud tingimustes. SH-SY5Y rakuliini diferentseeriti kolme erineva protokolli järgi, mille tulemusena saadi koliinergiliste, noradrenergiliste ning dopamiinergiliste omadustega rakukultuurid. Saadud rakukultuuride tundlikkust Aβ toksilisuse suhtes mõõdeti kahe erineva meetodiga: rakkude eluvõimelisust hinnati WST-1 testiga ning membraani läbilaskvust kontrolliti propiidiumi testiga. Tulemuseks saadi, et dopamiinergiline kultuur on vähem tundlik, kui koliinergiline või noradrenergiline kultuur, mis on kooskõlas olukorraga AT ajus, kus esimeste hulgas hukkuvad just koliinergilised ja noradrenergilised neuronid. Aβ kogunemist ja jaotust rakukultuurides visualiseeriti immunotsütokeemia abil ning peptiidi fibrillide olemasolu näidati elektronmikroskoopia abil. Täpsemalt uuriti koliinergilist rakukultuuri ning näidati, et toksiliseks agendiks on *in situ* fibrilleeruv Aβ42, mis käivitab apoptoosi ja indutseerib neuriitide hukkumist. Aβ40 peptiid ega ka Aβ42 peptiidist eelnevalt valmistatud „küpsed“ fibrillid rakkudele toksiliseks ei osutunud. Töös näidati, et diferentseeritud rakuliine võib kasutada edaspidiseks Aβ toksilisuse ning üldiste neurodegeneratsiooni mehhanismide uurimiseks ning uute ravimikandidaatide skriininguks.

# Appendix

## Publication I

Tiiman, Ann; Krishtal, Jekaterina; Palumaa, Peep; Tõugu, Vello (2015). In vitro fibrillization of Alzheimer's amyloid-β peptide (1-42). AIP Advances, 5, 092401.10.1063/1.4921071

# *In vitro* fibrillization of Alzheimer's amyloid-β peptide (1-42)

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The amyloid deposition in the form of extracellular fibrillar aggregates of amyloid-β (Aβ) peptide is a critical pathological event in Alzheimer's disease. Here, we report a systematic investigation of the effects of environmental factors on the kinetics of Aβ fibrillization *in vitro*. The effects of Aβ42 peptide concentration, temperature, pH, added solvents and the ratio of Aβ40 and Aβ42 on the peptide fibrillization under agitated conditions was studied. The analysis show that the rate of fibril growth by monomer addition is not limited by diffusion but by rearrangement in the monomer structure, which is enhanced by low concentrations of fluorinated alcohols and characterized by the activation energy of 12 kcal/mol. Fibrillization rate decreases at pH values below 7.0 where simultaneous protonation of His 13 and 14 inhibits fibril formation. The lag period for Aβ42 was only twofold shorter and the fibril growth rate twofold faster than those of Aβ40. Lag period was shortened and the fibrillization rate was increased only at 90% content of Aβ42.  [http://dx.doi.org/10.1063/1.4921071]

## ABBREVIATIONS

| | |
|---|---|
| AD | Alzheimer's disease |
| Aβ | amyloid-β |
| APP | amyloid precursor protein |
| HFIP | 1,1,1,3,3,3-hexafluoro-2-propanol |
| TFE | 2,2,2-trifluoroethanol |
| ThT | Thioflavin T |
| NP | nucleated polymerization |

## I. INTRODUCTION

The assembly of peptides and proteins into fibrillar aggregates plays an essential role in the onset of several pathologies known as amyloid diseases[1] including Alzheimer's disease (AD), Parkinson's disease, type II diabetes as well as prion diseases. Identification of the key steps and understanding the mechanism of amyloid fibril formation on the molecular level may reveal important information for the development of methods and drugs for the suppression and reversal of amyloidogenesis.

In this paper we have studied the effects of multiple environmental factors on the aggregation of amyloid-β 1– 42 (Aβ42) peptide, the main component of amyloid plaques, characteristic to AD. Aβ42 is more prone to aggregation than the most common form, Aβ40, and in many

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papers it has been considered to be responsible for amyloid plaque formation or at least for the initiation of the process. As a rule, fibrillization of amyloidogenic peptides and proteins *in vitro* is a self-propagating process characterized by a sigmoidal growth curve. The process starts with a relatively long lag-phase that is followed by the fast propagation of fibrillar material. According to the most popular model for Aβ fibrillization, the nucleated polymerization (NP) model,[2,3] the lag-phase is the time required for the formation of oligomeric nucleus large enough to be stable and grow due to the addition of monomers. However, recent studies have shown the importance of secondary nucleation events in the fibril growth process. For example the fragmentation of fibrils, can dominate in the propagation of amyloid growth.[4] Moreover, several recent studies have shown that amyloid formation *in vivo* is also initiated by the formation of a limited number of seeds that spread from a single nucleation site and start the formation of plaques e.g. the process involves a secondary nucleation step.[5,6]

Fibrillization is known to be enhanced by increasing temperature and affected by changes in pH, addition of organic solvents or other solutes as well as agitation of the reaction mixture. Recently, it has been shown that agitation enhances the fibrillization of Aβ42 peptide only in the initial exponential phase and this has been suggested to be related with the significant role of secondary nucleation.[7]

In order to elucidate the nature of the physico-chemical interactions involved in the fibrillization of Aβ peptides we have experimentally studied the effects of multiple environmental factors (temperature, pH, peptide concentration, ionic strength, and denaturing agents) on the kinetics of the Aβ42 fibril formation. The results suggest that the growth rate of Aβ42 fibrils is not under control of the diffusion of peptide molecules to the fibril end. The fibril growth is most likely limited by a monomolecular event that may be related to an "opening" of the conformation and exposition of hydrophobic surfaces. Destabilization of hydrophobic interactions by small amounts of solvents increased the fibrillization rate showing that hydrophobic interactions may stabilize the monomers in the solution. The decrease in the fibrillization rate at lower pH values is caused by the concurrent protonation of H13 and H14 residues that stabilizes the soluble monomer.

## II. EXPERIMENTAL PROCEDURE

### A. Materials

Lyophilized Aβ peptides (ultra-pure, recombinant) HFIP forms were purchased from rPeptide (Athens, USA). HEPES, Ultrapure, MB Grade was from USB Corporation (Cleveland, USA). 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), 2,2,2-trifluoroethanol (TFE), and Thioflavin T (ThT) were from Sigma Aldrich (St. Louis, USA). NaCl (extra pure), ammonium acetate, and ammonium were from Scharlau (Barcelona, Spain). All solutions were prepared in fresh MilliQ water.

### B. Sample preparation

Stock solution of Aβ peptides was prepared as follows: 1 mg of the peptide was dissolved in HFIP at a concentration 500 μM to disassemble preformed aggregates.[8] The solution was divided into aliquots, HFIP was evaporated in vacuum and the tubes with the peptide film were kept at −80° C until used. A 50 μg aliquot was assessed for amino acids in order to check the quality and quantity of the peptide. Before using the Aβ HFIP film was dissolved in water containing 0.02% $NH_3$ at a concentration of 10-20 μM. After 5 minutes incubation the Aβ stock solution was dissolved with buffer and used for experiments.

### C. Fluorescence Spectroscopy

Fluorescence spectra were collected on a Perkin-Elmer LS-45 fluorescence spectrophotometer equipped with a magnetic stirrer. Fibrillization was monitored using ThT fluorescence. If not otherwise stated, fresh Aβ stock solution was diluted in 20 mM HEPES and 100 mM NaCl, pH 7.4 containing 3.3 μM of ThT to a final concentration of 5 μM. 400 μl of each sample was incubated at

40 °C if not otherwise stated. ThT fluorescence was measured at 480 nm using excitation at 445 nm. The pH dependence was determined using 20 mM ammonium acetate as a buffer.

### D. Data analysis and kinetics of fibril formation

The kinetics of Aβ fibrillization could be described as sigmoid curves and the aggregation parameters were determined by fitting the plot of fluorescence intensity versus time to Boltzmann curve

$$y = \frac{A_2 - A_1}{1 + e^{(t-t_0)\times k}} + A_2 \quad (1)$$

where $A_1$ is the initial fluorescence level, $A_2$ – corresponds to the fluorescence at maximal fibrillization level, $t_0$– is the time when fluorescence is reached half maximum and $k$ – is the rate constant of the fibril elongation. The lag time is calculated as

$$\text{lag} = t_0 - 2/k \quad (2)$$

### E. Transmission electron microscopy (TEM)

An aliquot of 5 μl of sample was loaded on a Formvar-coated, carbon-stabilized copper grid (300 mesh from Ted Pella Inc., Redding CA). After 1 min, the excess solution was drained off using a Whatman filter paper. The grid was briefly washed and negatively stained with 5 μl of 2% uranyl acetate. The grid was air-dried and then viewed on a Tecnai G2 BioTwin transmission electron microscope (FEI, Japan) operating with an accelerator voltage of 80 kV. Typical magnifications ranged from 20,000 to 60,000×.

## III. RESULTS AND DISCUSSION

### A. Kinetics of Aβ fibrillization

The fibrillization of Aβ was followed by an increase in ThT fluorescence due to the binding of the dye to the fibrils. The representative fibrillization curve on Fig. 1 demonstrates a good fit between the Boltzmann equation used for fitting and experimental data that is confirmed by plotting the residuals versus time in the inset. Parallel measurements at 40 °C with 5 μM Aβ42 showed good reproducibility of the maximal ThT fluorescence 322 ± 17 and the fibril growth rate ($k$ = (1.35 ± 0.25) $\text{min}^{-1}$ (n=7)), however, the variation in the lag-period of the reaction was considerably larger (3.3 ± 2.0 min). The lower reproducibility of the lag period is most probably related with the stochastic nature of the formation of initial fibrils. The fibrillization of Aβ peptides is highly dependent on the agitation rate and conditions.[9] We have used conditions where further increase in the agitation rate does not speed up the process any more.[7,10]

A known drawback of the ThT method is the interference of added compounds or reaction conditions with ThT binding or its fluorescence,[11,12] thus, under certain circumstances the final ThT emission values under different conditions does not allow to compare the amount of fibrils formed. We have confirmed[11] that when the maximal ThT fluorescence intensity is achieved the solution does not contain monomeric Aβ42 peptide in detectable amounts. An interesting question is the mathematical model used for data fitting. Boltzmann equation describing a symmetric growth curve has been successfully used to describe the time curves of peptide fibrillization in several papers.[13–16] This equation also gave good descriptions for Aβ42 fibrillization and the parameters determined can be used in the analysis of various effects on the fibril formation and growth rates.

### B. Effect of peptide concentration

The kinetics of Aβ42 fibrillization was studied at peptide concentrations between 0.5 and 20 μM. The maximal level of ThT fluorescence showed nearly linear dependence from peptide

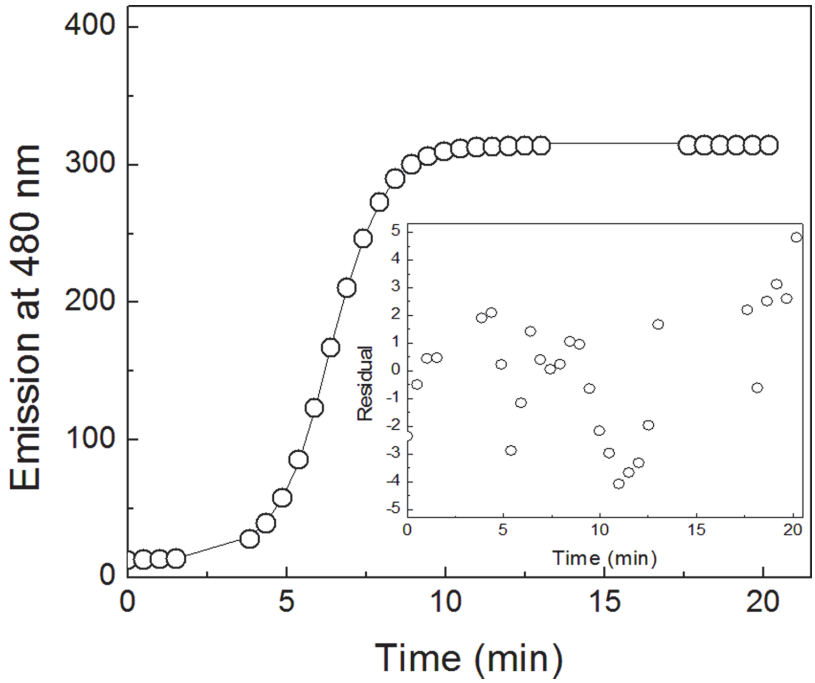


FIG. 1. **The fibrillization of Aβ peptide.** A representative fibrillization curve of the aggregation of 5 μM Aβ42 in 20 mM HEPES, 100 mM NaCl, pH 7.4 at 40 °C with continuous agitation. Solid line corresponds to fit of the data to Boltzmann equation (**eqn. (1)**). Inset: Residuals versus time.

concentration (Fig. 2), and the solutions did not contain Aβ monomers at the end of the process in detectable amounts.

The rate constant for fibril formation increased with an increase in the peptide concentration up to 3 μM, however, at peptide concentration above 3 μM its value ($k = 0.52 \pm 0.20$ min$^{-1}$ (Fig. 2)) remained constant. In the fibril elongation phase the rate-constant corresponds to the disappearance of monomers[17] and its independence of the peptide concentration means that this is a first-order (or most likely a pseudo-first order) process. It has been suggested that the rate constant can be independent of the peptide concentration when the equilibrium between the peptide fibrillization-competent and incompetent conformations in the solutions is shifted towards the latter[18] e. g. the rate of fibril elongation is limited by obtaining an addition-competent structure in solutions (or any other monomolecular process) and the concentration of nucleation centers or growing fibrils is sufficiently high to bind all the peptide molecules in favorable conformation. The fibrillization rate of an Aβ42 derivative with a methionine residue in the N-terminus was also found to be concentration dependent at concentrations below 5.8 μM.[15] However, in that study different agitation conditions were used and the overall process was also slower. The fibrillization models assuming prevalent primary nucleation predict an extremely sharp shortening of the lag phase with increasing peptide concentrations. Unfortunately, the lag phases showed relatively poor reproducibility in our experiments, however, the similarity of the curves obtained at low and high peptide concentrations shows that lag phase duration does not decrease with the peptide concentration in $n^{th}$ power (where n is the minimal number of peptide molecules in a fibrillization-competent oligomeric nucleus). The secondary nucleation dominance with stochastic formation of early aggregates on the surfaces surrounding the solution is in agreement with the relative independence of the lag phase on the peptide concentration. Alternatively, the rate of fibril growth may become almost independent

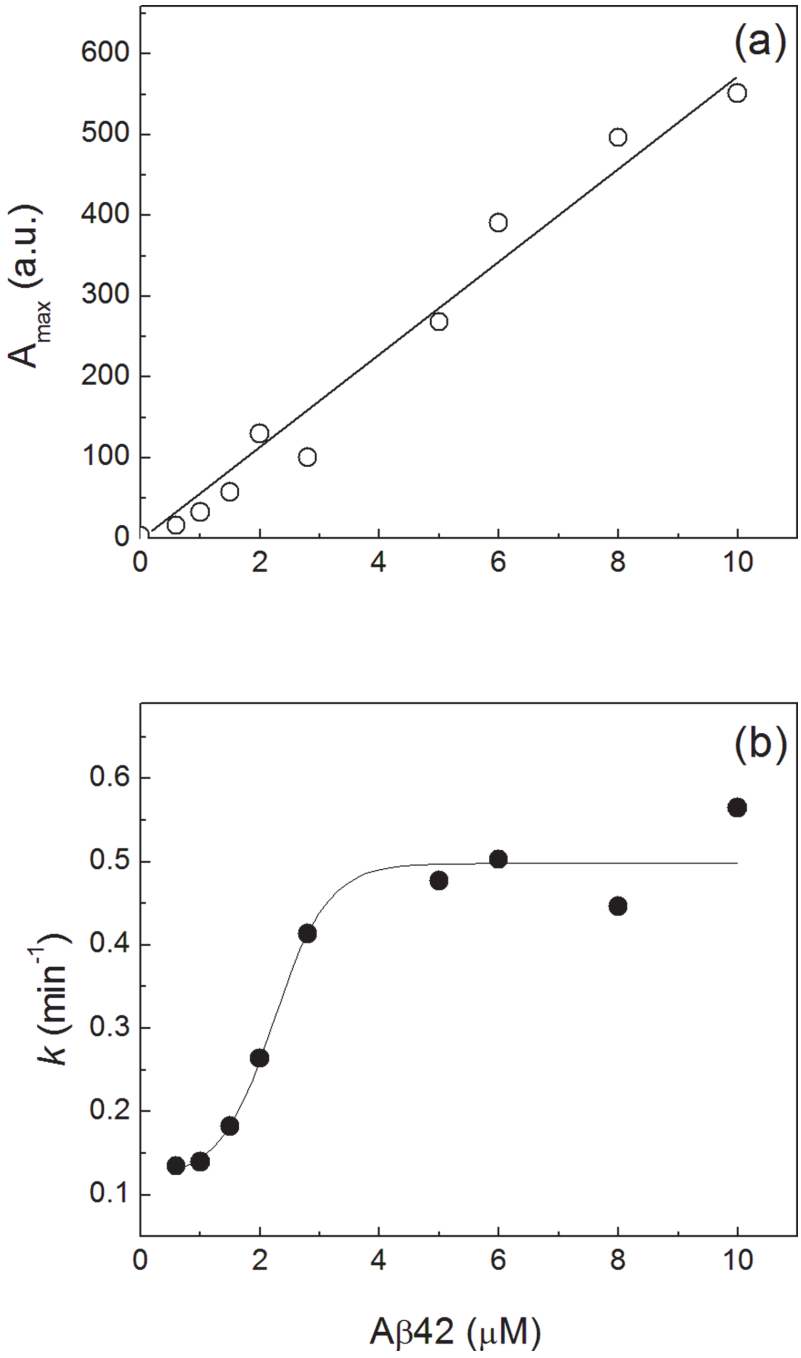


FIG. 2. **The effect of peptide concentration on the fibril formation.** Different concentrations of Aβ42 were aggregated in 20 mM HEPES, 100 mM NaCl, pH 7.4, with continuous agitation in the presence of 3.3 μM ThT at 25 °C. The values of maximal ThT emission at 480 nm (a) and *k* values (b) were calculated using Boltzmann equation **(eqn. (1))**.

of peptide concentration at "supercritical concentration" when oligomer population may become significant with respect to the total peptide concentration, and when aggregates can only grow by monomer addition.[19] However, this does not happen in our conditions since SEC analysis showed the presence of only monomeric peptide in the initial solutions up to peptide concentrations of 20 μM.

### C. Effect of temperature on the fibrillization of Aβ42

Aβ42 fibrillization was studied in the temperature range from 10 to 45 °C. The final level of ThT fluorescence was similar at all temperatures studied indicating that there is no drastic differences between the fibrils formed. The fibrillization rate constant increased and the lag time decreased with increasing temperature. The log k values showed a linear relationship in Arrhenius plot (Fig. 3) and the slope of the relationship corresponded to the activation energy 12 kcal/mole.

In general fibril formation is accelerated at higher temperatures.[20–22] Incubation of Aβ peptide at low temperature (4 °C) and physiological pH is known to lead to oligomerization, whereas fibrillization occurs at higher temperatures.[23] Our data confirm that higher temperatures favor fibrillization, moreover, the effect of temperature is characterized by a linear Arrhenius plot without temperature sensitive "switches" in the reaction mechanism. The $\Delta H^{\ddagger}$ value is in the same range with $\Delta H^{\ddagger}$ values determined for Aβ40 aggregation at acidic pH[17,22] and high peptide concentration. $\Delta H^{\ddagger}$ = 12 kcal/mole has been calculated from the rate of protofibril fibrillization at high peptide concentration,[20] a process that can be equivalent to fibril growth.

The relatively high $\Delta H^{\ddagger}$ value of the process clearly shows that fibrillization is not controlled by a diffusional event and must involve a conformational rearrangement. Thus, it can be concluded that the rate limiting step of fibril growth is a "lock" not a "dock" within the "dock and lock"

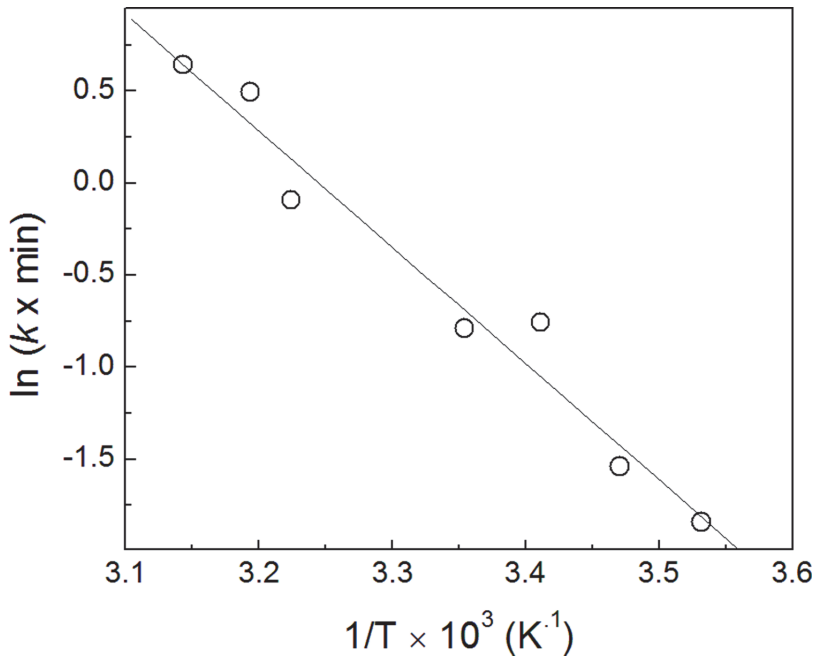


FIG. 3. **The effect of temperature on the fibril formation of Aβ42.** The aggregation of 5 μM Aβ42 in 20 mM HEPES, 100 mM NaCl, pH 7.4, with continuous agitation in the presence of 3.3 μM ThT at various temperatures. Line corresponds to Arrhenius plot.

model.[17,24] The incubation of Aβ40 at higher temperatures can result in reversible beta-sheet accumulation without aggregation,[25] thus, the high $\Delta H^{\ddagger}$ value can reflect the shift of the conformational equilibrium towards the more aggregation-competent peptide conformation. According to a recent model[26] the energy barrier of fibrillization does not correspond to the transition state but describes the recovery from off-pathway kinetic traps.

### D. Effect of solvents on the Aβ fibrillization

Effects of two fluorinated alcohols, 2,2,2-trifluoroethanol (TFE) and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), on Aβ42 fibrillization were studied. Both TFE and HFIP produced a bell-shape rate constant dependence of concentration (Fig. 4), with the maximal rate constant values at 5 % and 0.1 % of the solvent, respectively. In conformity with their relative hydrophobicity the effective concentration of TFE is higher than that of HFIP for the same effect. TFE had no effect on the ThT emission of preformed Aβ fibrils showing, that it does not interfere the ThT fluorescence, however, the fibrils formed in the presence of high TFE concentrations showed lower ThT fluorescence.

Fluorinated alcohols at high concentrations (> 40%) have been shown to break β-sheet structure, disrupt hydrophobic forces, and favor α-helical conformation of the peptide.[27–29] In the case of Aβ a considerably sharp transition of the peptide into the α-helix form occurs at TFE concentrations 15 to 25%.[30] We observed that under the conditions where α-helical structure becomes prevailing (at 20-25% TFE) fibrillization is almost completely suppressed. Thus, most likely the enhancement of fibrillization rate constant by TFE and HFIP arises from the destabilization of intramolecular hydrophobic interactions in the initial conformation. At higher concentration organic solvent stabilizes the conformation of the helical form, thus suppressing the formation of β-sheets. The effects of

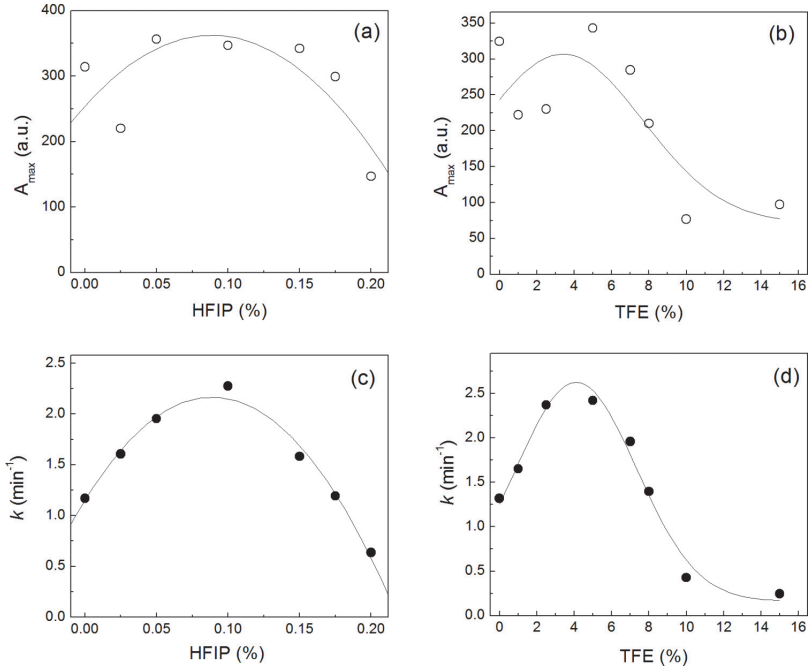


FIG. 4. **The effect of fluorinated alcohols on the fibril formation of Aβ42.** Aβ42 was incubated in 20 mM HEPES, 100 mM NaCl, pH 7.4, with continuous agitation in the presence of 3.3 μM ThT at 40 °C at various concentrations of TFE (a, b) and HFIP (c, d). The values of the ThT emission maximum at 480 nm (a, c), and $k$ (b, d) were calculated using Boltzmann equation (**eqn. (1)**).

HFIP and TFE are in agreement with the effects of peptide concentration and temperature indicating that the fibrillization rate is limited by intramoleculecular reorganization in the peptide molecule, and they do not support the involvement of hypothetical α-helical intermediates in the fibrillization process.

### E. Effect of pH on Aβ fibrillization

The effect of pH on Aβ fibrillization was studied on the wt Aβ42 and three His to Ala mutants (H6A, H13A and H14A). Fig. 5 shows that the fibrillization rate constant of the wild type peptide was independent of the pH in the range of 7-9, whereas, at lower pH fibrillization rate decreased significantly, which can be caused by the protonation of histidines. The local environment of histidines changes considerably during aggregation as shown by upfield shifts of the histidine 2H NMR signals[31] The lower fibrillization rate at lower pH where the histidines are protonated shows that it is not likely that they form salt bridges with aspartic acid residues in fibrils as suggested. In acidic solution the positively charged histidines can repel each other in structure of the forming fibril thus inhibiting the fibril formation. In order to find out which of the histidines are essential for Aβ fibrillization, the dependence of fibril elongation rate on the pH of the incubation medium was studied for three His to Ala mutants – H6A, H13A and H14A. The pH dependence of the fibrillization rate of H6A peptide was similar to that of the wild type Aβ suggesting that the protonation of H6 does not affect fibril formation. The fibrillization rates of H13A and H14A mutants were constant in the given pH range suggesting that only simultaneous protonation of both histidines H13 and H14 inhibits Aβ fibril formation.

### F. Fibrillization of the mixtures of Aβ42 with Aβ40

Aβ40 is the major form of the peptide in cerebral spinal fluid, however, the senile plaques consist mainly of Aβ42,[32] whereas *in vitro* studies show that they form mixed aggregates.[33] This phenomenon is difficult to explain considering that the peptides form interlaced amyloid fibrils

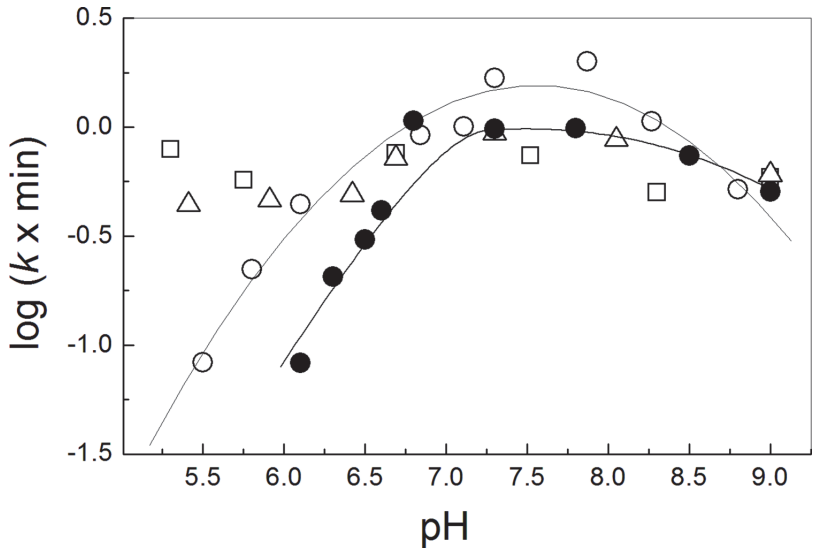


FIG. 5. **The effect of pH on the fibril formation of Aβ42.** The aggregation of 5 μM wt (●), H6A (○), H13A (□) or H14A (Δ) mutant of Aβ42 in 20 mM ammonium acetate, 100 mM NaCl with continuous agitation in the presence of 3.3 μM ThT at 25 °C at different pH values.

*in vitro*.[34] Our results showed that in the agitated solutions both peptides demonstrated similar fibrillization kinetics, the lag period for Aβ42 was only twofold shorter and the fibril growth rate twofold faster than that of Aβ40. The kinetic parameters of the fibrillization process depend on the ratio of Aβ40/42 in the mixture. Adding 10 % Aβ40 to Aβ42 did not affect the lag period, however 50% of Aβ40 elongated the lag period almost to the level observed with pure Aβ40. Importantly, 10 and 50 % Aβ42 did not increase the fibrillization rate that started to increase only when the Aβ42 content was 90%. The fibrils of Aβ40, Aβ42, and their mixtures showed similar structures in transmission electron microscopy (Fig. 6). It has been demonstrated earlier in experiments in quiescent solutions that Aβ40 inhibits Aβ42 fibrillization.[35,36] Considering that Aβ40 but not Aβ42 fibrils grow slower in quiescent solutions[7] the different behaviour of Aβ40/Aβ42 mixtures observed in different papers can be caused by different agitation conditions that is in agreement with the protective effect of Aβ40 in biological context.[37]

### G. Effect of denaturating agents on the Aβ fibrillization

Increasing concentrations of denaturating agents GdnCl (Fig. 7(a), 7(c)) and SDS (data not shown) decreased the fibrillization rate and also the final level of ThT fluorescence. As they also had a noticeable effect on the fluorescence of preformed fibrils, their effect on Aβ fibrillization at

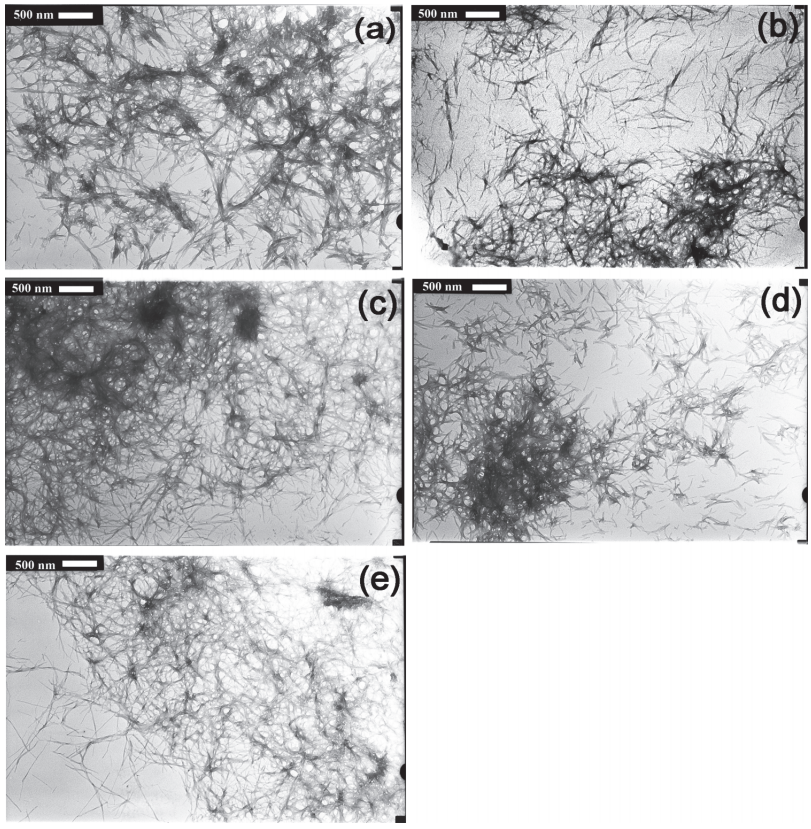


FIG. 6. **TEM images of Aβ samples.** The white bar on the pictures represents 500nm. 10μM Aβ40 (a); 10μM Aβ42 (b). Mixtures with total concentration 10μM and Aβ42 to Aβ40 ratio of 1:9 (c);5:5 (d); and 9:1 (e).

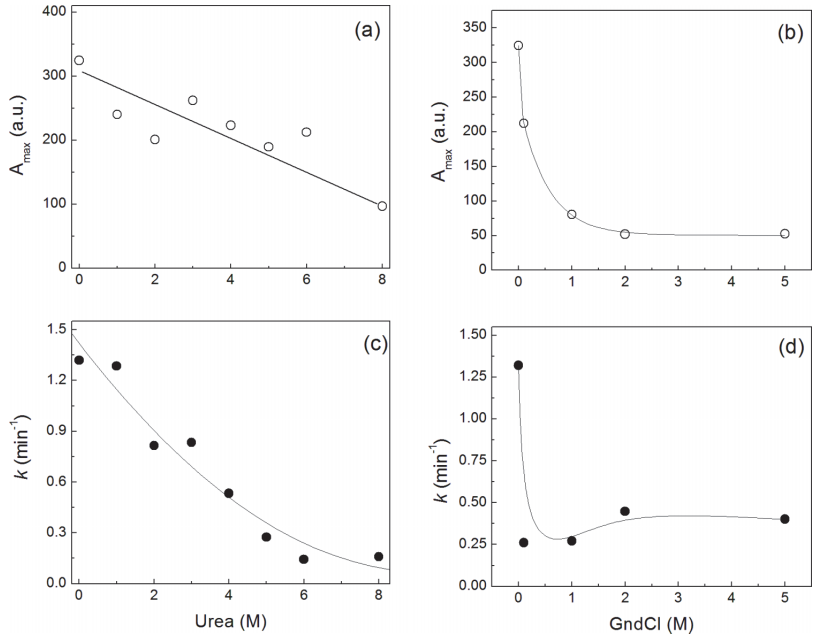


FIG. 7. **The effect of urea ((a) and (c)) and GndCl ((b) and (d)) on the fibril formation.** Aβ42 was aggregated in 20 mM HEPES, 100 mM NaCl, pH 7.4, with continuous agitation in the presence of 3.3 μM ThT at 40 °C with various urea or GndCl concentrations. ThT emission maximums at 480 nm (a) and (b), and $k$ values (c) and (d) were calculated using Boltzmann equation **(eqn. (1))**.

higher concentrations cannot be reliably studied by this method. However, both denaturating agents inhibited the fibrillization of Aβ42 peptide already at low concentrations.

In contrast urea had no effect on the final level of ThT fluorescence up to 6 M, thus, even 6 M does not shift the fibrillization equilibrium to higher free peptide concentrations. Below these concentrations urea had an inhibitory effect only on the fibrillization rate constant (Fig. 7). The inhibitory effect of urea on Aβ40 fibrillization has been demonstrated earlier at very high peptide concentrations[38] and therefore urea is not an efficient reagent for the prevention of fibrillization of peptide samples or solubilizing the fibrils.

### In vitro fibrillization as a model process for amyloid formation

*In vitro* fibrillization kinetics is studied in order to get valuable information about the characteristics of the process and to find clues for putative strategies to suppress the amyloid formation processes. Fibrillization consist of at least four phases where the dominating processes are (i) formation of initial fibrillization centers where fibrils start to grow (ii) their propagation; (iii) growth of fibrils and (iv) fibril maturation, respectively. The fibril growth corresponds to the *in vivo* situations where amyloid is already started to form in the brain of an AD patient. It seems that in this case the process can be suppressed by lowering the peptide concentration and stabilizing the peptide in the soluble state by the administration of compounds that form complexes with the peptide molecule. In this phase the *in vitro* process is a good model for the amyloid growth *in vivo*. The main factor determining the duration of the first phases is agitation that can multiply the number of growth centers, fibril ends, by rupture of the long fibrils. The initial stages of the reaction are difficult to study quantitatively since the variation in the lag periods is considerably high. Nevertheless, it is clear that the formation of the initial fibril is not completely stochastic. Hellstrand et al. studied the

concentration dependence of Aβ fibrillization and concluded that Aβ fibril formation arises from a sequence of events in a highly predictable manner.[15] Recently it was shown that the primary nucleation of α-synuclein fibrillization is enhanced more than three orders of magnitude by lipid bilayers.[39] Most likely the non-stochastic formation of primary seeds can occur on interfaces also in the case of *in vitro* studies of Aβ fibrillization. As a rule the fibrillization *in vitro* is initiated by preformed fibrillary seeds or by extremely high peptide concentrations where the solution contains nonfibrillar peptide aggregates that can transform to growing fibrils. Whether these mechanisms of formation of primary fibrils model of any processes in the brain is not known, the amyloid formation in the brain can also be triggered by other mechanisms such as for example metal induced Aβ aggregation.[40]

## IV. CONCLUSIONS

Fibrillization of Aβ consist of at least four phases (i) formation of initial fibrillization centers; (ii) their propagation; (iii) growth of fibrils; and (iv) fibril maturation. The effects of environmental factors on the kinetics of Aβ fibrillization in the agitated solutions suggest that:

1. The relatively small effect of peptide concentration on the Aβ fibrillization is not in agreement with the model suggesting dominance of primary nucleation in the bulk solution.
2. The physiological 10% content of Aβ42 in the Aβ solution does not significantly enhance fibrillization. The fibrillization of Aβ42 in agitated solutions is only 2.5 times faster than that of Aβ40.
3. The effects of temperature and solvents show that the fibril growth rate in the stationary phase is limited by conformational rearrangement of the peptide molecule during the binding to the fibril. Due to the relatively high $\Delta H^{\ddagger}$ value the fibrillization is very slow at low temperatures.
4. The fibrillization rate is decreased at pH below 6.5 due to the simultaneous protonation of His13 and His14.
5. Guanidinium chloride is a strong and urea is a very weak inhibitor of Aβ fibrillization.

## ACKNOWLEDGEMENTS

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## Publication II

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PLOS ONE

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

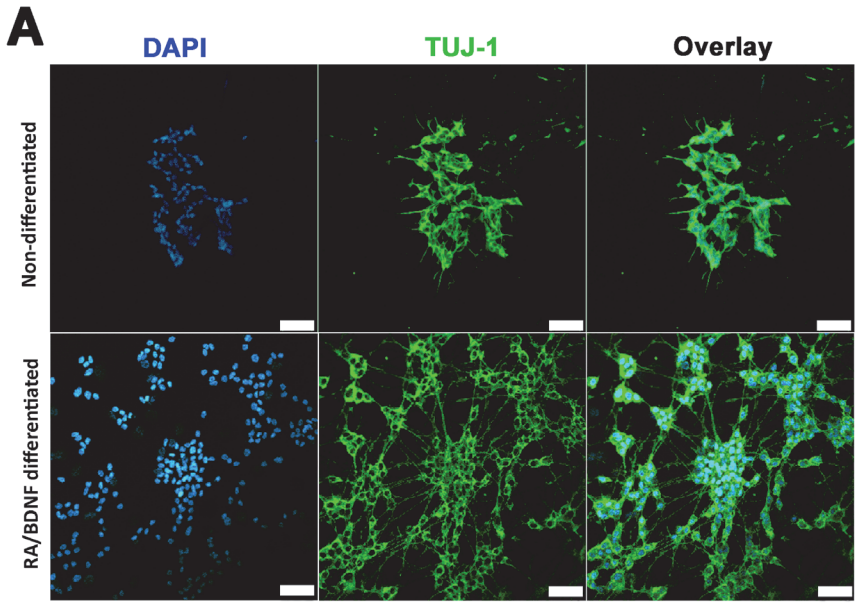


**Fig 1. RA/BDNF differentiated cells establish a neuron-like phenotype with long neurites.** (A) Immunocytochemistry of non-differentiated and RA/BDNF differentiated SH-SY5Y cells for DAPI (blue; left), anti-TUJ-1 (green; middle). Scale bar 50 μm.

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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

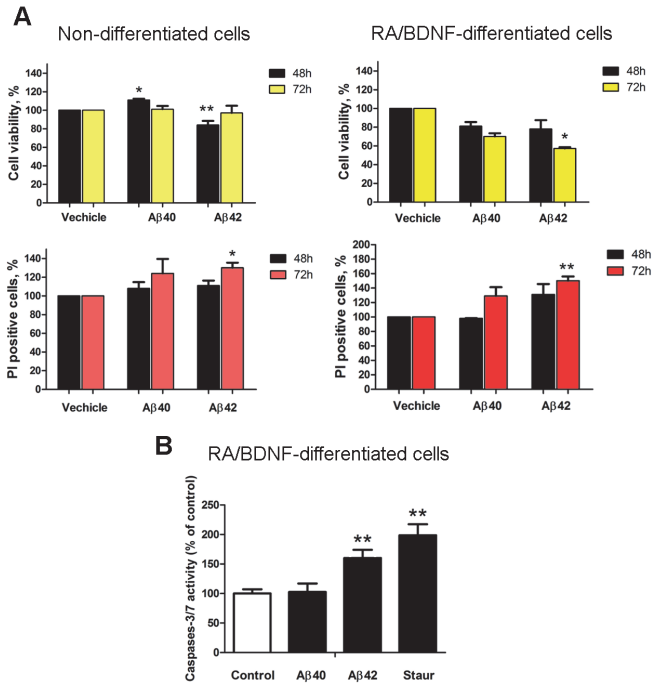


**Fig 2. Aβ reduces viability and activates caspases-3/7 in differentiated SH-SY5Y cells.** (A) Cell viability was measured with the WST-1 test and membrane integrity was measured using propidium iodide 48h and 72h after incubation with 20μM peptides. The figure displays the mean± SEM; at least $n = 3$ 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

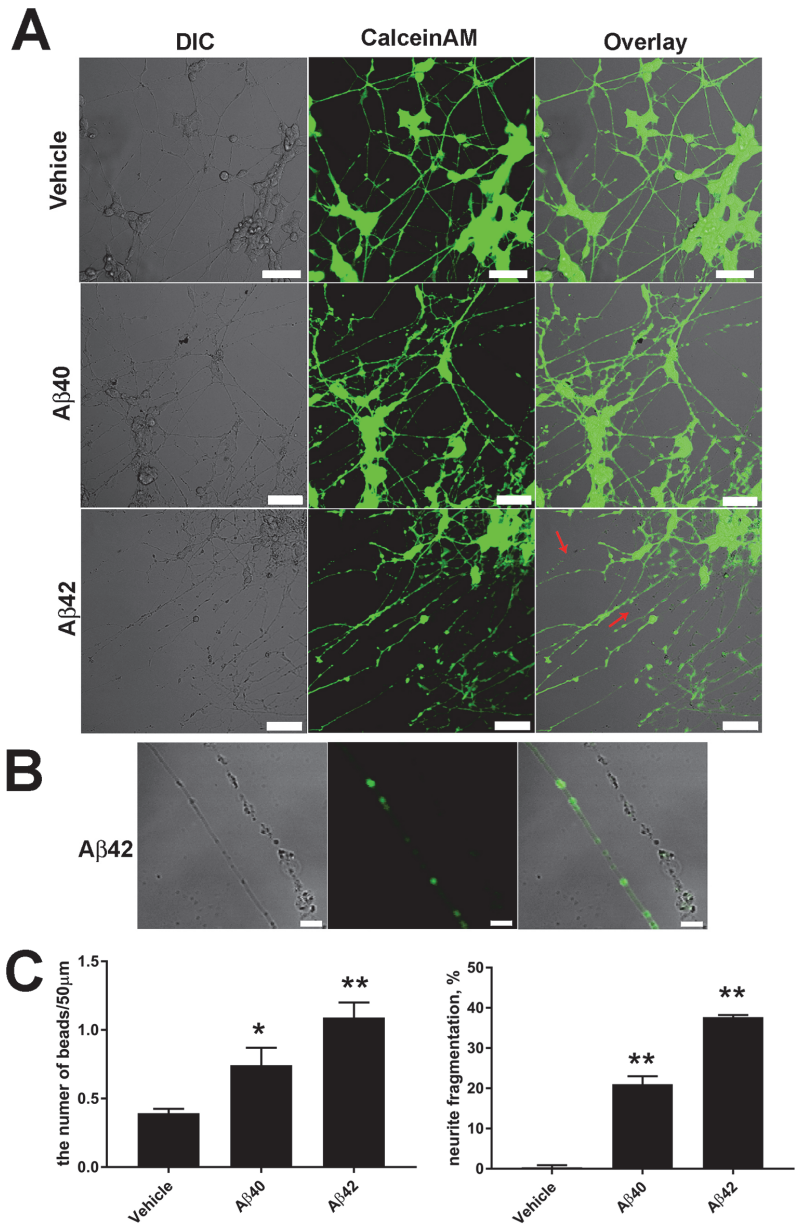


**Fig 3. Aβ40 and Aβ42 induce pathological changes in neurite morphology after 72 h.** (A) RA/BDNF differentiated live cell imaging for differential interference contrast (right), CalceinAM fluorescence (green; middle). Red arrows indicate fragmented neurites. Scale bar 20µm. (B) Greater magnification 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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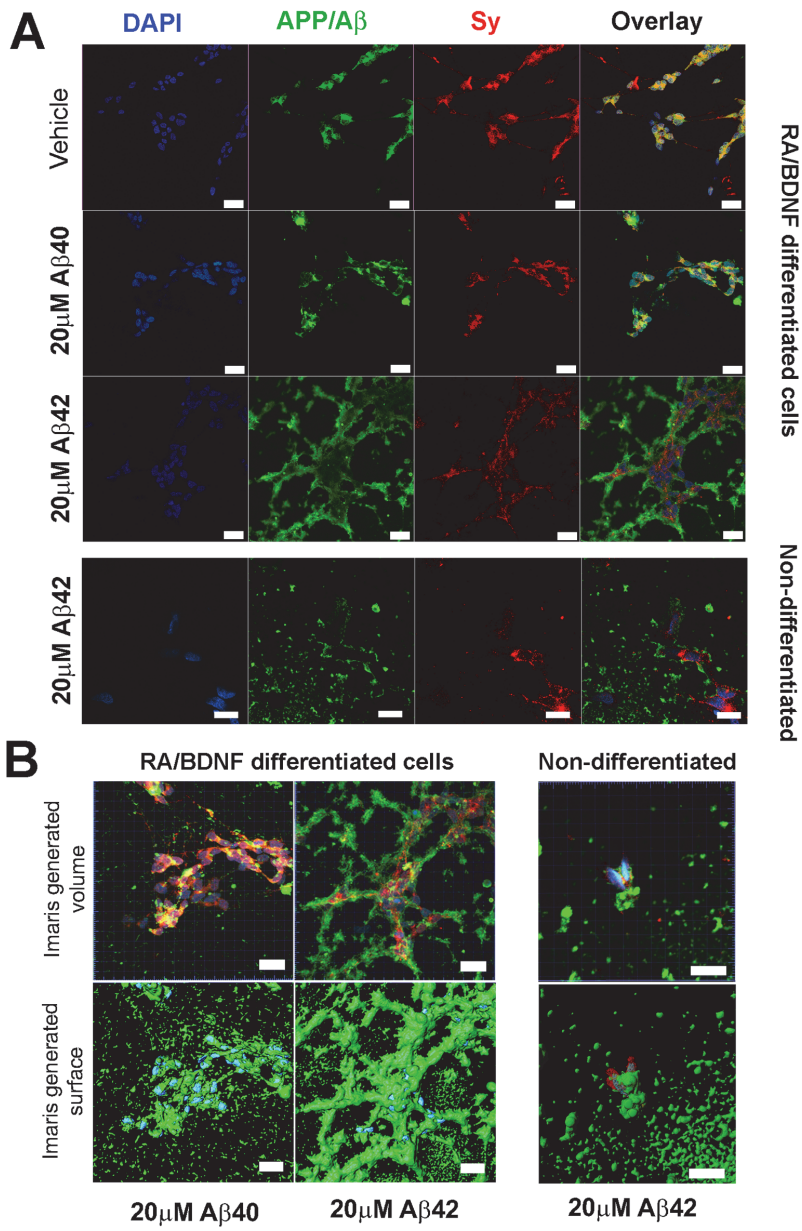


**Fig 4. Aβ42 covers cell bodies and neurites.** (A) Immunocytochemistry of non-differentiated and RA/BDNF differentiated SH-SY5Y cells after a 24h (Aβ42) and a 48h incubation (Vehicle; Aβ40) for DAPI (blue; left), anti-APP/Aβ (green; left middle), anti-Synaptophysin1 (red; right middle). Scale bar 20μm. (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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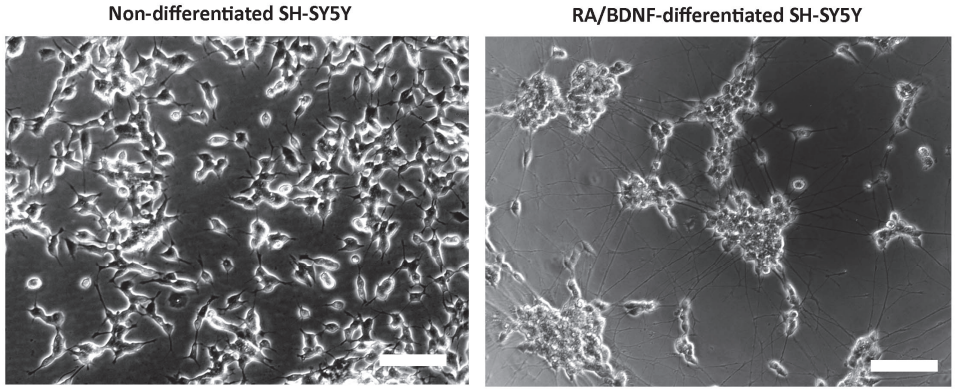
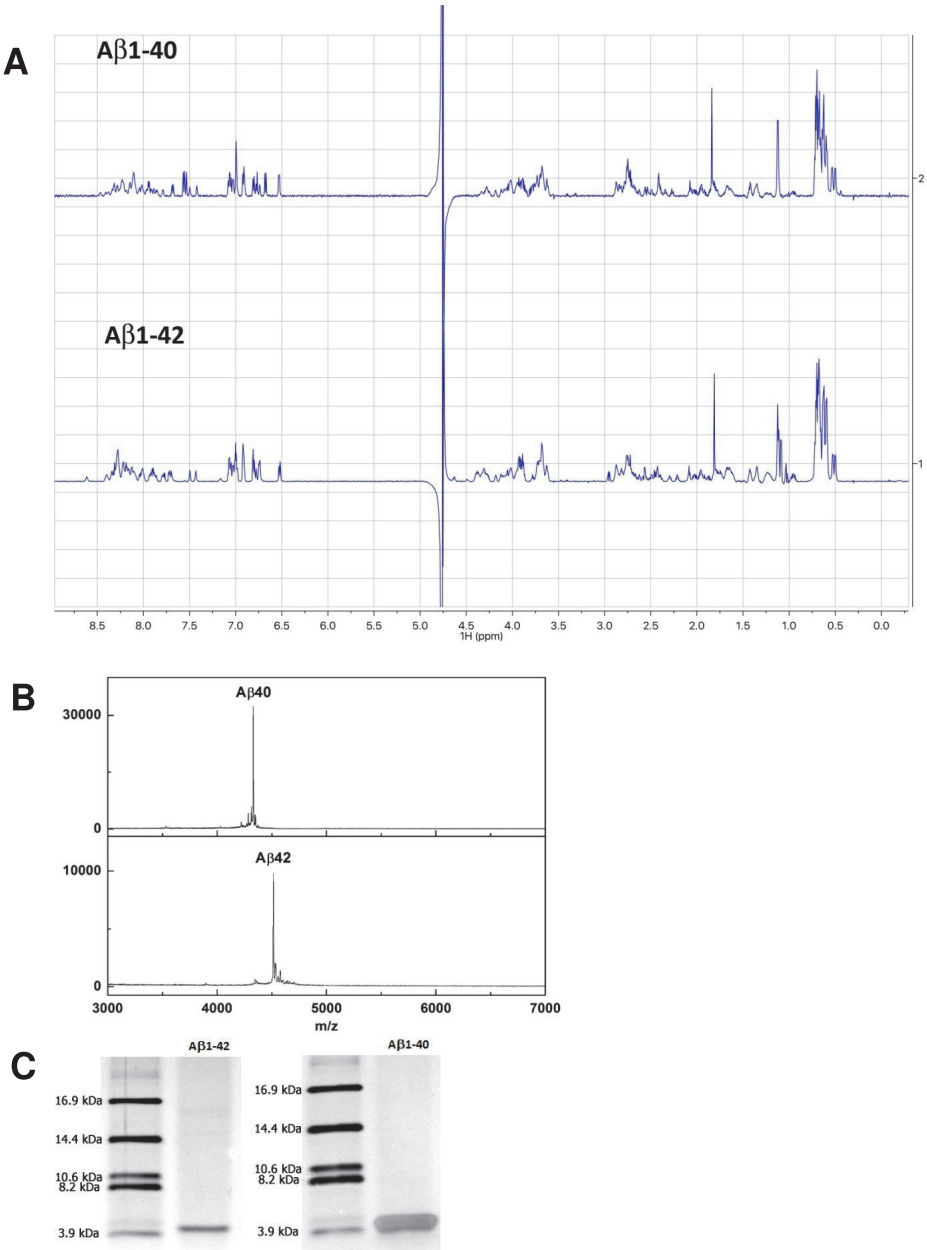
## S1 Fig.




**S1 Fig. Representative photograph of non-differentiated and RA/BDNF differentiated cells in phase contrast**[1]. Scale bar 100μM.

[1] Images obtained with Zeiss Axiovert 200M, 20x objective.

## S2 Fig.


A
Aβ1-40
Aβ1-42
2
1
8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
1H (ppm)
B
Aβ40
30000
0
Aβ42
10000
0
3000 4000 5000 6000 7000
m/z
C
Aβ1-42
Aβ1-40
16.9 kDa
14.4 kDa
10.6 kDa
8.2 kDa
3.9 kDa
16.9 kDa
14.4 kDa
10.6 kDa
8.2 kDa
3.9 kDa

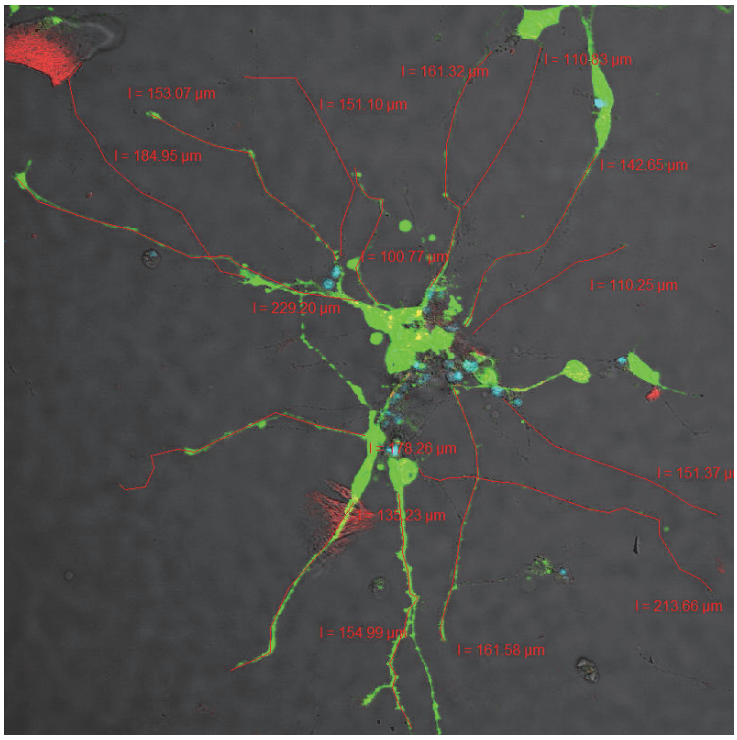
**S2 Fig. Amyloid beta quality control: NMR[2] spectra (A); MALDI-MS[3] (B); SDS-PAGE[4] (C)**

[2] Nuclear magnetic resonance (NMR) spectra were recorded at 278K and pH 7.4 in a Shigemi 5mm PMS3 tube with the spectrometer frequency of 800.13MHz. The length of the calibrated 90° pulse was set to8.9 $\mu$s (the total time of measurment was 7 min with the number of scans at 128) . The sample contained peptide at a concentration of 70 $\mu$M and was buffered with 50mM potassium phosphate and 5mM sodium hydroxichloride. The NMR experiments were performed on an Avance III 800 MHz spectrometer (Bruker, Karlsruhe, Germany), equipped with the 5-mm standard probe TXI 800 MHz S4 with Z-gradient.

[3] Matrix-assisted laser desorption mass spectrometry. Spectra were acquired by *Voyager-DE™ STR Biospectrometry Workstation* in linear mode using automated program. Instrument parameter/settings: accelerating voltage 25 000 V; mass range (m/z) 1500-10 000 Da; delay time 485 ns; grid voltage 93%; laser intensity 2200 V

[4] Sodium dodecyl sulfate poly acrylamide gel electrophoresis (SDS-PAGE) was performed using *Mini-PROTEAN TetraSystem* (Bio-Rad). Samples were mixed with loading buffer (0.36 M Bistris, 0.053 M Bicine, 15% glycerol, 1% SDS, 0.004% bromophenol blue), maintained at room temperature, applied to Bicine-Tris 15%T/5%C gel and resolved in a cathode buffer with 0.25% SDS (110V). Gels were fixated in glutar aldehyde/borate buffer solution for 45 minutes and stained with silver according to a protocol in Ref 1. Dunn, M.J. and S.J. Crisp, *Detection of proteins in polyacrylamide gels using an ultrasensitive silver staining technique.* Methods Mol Biol, 1994. **32**: p. 113-8.

## S3 Fig.

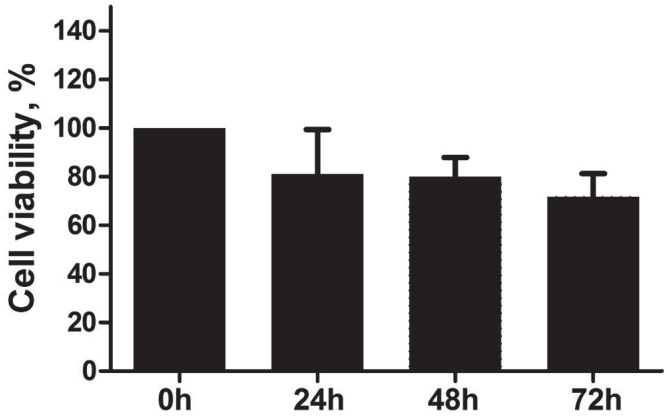



**S3 Fig. Representative snapshot of the process of the evaluation of neurite degeneration**[5]. The image is the overlay of three channels: DIC, Calcein AM (green), DAPI (blue).

[5] Photomicrographs of at least 12 random areas of neurites were taken using Zeiss Duo 510 META with 20X objective and 63X objective with oil immersion. Neurites were stained with CalceinAM (calcein-acetoxymethyl ester) for the analysis. General morphology of neurites was obtained with differential interference contrast (aperture DIC2) technique. The microscopy experiments were performed in an incubation chamber at $37^0$C in the presence of 5% $CO_2$.

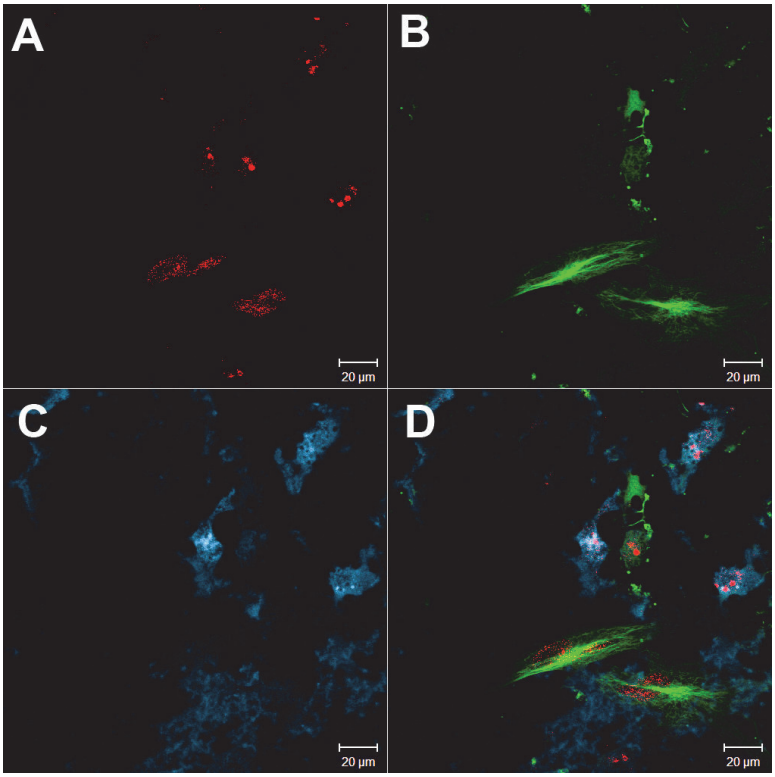
Photomicrographs of neurites in randomly selected areas were stored and processed using LSM Image Browser software. The method of evaluation of neurite degeneration was adopted from Ref. 2. Kawataki, T., et al., *Neuronal maturation-associated resistance of neurite degeneration caused by trophic factor deprivation or microtubule-disrupting agents.* Brain Res, 2008. **1230**: p. 37-49. 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.

## S4 Fig.




**S4 Fig. WST-1 test results on non-differentiated cells without serum** (See Materials and Methods). Data are shown as mean± SEM

## S5 Fig.

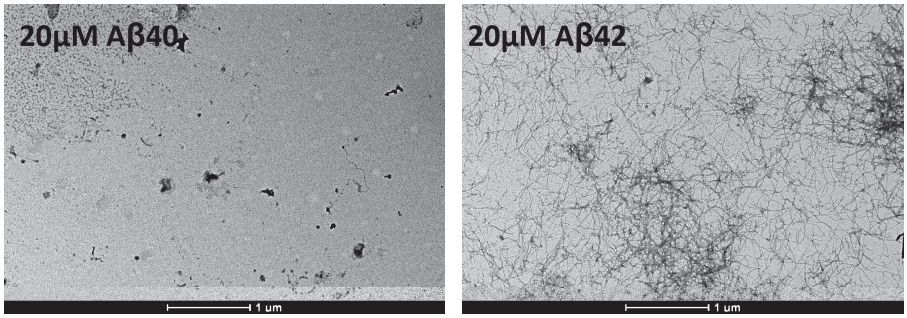



**S5 Fig. Representative photograph of RA-differentiated SH-SY5Y cells after incubation with 20µM Aβ42**. *In case of RA differentiation cell culture is not enough homogenic. In the photograph several cells are not amyloid beta-affected: microtubules are intact. At the same time, nuclear fragmentation and microtubule disruption can be detected.* (A) Nuclei staining with PI[6] (red) showing nuclear fragmentation (B) βIII-tubulin staining (green) showing different cell types, (C) Fluorescamine-stained Aβ42[7] (blue), (D) merged image. Magnification: 630X, scale bar: 20µm

[6] Methanol-fixed cells were stained with propidium iodide/RNAse solution for 10 min.

[7] Peptides were stained with fluorescamine (FC, Sigma) for microscopy. FC is a non-fluorescent dye that reacts with primary amines to form a fluorescent product. Defibrillized peptides were dissolved in 10 mM NaOH and then FC stock in acetonitrile (Sigma) was added in 2-fold higher concentration than peptide concentration. The mixture was incubated for 10 min at RT in dark and afterwards diluted in 40 mM HEPES/200 mM NaCl buffer (pH 7,3) for suitable concentration and applied on cell culture.

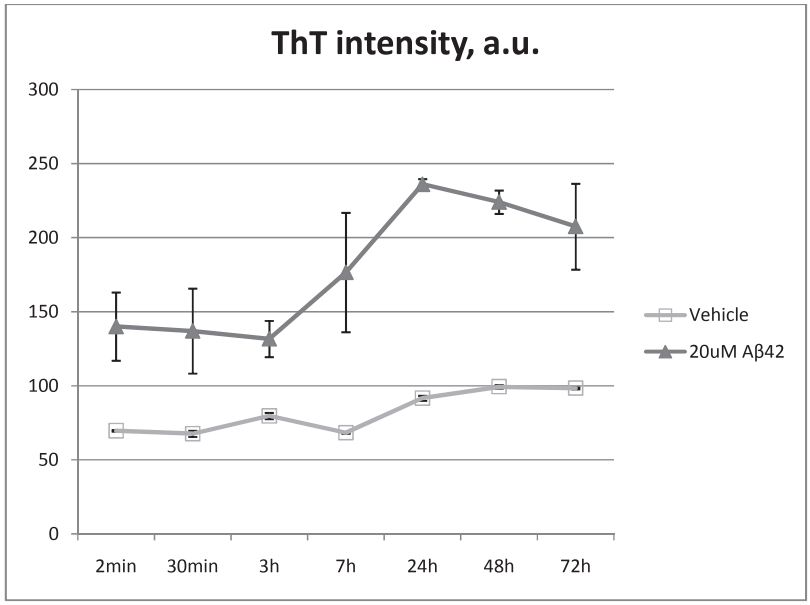
## S6 Fig.




**S6 Fig. Transmission electron microscopy[8] of cell medium after a 48 h incubation with amyloid peptides**. Scale bar 1 µm.

8 Transmission Electron Microscopy Imaging An aliquot of 5 µl of sample was loaded on a Formvar-coated, carbon-stabilized copper grid (300 mesh from Ted Pella Inc., Redding CA, USA). After 1 min, the excess solution was drained off using a Whatman filter paper (Thermo Fisher). The grid was briefly washed and negatively stained with 5 µl of 2% uranyl acetate. The grid was air-dried and the transmission electron microscopy (TEM) images were recorded on a Tecnai G2 BioTwin transmission electron microscope (FEI, Japan) operating with an accelerator voltage of 120 kV, 1.53 nA. Typical magnifications ranged from 4800x to 18 500x.

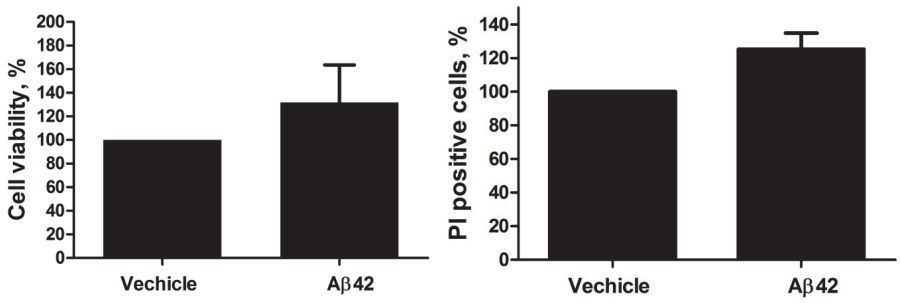
## S7 Fig.




**S7 Fig. Detection of amyloid aggregation by Thioflavin T in the cell medium**[9]. Vehicle is the HEPES buffer without peptide (See Materials and Methods). The error bars are displayed with ±SD; n=3

[9] Aβ stock mixtures (See materials and methods) were diluted in DMEM without serum to a final peptide concentration of 20 μM and 5 μM Thioflavin T was added. The white 96 well plate was used for the experiments and incubated in the cell incubator at 37°C 5% CO2. The fluorescence of binded ThT were measured by a Tecan Microplates reader, series 750 (ex 405nm em 485nm).

## S8 Fig.




**S8 Fig. Cell viability after a 72h incubation with 20μM previously formed fibrils**[10] **measured with the WST-1 test and membrane integrity counted with the propidium iodide permeabilization tests** (See Materials and Methods). Data are shown with the mean± SEM, n=3.

[10] The fibrills were performed according to the protocol in Ref 3. Tiiman, A., et al., *In vitro fibrillization of Alzheimer's amyloid-beta peptide (1-42).* AIP Advances, 2015. **5**(9): p. 092401. After fibrillization detection, the solutions were collected and stored overnight at room temperature, then centrifuged at 10 000rcf for 10min at 4°C. The pellets were resuspended in the buffer (See Materials and Methods) and applied on cells.

## S1 Table: Non-differentiated SH-SY5Y cells, cell viability WST-1 test.

| | 48h | | 72h | |
|---|---|---|---|---|
| Vehicle | Aβ40 | Aβ42 | Aβ40 | Aβ42 |
| 100% | 112.7 | 83.9 | 96.9 | 96.7 |
| | 114.8 | 88.9 | 99.8 | 111.1 |
| | 106.1 | 91.2 | 91.2 | 83.7 |
| | 111.0 | 70.6 | 112.1 | |
| | 109.1 | | 106.8 | |
| Average | 110.7 | 83.6 | 101.4 | 97.1 |
| SEM | 1.5 | 4.6 | 3.7 | 7.9 |

## S2 Table: Non-differentiated SH-SY5Y cells, propidium iodide test.

| | 48h | | 72h | |
|---|---|---|---|---|
| Vehicle | Aβ40 | Aβ42 | Aβ40 | Aβ42 |
| 100% | 118.2 | 101.2 | 154.5 | 119.5 |
| | 95.0 | 117.1 | 102.0 | 133.1 |
| | 110.4 | 96.3 | 116.6 | 138.3 |
| | | 117.1 | | |
| | | 125.1 | | |
| Average | 107.9 | 111.4 | 124.4 | 130.3 |
| SEM | 6.8 | 5.4 | 15.6 | 5.6 |

## S3 Table: RA/BDNF-differentiated SH-SY5Y cells, cell viability WST-1 test.

| | 48h | | 72h | |
|---|---|---|---|---|
| Vehicle | Aβ40 | Aβ42 | Aβ40 | Aβ42 |
| 100% | 89.8 | 81.5 | 67.1 | 54.9 |
| | 82.0 | 109.4 | 77.0 | 57.4 |
| | 71.7 | 38.4 | 66.2 | 59.6 |
| | | 69.0 | | |
| | | 80.2 | | |
| | | 86.9 | | |
| Average | 81.2 | 77.6 | 70.1 | 57.3 |
| SEM | 4.6 | 9.5 | 3.4 | 1.3 |

## S4 Table: RA/BDNF-differentiated SH-SY5Y cells, propidium iodide test.

| | 48h | | 72h | |
|---|---|---|---|---|
| Vehicle | Aβ40 | Aβ42 | Aβ40 | Aβ42 |
| 100% | 97.7 | 137.5 | 97.9 | 154.2 |
| | 98.5 | 183.9 | 133.3 | 158.4 |
| | 97.1 | 100.0 | 157.3 | 138.6 |
| | | 119.4 | 127.9 | |
| | | 112.8 | | |
| Average | 97.7 | 130.7 | 129.1 | 150.4 |
| SEM | 0.4 | 14.6 | 12.2 | 6.0 |

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

| Vehicle | Aβ40 | Aβ42 | Staur |
|---|---|---|---|
| 100% | 90.4 | 146.8 | 192.0 |
| | 110.7 | 175.0 | 227.1 |
| | 109.8 | 173.7 | 202.2 |
| | 117.8 | 158.6 | 198.1 |
| | 84.1 | 146.9 | 203.1 |
| | | | 169.7 |
| Average | 102.6 | 160.2 | 198.7 |
| SD | 14.5 | 13.8 | 18.6 |

## S6 Table: The number of beads per 50μM of neurite length after 72h with 20μM peptide.

| | Vehicle | Aβ40 | Aβ42 |
|---|---|---|---|
| | 0.4 | 0.9 | 1.2 |
| | 0.4 | 0.7 | 1.0 |
| | 0.4 | 0.7 | 1.1 |
| Average | 0.4 | 0.8 | 1.1 |
| SEM | 0.0 | 0.1 | 0.1 |

## S7 Table: Proportion of fragmented neurites per area after 72h with 20µM peptide.

| | Vehicle | Aβ40 | Aβ42 |
|---|---|---|---|
| | 1 | 23 | 38 |
| | 0 | 21 | 37 |
| | 0 | 19 | 38 |
| Average | 0.6 | 22.4 | 37.6 |
| SEM | 0.6 | 1.0 | 0.4 |

## References:

1. Dunn, M.J. and S.J. Crisp, *Detection of proteins in polyacrylamide gels using an ultrasensitive silver staining technique.* Methods Mol Biol, 1994. **32**: p. 113-8.
2. Kawataki, T., et al., *Neuronal maturation-associated resistance of neurite degeneration caused by trophic factor deprivation or microtubule-disrupting agents.* Brain Res, 2008. **1230**: p. 37-49.
3. Tiiman, A., et al., *In vitro fibrillization of Alzheimer's amyloid-beta peptide (1-42).* AIP Advances, 2015. **5**(9): p. 092401.

## Publication III

Krishtal, J.; Metsla, K.; Bragina, O.; Tõugu, V.; Palumaa, P. (2019). Toxicity of amyloid beta peptides varies depending on differentiation route of SH-SY5Y cells. J Alzheimers Dis. 2019;71(3):879-887; DOI 10.3233/JAD-190

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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 predifferentiated 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

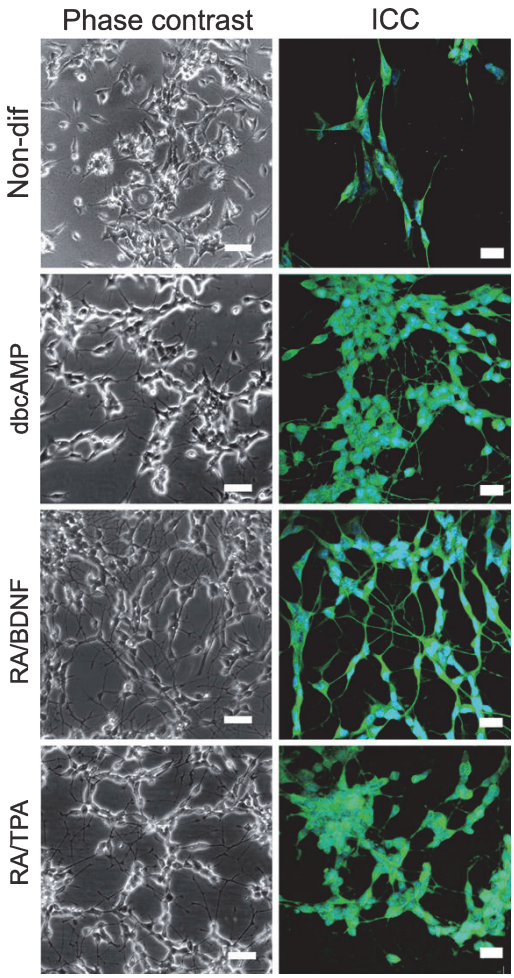


Fig. 1. Visualization of the differentiated SH-SY5Y cells. Phase contrast (left column) images obtained with Zeiss Axiovert 200 M, Axiocam MRc5 with 20x A-Plan with Ph2. Scale bar 50 μm. Immunohistochemistry for TUJ (right column) with counterstained nuclei (DAPI) were visualized with the confocal microscope 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 ($1.1 \pm 0.1$ 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 $1.8 \pm 0.2$ 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 ($1.94 \pm 0.1$ fold increase over control) (Fig. 2D), incubation with 10 μM $A\beta_{42}$ caused $1.37 \pm 0.1$ 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 ($59.4 \pm 5.3$ %, $p < 0.0002$) 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 $1.8 \pm 0.2$ fold increase over control, whereas 20 μM $A\beta_{42}$ displayed lower $1.6 \pm 0.2$ 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

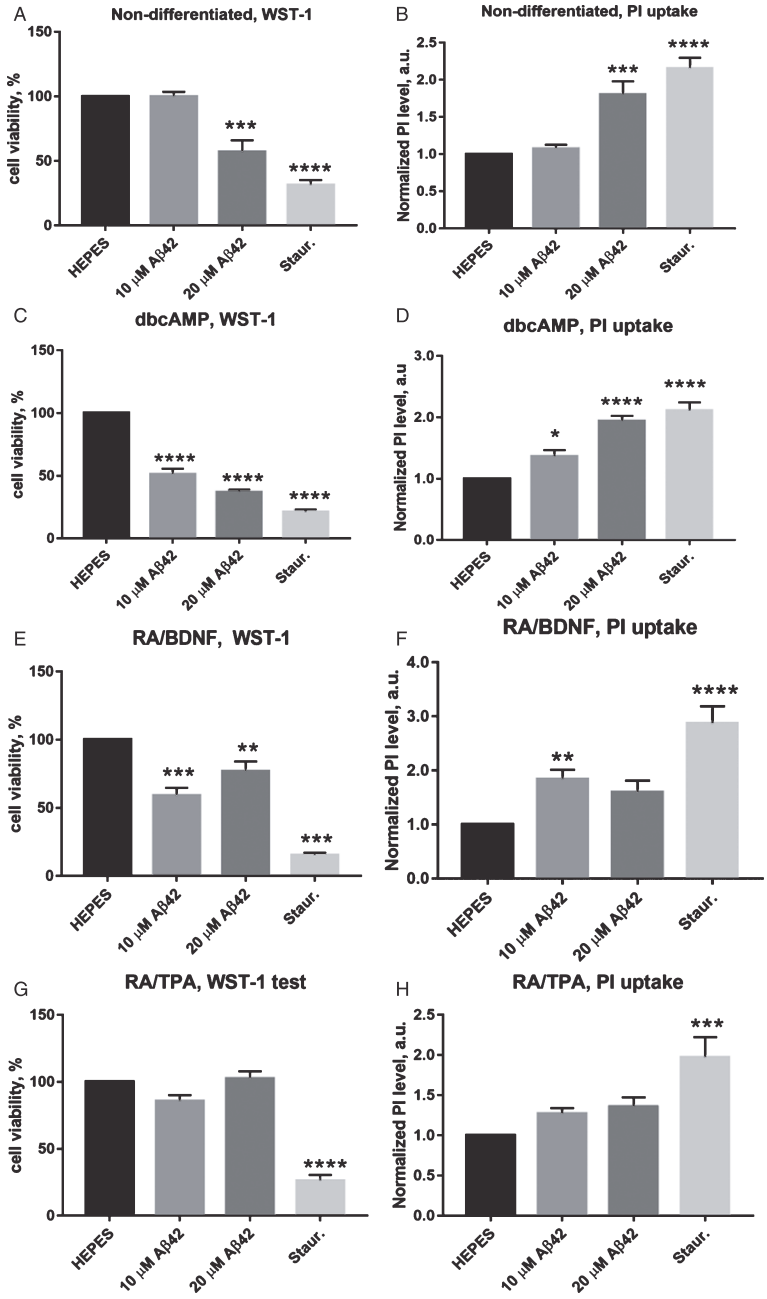


Fig. 2. Quantitation of viable and dead cells after 48 h incubation with $A\beta_{42}$. WST-1 viability test results are on panels A, C, E and G. PI uptake estimation results are on panels B, D, F and H. Negative control: HEPES buffer; positive control: staurosporine. The figure displays the mean ± SEM; $n = 4$ 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

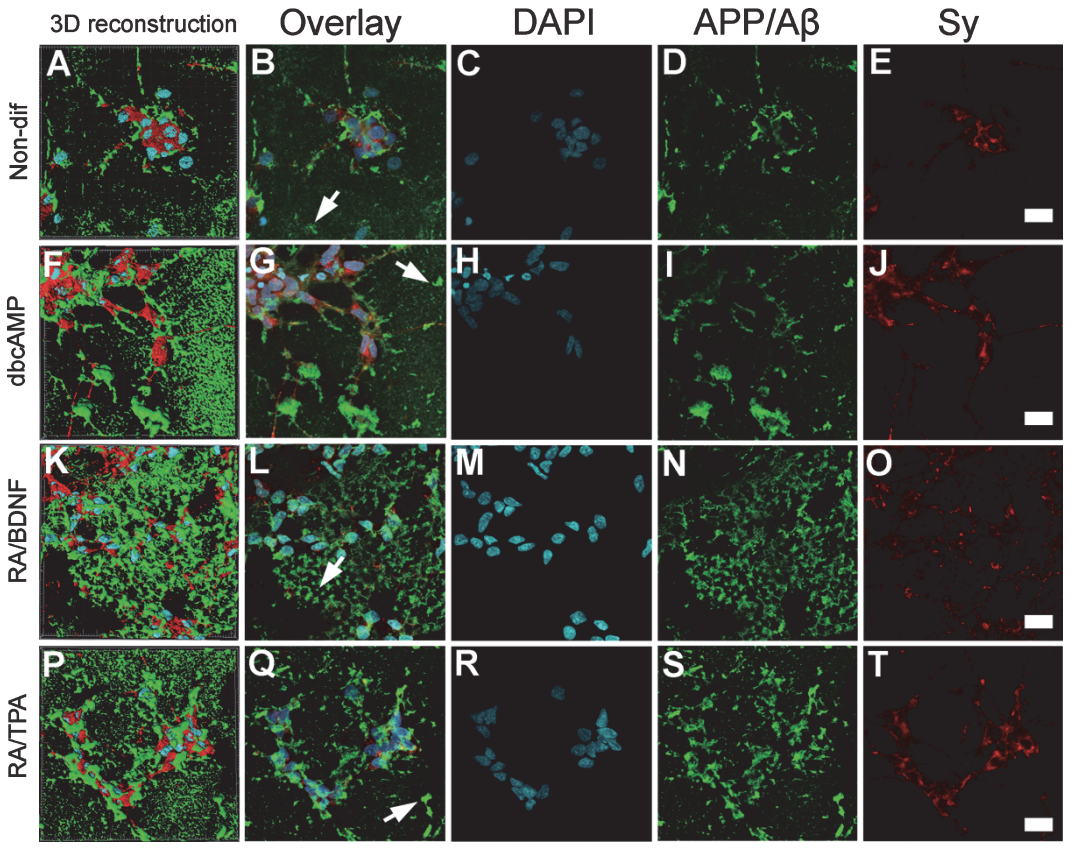


Fig. 3. Distribution of Aβ in cell cultures after 24 h incubation. Immunocytochemistry of 10 μM $A\beta_{42}$-treated SH-SY5Y cells for APP/Aβ (panels D, I, N, and S) and Sy (panels E, J, O, and T) with DAPI counterstained nuclei (panels C, H, M, and R) visualized with the confocal microscope Zeiss Duo 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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## 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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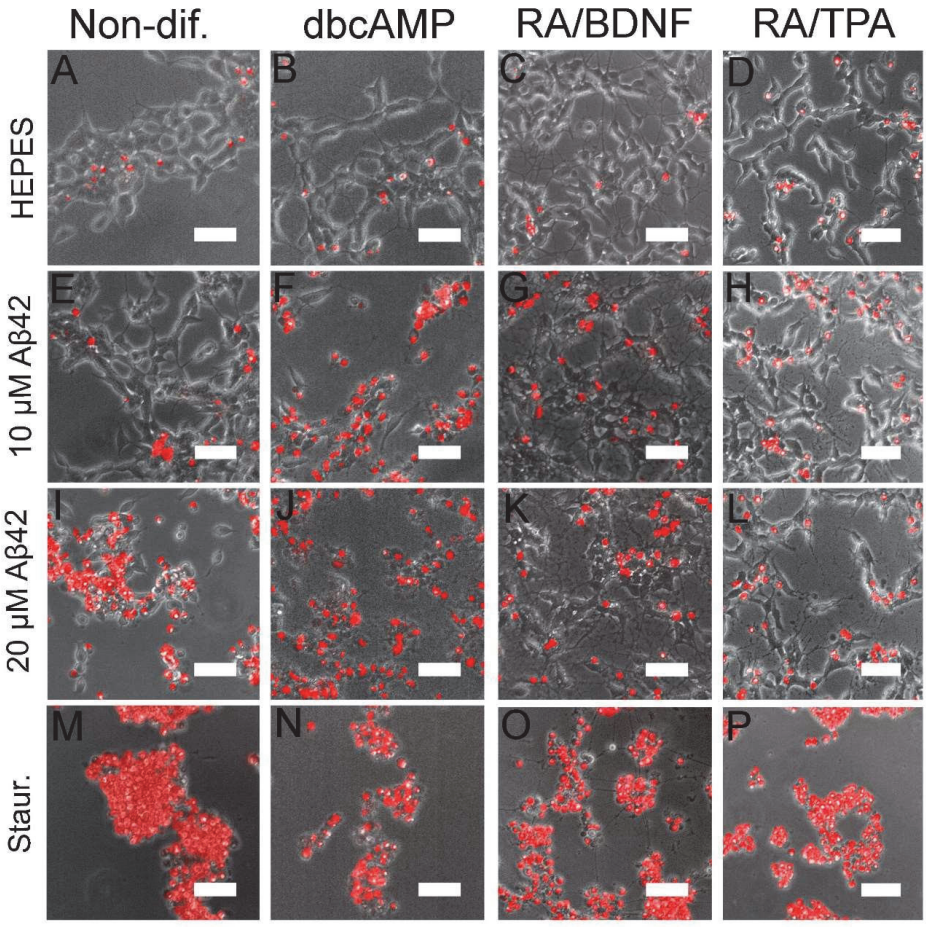
**Supplementary:**

**Supplementary figure 1:** Phase contrast overplayed with fluorescence of PI after 48h incubation with Aβ42 peptide, Non-differentiated, dbcAMP, TPA and BDNF-differentiated SH-SY5Y cells. A-D: Negative control- HEPES; E-H: 10 μM Aβ42; I-L: 20 μM Aβ42; M-P: Positive control- 1 μM staurosporine. Images were acquired with Zeiss Axiovert 200M, Axiocam MRc5 with 20x A-Plan with Ph2. Scale bar 50μm.



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Krishtal, J.; Metsla, K.; Bragina, O.; Tõugu, V.; Palumaa, P. (2019). Toxicity of amyloid beta peptides varies depending on differentiation route of SH-SY5Y cells. J Alzheimers Dis. 2019;71(3):879-887; DOI 10.3233/JAD-190705

Kubo A-L., Vasiliev G., Vija H., Krishtal J., Tõugu V., Kahru A., Bondarenko O. M. Surface carboxylation or PEGylation decreases CuO nanoparticles' cytotoxicity to human cells in vitro without compromising their antibacterial properties (Submitted)

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Kristel Metsla, Batchelor's degree, 2015, (sup) Vello Tõugu; Jekaterina Krishtal; *„Alzheimeri amüloid-β peptidide toksilisus diferentseeritud inimese neuroblastoomi rakkudele".* Tallinn University of Technology School of Science, Department of Chemistry and Biotechnology

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Märts 2019 posterettekanne „Cell differentiating agents alter sensitivity of SH-SY5Y cells to amyloid beta"; 14th International Conference on AD/PD, Lisbon, Portugal

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Tiiman, A., A. Noormägi, M. Friedemann, J. Krishtal, P. Palumaa and V. Tõugu (2013). „Effect of agitation on the peptide fibrillization: Alzheimer's amyloid-beta peptide 1-42 but not amylin and insulin fibrils can grow under quiescent conditions." J PeptSci19(6): 386-391. DOI: 10.1002/psc.2513

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Krishtal, J.; Metsla, K.; Bragina, O.; Tõugu, V.; Palumaa, P. (2019). Toxicity of amyloid beta peptides varies depending on differentiation route of SH-SY5Y cells. J Alzheimers Dis. 2019;71(3):879-887; DOI 10.3233/JAD-190705

Kubo A-L., Vasiliev G., Vija H., Krishtal J., Tõugu V., Kahru A., Bondarenko O. M. Surface carboxylation or PEGylation decreases CuO nanoparticles' cytotoxicity to human cells in vitro without compromising their antibacterial properties (Submitted)

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