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Screening of plant-based natural products for reactive astrogliosis in Alzheimer's disease

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Abstract

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder, that involves the deposition of extracellular amyloid-beta (Aβ) plaques, the intracellular aggregation of hyperphosphorylated tau as well as the onset of chronic neuroinflammation along with synaptic dysfunction. Despite extensive research, effective treatment options remain elusive. Astrocytes, key regulators of neuroinflammation and Aβ processing, are emerging as promising therapeutic targets. The accumulation of pyroglutamylated Aβ (Aβ3(pE)-42) in astrocytes triggers glial activation and the release of pro-inflammatory cytokines, ultimately contributing to early synaptic dysfunction in AD. However, the mechanisms of Aβ3(pE)-42 uptake to astrocytes are unknown and pharmacological interventions interfering with this process are lacking.

Plant-based natural products, such as polyphenols, hold promise for AD therapy due to their anti-inflammatory and antioxidant properties. This study aimed to identify plant substances modulating astrocytic responses to Aβ3(pE)-42. Optimal culture conditions for primary astrocytes were established, and astrocytic Aβ3(pE)-42 uptake was confirmed without significant changes in reactivity markers. A screening assay identified five polyphenols (quercetin, epicatechin, rutin, apigenin, resveratrol) that reduce Aβ3(pE)-42 uptake, with quercetin showing protective effects against early synaptic loss in neuron-glia co-cultures and long-term potentiation impairment in acute mouse hippocampal slices. Taken together, this work highlights the potential of plant-derived polyphenols in mitigating astrocyte-related cellular dysfunctions in AD.

Keywords

Astrocytes, Alzheimer's disease, amyloid-β, quercetin, synaptic plasticity

Abbreviations

Aβ	- Amyloid-β
Aβ3(pE)-42	- Pyroglutamylated amyloid-β
AD	- Alzheimer's disease
APP	- Amyloid precursor protein
BBB	- Blood-brain-barrier
CSF	- Cerebrospinal fluid
CNS	- Central nervous system
CREB	- cAMP response element-binding protein
DAPI	- 4',6-Diamidin-2-phenylindol
DIV	- Day in vitro
DMEM	- Dulbecco's Modified Eagle Medium
DMSO	- Dimethyl sulfoxide
fEPSP	- Field excitatory postsynaptic potential
FOV	- Field of view
GFAP	- Glial fibrillary acidic protein
HEK-293T	- Human embryonic kidney-293T cells
HFIP	- 1,1,1,3,3,3-Hexafluoro-2-propanol
IBA1	- Ionized calcium-binding adapter molecule 1
IFNγ	- Interferon-γ
IL1α	- Interleukin 1α
LPS	- Lipopolysaccharide
LTP	- Long-term potentiation
MCI	- Mild cognitive impairment
NF-κB	- Nuclear factor-kappa B
NLRP3	- NOD-like receptor family pyrin domain containing 3
NMDAR	- N-methyl d-aspartate receptor
NO	- Nitric oxide
OHSC	- Organotypic hippocampal slice cultures
PET	- Positron emission tomography
PFA	- Paraformaldehyde
PI	- Propidium iodide
PTM	- Post-translational modification
ROI	- Region of interest
ROS	- Reactive oxygen species
STAT1	- Signal transducer and activator of transcription 1
TNFα	- Tumor necrosis factor α
TGF-β1	- Transforming growth factor β1
WHO	- World Health Organization

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1. Introduction

1.1 Alzheimer's disease

1.1.1 Epidemiology, clinical characteristics and diagnostics

Alzheimer's disease (AD) is a progressive, neurodegenerative disease and as the most common cause of dementia still a devastating diagnosis for patients and their families. Clinically, AD is characterized by progressive cognitive decline, impaired episodic memory, decreasing spatial and temporal orientation, impaired communication skills resulting in a loss of personality, absolute dependency on constant care and ultimately a reduced life expectancy [1, 2]. Although the disease was first described by the German psychiatrist Alois Alzheimer about 100 years ago, the etiology and pathogenesis of sporadic AD are still under debate and the disease remains incurable. With the worldwide increase of life expectancy and with AD as the seventh leading cause of death globally, the number of people at risk increase rapidly, making the disease one of the leading contributors to rising health care costs [3, 4, 2]. AD follows a prolonged, progressive disease course that begins with pathophysiological changes in the brains of affected individuals years before any clinical manifestations are observed. It elicits neuropathological hallmarks such as the deposition of extracellular amyloid-beta (Aβ) plaques, the intracellular aggregation of hyperphosphorylated tau protein in neurofilament tangles, chronic neuroinflammation, neuronal dysfunction and subsequent cognitive decline [2, 5, 1, 6, 3].

Patients presenting with AD symptoms generally undergo a variety of different tests, including basic neurological examination, cognitive short (MMST, MoCa, DemTect etc.) and long (CERAD, ADAS-cog, SIDAM etc.) tests to evaluate severity, progression and also propose differential diagnoses. Besides clinical and cognitive evaluation, the so-called ATX-(N) classification system uses highly accurate fluid and neuroimaging biomarkers to diagnose AD more precisely [7]. ATX-(N) is a precise classification method, that categorizes patients based on biomarkers reflecting key AD pathophysiological features, namely Aβ deposition (A), tau-mediated pathophysiology (T) and neurodegeneration (N). The X represents novel candidate biomarkers involved in neuroinflammation, synaptic dysfunction or blood-brain-barrier (BBB) alterations. The ATX-(N) criteria combine different imaging and fluid (Cerebrospinal fluid (CSF), blood serum) biomarkers, with (A) biomarkers including cortical amyloid-positron emission

tomography (PET) or low CSF Aβ42, (T) including tau-PET and CSF phosphorylated tau levels and (N) represented by brain atrophy on MRI, ^{18}F-Fluordesoxyglukose (FDG) -PET or CSF total tau. The category (N) is still evolving with the possibility of including emerging biomarkers, e.g., of reactive astrogliosis in the near future. The ATX-(N) classification system was primarily designed to establish a common language in the research community and is therefore not directly applicable to clinical practice. Nonetheless, it summarizes and provides a structured overview over the main diagnostic criteria of AD [7, 5, 8, 3].

1.1.2 Pathophysiology and amyloid hypothesis

Although the exact pathophysiological mechanisms of AD are still not completely understood, the so called "amyloid-hypothesis" currently presents a promising explanation. In its strongest form it states, that oligomerization and deposition of Aβ in the brain is the initiating event of AD pathogenesis and thus causative for disease onset and progression. Some of the most severe consequences of extracellular Aβ accumulation include neuronal and glial cytopathology in brain regions serving memory and cognition, ultimately leading to cell death and progressive cognitive impairment [9, 6, 1, 2]. Aβ is a cleavage product of the amyloid precursor protein (APP), which, when cleaved by β- and γ-secretases in the so-called "amyloidogenic pathway" results in the formation of Aβ1-40 and Aβ1-42, with 40 and 42 amino acids in length respectively. After cleavage, Aβ monomers often aggregate forming di- and trimers, small oligomers, protofibrils, fibrils and finally the insoluble amyloid plaques, characteristically found as deposits in the brains of AD patients. Although some Aβ peptides are believed to be essential for neuronal survival in picomolar concentrations, small soluble oligomers appear to be the most toxic species with the potential to spread throughout the entire brain, thus contributing to disease progression [2, 10, 11].

One key pathophysiological element of AD is the dyshomeostasis of Aβ production and clearance resulting in intracerebral aggregation of Aβ fibrils in senile plaques. While the gradually rising levels of Aβ in familial AD are generally caused by defined genetic mutations triggering an increased Aβ production, the more common sporadic forms of AD are largely driven by a failure of Aβ clearance [6]. Aβ clearance depends on different pathways including enzyme degradation, removal by glia, crossing of the BBB and interstitial fluid or CSF transportation, all of which can be altered in aged and diseased brains. It has for example been

shown that the expression of different blood efflux transporters in the BBB was reduced in AD brain tissue, while certain influx transporters showed elevated expression, which might ultimately contribute to intracerebral Aβ accumulation [10, 12].

1.1.3 Posttranslational modifications (PTMs) of Aβ

Although there is little doubt among researchers that the amyloid hypothesis is central to the pathology of AD, targeting amyloid plaques with various therapeutic approaches has not yet led to a successful cure of the disease. Additionally, different studies suggest, that the total amyloid load in the brain does not directly correlate with the individual's cognitive capabilities [13–15]. One possible explanation focuses on the involvement of post-translationally modified forms of Aβ in the progression of the disease. Common transformations of Aβ include isomerization, truncation, pyroglutamylation, phosphorylation, nitration, racemization and more ***(Fig. 1)***. Many of these modifications increase the protein's neurotoxicity, amyloidogenicity or tendency to aggregate [1, 16]. One of the most common PTMs of Aβ is the pyroglutamylated Aβ3(pE)-42, which is formed via n-terminal truncation and subsequent cyclization of the free glutamate residue on position 3, catalyzed by an enzymatic reaction of the glutaminyl cyclase [13, 17]. Aβ3(pE)-42 has been described as a major constituent of Aβ deposits associated with human AD while being absent in normal aging. Further, the protein has been shown to seed highly toxic co-oligomers with conventional Aβ1-42 and to enhance neuronal loss and synaptic dysfunction in a transgenic mouse model that exhibits an increased Aβ3(pE)-42 load [13, 18]. Compared to Aβ1-42, Aβ3(pE)-42 oligomers have even been shown to cause stronger microglia activation and astroglia proliferation and to exhibit higher astrocytic secretion of pro-inflammatory tumor necrosis factor α (TNFα) *in vitro* [19]. Also, due to its unique biophysical properties, Aβ3(pE)-42 is prone to aggregation and formation of fibrillar species and more resistant to degradation [20, 18, 13, 21]. Therefore, n-terminally modified Aβ species represent relevant therapeutic

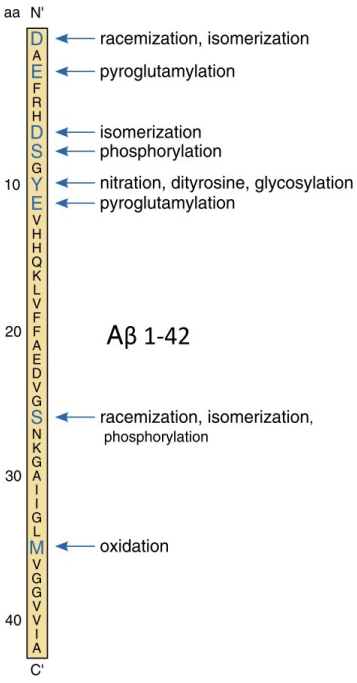

Fig. 1: Different PTMs of Aβ1-42*. Blue letters indicate the positions of commonly modified amino acids in Aβ1-42. Cartoon from Kummer and Heneka, 2014.*

targets to fight the onset and progression of AD. Different groups of researchers have for example developed monoclonal Aβ3(pE)-42 antibodies, that demonstrated beneficial effects in terms of lower Aβ3(pE)-42 levels and the reduction of pre-existing amyloid plaques in the hippocampi of transgenic AD mice [22–24].

1.1.1 Neuroinflammation

In the past it has often been reported that AD neuropathology does not necessarily correlate with the clinical disease manifestation and that brain lesions and plaque load considerably overlap in demented and non-demented patients. It is therefore likely, that factors other than Aβ aggregation and tau pathology contribute to AD progression. After viewing inflammatory processes as secondary effects triggered by plaques and tau tangles in AD brains for years, it is nowadays emerging that chronic, sterile neuroinflammation plays an important role in disease progression of AD [25, 1, 10, 26].

It is well established that glial cells have the ability to internalize intracerebral Aβ aggregates [27–30]. Additionally, growing evidence suggests an early invasion of glia to senile Aβ plaques as well as inflammation-associated gene profiles and activity following Aβ ingestion in disease-affected brain areas [25, 1, 31]. As a result, astrocytes and microglia display characteristic features such as morphological changes, increased proliferation and migration, but also a shift in gene expression profiles and production of pro-inflammatory metabolites, which in turn promote neuronal damage and synaptic dysfunction [32–37]. Moreover, prolonged glial activation impairs phagocytic functions while the production of pro-inflammatory metabolites is not affected, which might contribute to an exacerbation of AD pathology [38, 31, 39–41]. In a previous study our group demonstrated that, following ingestion by astrocytes, Aβ3(pE)-42 potently induces glial release of the pro-inflammatory cytokine TNFα and thereby drives synaptic loss and dysfunction [19]. Moreover, it has been shown that AD-associated PTMs of Aβ are induced by an inflammatory milieu [42], thus making Aβ3(pE)-42 part of a vicious cycle in the development of neuroinflammation.

Besides its negative implications, neuroinflammation also serves beneficial functions. As first-line response of the immune system, neuroinflammation is an essential protective response for tissue repair after injury, infection or trauma. The glial release of pro-inflammatory mediators also serves the purpose of attracting further immune cells, which aid to eliminate

the cause of injury, prevent further spreading and support neural tissue repair. And, the inflammatory response can resolve over time if acute triggers recede [43, 25, 44].

Supporting the hypothesis of an inflammatory component in AD pathogenesis, research also showed that patients with long-term anti-inflammatory drugs (non-steroidal anti-inflammatory drugs – NSAIDs) for treatment of other diseases, such as rheumatoid arthritis, showed significantly better performances in cognitive testing as well as slower cognitive decline than AD patients without NSAIDs [10, 45]. Apart from its importance as an early defense mechanism of the immune system, chronic neuroinflammation appears to be directly involved in the development and progression of AD by driving neurodegeneration [39], hence understanding and controlling this phenomenon to prevent the disease is of great interest.

1.1.2 Current developments in AD therapy

Despite the immense efforts in the field over the last decades, there are still no satisfying therapeutical interventions, that reliably stop AD progression. Nevertheless, new pathophysiological approaches and novel drugs are being tested continuously.

The concept of prevention represents an invaluable first-line therapeutic approach in numerous chronic diseases, especially since it is known that e.g. cardiovascular risk factors and unhealthy life style are associated with an increased risk of dementia [46]. Concordantly, the World Health Organization (WHO) recently released guidelines to decrease the risk of cognitive decline and dementia, which include physical activity and a healthy diet but also the reduction of risk factors such as obesity, tobacco, alcohol, hypertension and diabetes [47]. Also, the Finnish FINGER study, a largescale, long-term, randomized controlled trial, showed that a multidomain lifestyle-based intervention can reduce the risk of cognitive impairment with a combination of healthy balanced nutrition, physical exercise, cognitive training, social activities, and vascular and metabolic risk management. The trial showed benefits on cognition, even in people with genetic susceptibility to AD [48].

Pharmacological interventions with the capability to enhance cognitive capabilities encompass the standard of care for many patients with AD. Two approved substance groups include cholinesterase inhibitors, such as Donepezil, Galantamine or Rivastigmine, which increase the acetylcholine concentration in the synaptic cleft and memantine, an N-methyl d-aspartate receptor (NMDAR) antagonist, that prevents overactivation of NMDAR and thereby

restores its normal activity [24, 10, 2]. These drugs are mostly effective in mild to moderate disease stages, while only Donepezil is also approved in late-stage AD [2, 10]. Additionally, drugs, such as 5-HT_{2A} receptor agonists, atypical antipsychotics or selective serotonin reuptake inhibitors have been shown to be effective for treating neuropsychiatric co-symptoms of AD including AD-related psychosis, agitation or disturbed sleep patterns [1, 2, 10, 10, 3].

While the before-mentioned cognition enhancing drugs and co-therapies are potently reducing AD-related symptoms, current research is mainly devoted to so-called disease-modifying therapies, which aim to directly interfere with the course of the disease. With Aβ as a key player of AD pathogenesis, different drugs with effects on Aβ formation and degradation, neutralization of toxic Aβ species or inhibition of Aβ aggregation have been established.

Currently, there are three antibody-based drugs available for the treatment of AD (October 2024), while many more are being tested in clinical trials [2, 49, 50]. Aducanumab is a human IgG1 monoclonal antibody against a small epitope localized on the n-terminus of Aβ. By binding Aβ in both the oligomeric and fibrillar forms but not in the monomeric form, the antibody promotes the elimination of harmful plaques in the brains of AD patients. Accordingly, several studies showed the presence of smaller plaques in PET imaging and demonstrated a reduction in cognitive decline in patients with mild cognitive impairment (MCI) compared to a placebo group. Aducanumab was first approved in the US in June 2021, while approval was never granted in Europe. [49, 51]. Secondly, Lecanemab, another humanized IgG1 antibody, was approved in the US in January 2023. By binding to large soluble Aβ protofibrils, it was shown to lower brain amyloid levels and reduce cognitive and functional decline in patients with MCI and early AD [50, 51]. Lecanemab was recommended for approval by the European Medicines Agency (EMA) in November 2024 [52]. Lastly, the antibody Donanemab targets insoluble N-truncated pyroglutamylated Aβ species and only binds to established plaques but not to soluble forms of Aβ [53, 51]. Its effects are suggested to be greatest in early disease stages. A phase 2 clinical trial showed that Donanemab reduced Aβ plaques in the brain and revealed a statistically significant slowing of cognitive decline in patients with early AD [53, 51]. The antibody was currently approved in the US on 2 July 2024 and in the UK on 23 October 2024, while the approval in the EU is still pending (October 2024).

1.2 Astrocytes in health and disease

1.2.1 Astrocyte function in CNS homeostasis

As highly specialized glial cells, astrocytes fulfill essential regulatory functions in brain health and homeostasis. Next to microglia and oligodendrocytes they make up about 20-40% of glial cells in the human CNS [8]. Glial cells get their name from the Greek word for “glue”, which refers to their originally assumed function to simply stabilize the neurons in the CNS. However, nowadays it is well established that astrocytes comprise far more fundamental functions as “key homeostatic cells” [8, 43]. The star-shaped glia are for example involved in the homeostasis of neurotransmitters and neuromodulators, metabolic support of surrounding CNS cells or regulation of extracellular pH. Astrocytic end-feet are able to wrap blood vessels and stabilize endothelial connections, thereby supporting the stabilization of cerebral blood flow and BBB integrity. In addition, they play an essential role in communication with neurons, regulation of synapse formation and function and thus synaptic plasticity (***Fig. 2***). Astrocytes have been seen in diverse and heterogenous morphological states with many different molecular subtypes, depending on their respective functions and brain region. The most common categorization is into white matter fibrous astrocytes and grey matter protoplasmic astrocytes [8, 43, 54, 39].

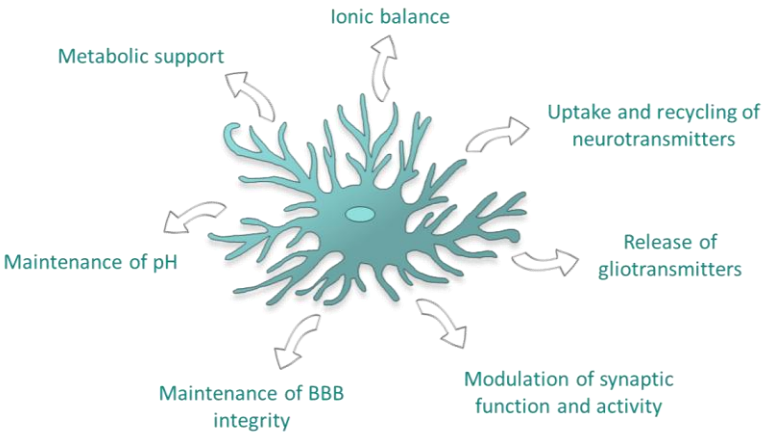

Figure 2: A selection of astrocyte physiological functions.

1.2.2 Reactive astrogliosis

Astrocytes play a crucial role in the brain’s defense mechanisms against harmful stimuli, external stressors or pathogens through an activation process referred to as ‘reactive astrogliosis’. A recent collective review defines reactive astrogliosis as “a process whereby astrocytes engage in molecularly defined programs involving changes in transcriptional regulation, as well as biochemical, morphological, metabolic, and physiological remodeling,

which ultimately result in gain of new function(s) or loss or upregulation of homeostatic ones, in response to pathology" [32]. They also describe reactive astrogliosis to vary depending on the type, severity, time, and duration of the insult. One marker commonly used to visualize reactive astrogliosis is glial fibrillary acidic protein (GFAP), the most abundant intermediate filament protein of the astrocytic cytoskeleton. Among the specific molecular changes, astrocytes undergo in the context of neurodegenerative diseases and normal aging are GFAP gene upregulation and increased protein levels, which has been confirmed in transgenic AD mouse models as well as in human AD cases. This molecular shift induces astrocytic growth and hypertrophic cellular processes [39, 43, 54, 25, 37].

The spectrum of reactive astrocytes has lately been considered to range between two extreme states: the neurotoxic, pro-inflammatory A1, which is triggered by neuroinflammatory insults, and the neuroprotective, anti-inflammatory A2, which is related to ischemia. However, these two different reactive states most likely coexist as part of a heterogeneous population. Thus, reactive astrogliosis may have a pro- or anti-inflammatory effect depending on the predominant signaling pathways and pathological triggers [25, 55, 39].

1.2.3 Astrocytes in AD

Numerous studies suggest a central role of reactive astrogliosis in the pathogenesis of AD and other neurodegenerative diseases [8, 56, 43, 57, 37]. In an attempt to contain the spread of inflammation, astrocytes have been shown to localize around Aβ plaques and to phagocytose and degrade Aβ deposits *in vitro* and *in vivo* [27–30]. Research also suggests that Aβ clearance by astrocytes significantly reduces the toxic load in AD brains and might therefore be a factor keeping Aβ plaques and thus AD progression from spreading throughout the whole brain in early stages of the disease [58]. It was even proposed, that reactive astrogliosis might precede amyloid deposition and tau hyperphosphorylation in the early cellular phase of the disease, making it a potential predictor for AD progression [59, 8].

On the other hand, reactive astrogliosis also seems to be a key player in the development and progression of neuroinflammation. Upon Aβ ingestion, the cells undergo distinct molecular changes which ultimately upregulate the release of pro-inflammatory mediators, such as TNFα, interleukin 1β (IL-1β) or interleukin 6 (IL-6), which contributes to a detrimental cascade eventually leading to synaptic dysfunction and neurodegeneration. [8, 32, 43, 54]. The PTM

Aβ3(pE)-42 has even been shown to cause significantly higher amounts of TNFα in primary astrocytes as compared to full-length Aβ and the conditioned medium from said astrocytes was able to induce synapse loss, synaptic dysfunction and decrease of regulatory transcription factors only in the case of Aβ3(pE)-42, which could be rescued with a TNFα antibody. Additionally, Aβ3(pE)-42 conditioned medium from cultures depleted of astrocytes did not induce synapse loss, once again underlining the role of astrocytes in the disease cascade [19]. Experimental evidence suggests that inflammatory processes directly correlate with Aβ production and thus the overall amyloid burden in the CNS. Thus, it has been shown, that pro-inflammatory cytokines are able to stimulate Aβ formation by astrocytes [60, 57]. Moreover, astrocytes seem be essential for the Aβ-induced tau phosphorylation in neurons, another important hallmark of AD pathogenesis [61]. ***Fig. 3*** illustrates the pathophysiological cascade of reactive astrogliosis in the context of AD.

Overall, reactive astrogliosis appears to be an essential CNS immune response to maintain brain homeostasis, limit brain injury and promote cell survival for as long as possible, with astrocytes serving as "resilient frontline soldiers" [8]. However, under prolonged stress conditions their response might become dysfunctional or even harmful for CNS integrity and functionality and promote AD onset and progression.

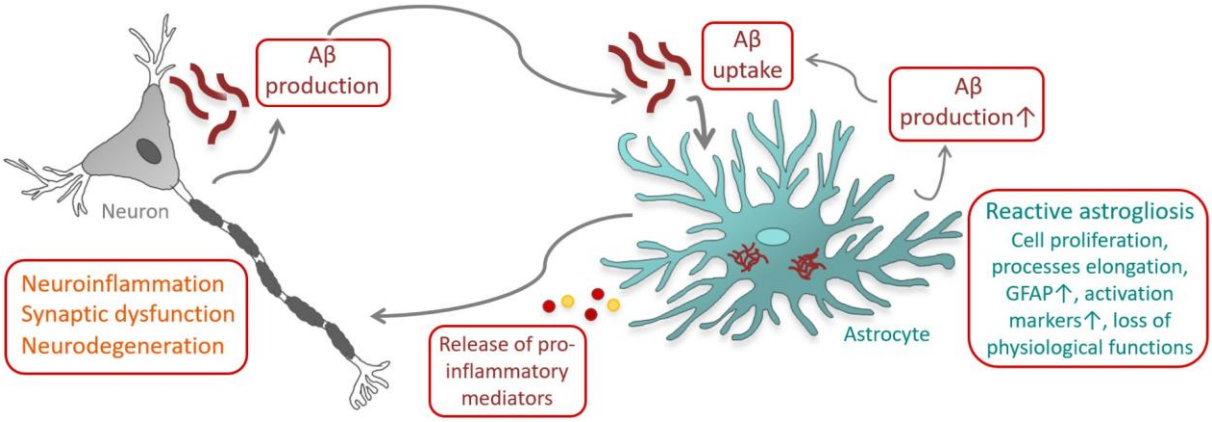

Figure 3: Representative scheme outlining the disease cascade of reactive astrogliosis and subsequent neuroinflammation in AD. *Astrocytes internalize soluble Aϐ oligomers and consequently undergo molecularly defined programs causing transcriptional, biochemical, morphological, metabolic, and physiological alterations. The result is an increased astrocytic production of Aϐ as well as an enhanced release of pro-inflammatory mediators, which ultimately promote neuroinflammation, synaptic dysfunction and neurodegeneration. AD = Alzheimer's disease; Aϐ = amyloid beta.*

1.3 Problems with Aβ-targeting therapeutic strategies and alternative approaches

1.3.1 Why have Aβ-targeting therapeutic strategies failed in the past?

As a fatal neurodegenerative disease affecting millions of people worldwide and with increasing life expectancies, AD will not only boost healthcare costs but also impact the quality of life of even more people in the coming decades. Regrettably, to this day there is still no known curative treatment to halt the progression of the disease [6]. It is therefore of great importance to not only develop new treatments with disease-modifying outcomes, but also to better understand the complex pathophysiology of AD.

Although various innovative strategies to fight the disease have been developed in the past, only very few phase three clinical trials have shown statistically significant benefits in terms of pre-specified clinical endpoints. Many explanations may be offered for this lack of success**.** For one, the timing of drug administration seems to be of great importance. In PET imaging studies, Aβ fibrils have been visualized up to 15 years before disease onset and aberrant Aβ levels in the CSF were detectable even up to 25 years before first symptoms occurred [62, 63]. Therefore, the time for administering Aβ drugs might currently occur after the therapeutic window has already closed and the disease cascade is already in a state of no return. Other explanations for failed drug trials comprise poorly designed trials or incomplete knowledge of the complex pathophysiological mechanisms of AD [60]. Additionally, with the development of Aβ-targeting therapies, the possible loss of Aβ physiological functions, including the enhancement of long-term potentiation (LTP), inhibition of oxidative stress, maintenance of BBB integrity, antimicrobial activity or stimulation of neuronal differentiation and survival [10, 37] need to be taken into account and have limited clinical success in the past.

1.3.2 Alternative approaches

Instead of focusing on a single step of the Aβ pathway, combining drugs that target different stages of Aβ metabolism (such as β-secretase inhibitors or γ-secretase modulators that interfere with Aβ production, or antibody therapies that promote Aβ degradation), as well as targeting different proteins (such as Aβ, tau, cholinesterase, or NMDA receptors), could present new opportunities for future AD therapy [10].

Since growing evidence also suggests that Aβ deposition in AD is caused by a complex dyshomeostasis of Aβ metabolism, inflammation, glucose metabolism and complex neurovascular dysfunction, recent approaches focus on the administration of mechanism-based therapies targeting multiple effector molecules and pathways at the same time [3, 2]. This concept is for example well recognized in Traditional Chinese Medicine, where substances with a broad spectrum of action and mild side effects have been in use for thousands of years. Although their mode of action is not completely understood, they show preventive and therapeutic effects in many chronic diseases by regulating inflammation, cell growth and differentiation, oxidative stress or glucose metabolism [64–67]. Among these substances are plant derived agents such as resveratrol, which is known to reduce Aβ generation by inhibiting the activity of β- and γ-secretases, to stimulate Aβ clearance and to increase the permeability of the BBB. Rutin is another plant metabolite, which has been shown to recruit microglia for promotion of Aβ clearance, to inhibit the activity of β-secretase and Aβ-induced neuronal depolarization and to reduce neuroinflammation by downregulating pro-inflammatory cytokines [68–70, 10]. Hence, some of these natural products might present interesting therapeutical candidates for the fight of AD.

1.3.3 Astrocytes as therapeutic targets

As astrocytes have been found to be involved in a variety of pathological processes in AD, such as above-mentioned changes in neuroinflammation, oxidative stress or Aβ generation and degradation, they have been proposed as intriguing new therapeutic targets. Although the exact relation between neuroinflammation, oxidative stress, astrocytes, and AD is still unclear, experimental evidence points towards an interplay in the pathophysiology of the disease.

Recently, several astrocyte-targeting approaches have shown promising results in *in-vitro* studies, animal models, and preclinical AD research [71, 60]. Some authors for example propose drugs that directly interfere with astrocyte reactivity and thus inhibit the formation of reactive astrocytes. Ideas in this field are neutralizing antibodies against pro-inflammatory cytokines such as TNFα, interleukin 1α (IL1α) or complement factor C1q [39]. Since astrocyte activation in neurodegenerative diseases has been shown to be linked to the nuclear factor-kappa B (NFκB) pathway and the release of complement factor C3, drugs controlling NFκB or C3 might hold therapeutic potential as well [72]. Although *in-vitro* experiments have shown

interesting results, human studies are still missing. One reason is the involvement of NFκB in synapse formation and plasticity and hence the uncertainty, whether NFκB inhibition might also have negative effects on CNS physiological functions and regeneration [39].

Several Aβ receptors could be identified on the surface of astrocytes and revealed a range of toxic effects, such as the production of excess Aβ or nitric oxide (NO) as well as increased synaptotoxicity. Exemplary astrocytic Aβ receptors include the α7-nicotinic acetylcholine receptor (α7-nAChR), the calcium-sensing receptor, the S100β receptor and the metabotropic glutamate receptor 5 (mGluR5) [73]. Interestingly, antagonists or modulators of these receptors can recover cognitive abilities, modify neurobiological changes or even reduce neuroinflammation, oxidative stress and reactive astrogliosis in cell cultures, animal models and human studies and might thus represent further therapeutic targets [73, 72, 54].

Moreover, the increased astrocytic production of pro-inflammatory cytokines and free radicals in response to Aβ suggests therapeutic strategies with novel compounds that exhibit anti-inflammatory and anti-oxidant properties [74, 60]. Since many secondary plant compounds are known to possess anti-inflammatory and anti-oxidative properties, they might also serve as valuable drugs in reducing Aβ-induced neuroinflammation by astrocytes [75, 60]. For example curcumin, a natural phenol that is commonly used as a spice, has been shown to reduce Aβ formation and decrease neurotoxicity in the brain, to improve spatial memory deficits and to attenuate inflammatory responses in astrocytes in an AD mouse model and primary rat neuron- glial co-cultures [76].

1.4 Secondary plant substances and their therapeutic potential

Plants and substances extracted from plants have been in use as medical agents for thousands of years and might even be the oldest known pharmaceuticals in the history of mankind. They contain numerous health-befitting secondary metabolites, including polyphenols and their subgroups flavonoids and non-flavonoids, carotenoids, mono- and triterpenes, phytoestrogens and many more. Plant substances and especially polyphenols are in the focus of research due to their known anti-inflammatory, anti-oxidant, anti-cancerogenic, anti-diabetic, anti-proliferative, anti-hypertensive and anti-hypercholesterogenic properties. Especially their capabilities to prevent oxidative stress and to reduce neuroinflammation are thought to be among the reasons for their beneficial effects in neurodegenerative diseases

and cognitive decline [64–67]. Additionally, plant-based substances are usually well tolerated and well accepted treatment options by the general public and some are even available as over-the-counter pharmaceuticals and nutritional supplements [64, 77, 78].

Natural hallmarks of all polyphenols are aromatic rings and hydroxyl groups, which enable polyphenols to scavenge free radicals and reduce harmful oxidative stress, ultimately leading to their beneficial effects associated with aging and chronic degenerative diseases [64, 67]. However, plant polyphenols have also been shown to directly protect neurons from neurotoxins such as Aβ, thereby suppressing neuroinflammation and promoting memory, learning and cognitive function [77, 78]. Two recent US studies with large cohorts confirmed these beneficial effects in prospective study-designs. Holland et al. followed up on 961 participants (mean baseline age = 81.4 y; 75% female) for an average of 6.9 years. They thoroughly assessed dietary intake of flavonoids and annually evaluated cognitive abilities. As a result they found that higher dietary intake of flavonols was associated with a slower rate of cognitive decline in global cognition and multiple cognitive domains including episodic memory, semantic memory, perceptual speed, and working memory, all of which are typically impaired in AD [66]. Shishtar et al. analyzed data from 2801 patients (mean baseline age = 59.1 y; 52% female) over a mean of 19.7 years and also assessed intake of different flavonoids. They observed lower risks of AD and AD-related dementias associated with higher dietary flavonoid intake [67]. Both studies adjusted for common AD risk factors, including age, sex, education, physical activity, smoking, vascular disease and APOE-ε4 status. Similar results were observed in an animal model of AD. Upon intraperitoneal flavonoid injection for 3 months, researchers observed decreased extracellular Aβ deposition, tauopathy, reactive astrogliosis and microgliosis in the hippocampus and the amygdala in a transgenic AD mouse model. Additionally, performance in learning and spatial memory tasks improved significantly, suggesting beneficial effects in histological hallmarks and cognitive functions [79]. Conclusively, research from human and animal studies clearly highlights the beneficial properties of plant secondary metabolites, which is why they might present intriguing new therapeutic targets in fighting AD-related amyloidosis, reactive astrogliosis, chronic neuroinflammation and neuronal dysfunction. Additionally, these findings underline the importance of a balanced, plant-based diet with sufficient intake of secondary plant metabolites for healthy neuronal aging.

1.5 Aims of the thesis

Compelling evidence suggests a link between AD pathogenesis, chronic neuroinflammation and reactive astrogliosis. Moreover, the accumulation of the most prevalent posttranslationally modified form of Aβ1-42, pyroglutamylated Aβ3(pE)-42, in astrocytes is directly linked to glial activation and the release of pro-inflammatory cytokines, which in turn contribute to early synaptic dysfunction in AD. Given their numerous beneficial anti-inflammatory and anti-oxidant properties, secondary plant substances are promising pharmaceutical candidates for targeting Aβ3(pE)-42 induced reactive astrogliosis and neuroinflammation. However, since the exact molecular mechanisms underlying these positive effects are largely unknown, the aim of this thesis was to develop a screening assay to identify plant substances, which show protective properties on primary astrocytes upon exposure to Aβ3(pE)-42.

This thesis is divided into two major parts.

1. In the first part, I established a culturing system to study primary astrocytes and assess their response to various stimuli *in vitro*. The main goal was to determine optimal culturing conditions under which astrocytes would exhibit a reactive phenotype upon stimulation with Aβ3(pE)-42. To achieve this, I cultured astrocytes at different densities, with different compositions of culture media and evaluated their response to Aβ3(pE)-42 oligomers under various conditions.
2. In the second part of this work my goal was to establish a simple screening assay to identify substances from the Natural Product Library at the Leibniz Institute of Plant Biochemistry in Halle (Saale) capable of modulating astrocytic responses to Aβ3(pE)-42. To achieve this, I had to find a suitable readout parameter that would be time- and cost-efficient for high-throughput screening of substances. The experiments involved analyzing various astrocyte reactivity markers, such as GFAP, STAT1, NLRP3, and NFκB-p65, as well as the uptake quantity of Aβ3(pE)-42 by primary astrocytes. I tested 17 different plant-derived substances for their effects on Aβ3(pE)-42 uptake by primary astrocytes, and ultimately, one candidate was further evaluated for its potential to efficiently protect against early synapse loss and dysfunction following exogenous Aβ3(pE)-42 application.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals

The chemicals used in this study were obtained from Baxter Deutschland GmbH, Calbiochem, Clontech/Takara, Gibco Life Technologies, Invitrogen, Merck Millipore, Roche, Roth, Serva and Sigma-Aldrich. Buffers and solutions for protein biochemistry and molecular biology were prepared using deionized water (SeralpurProCN®, Seral) and Milli-Q water (Milli-Q®, Millipore).

2.1.2 Aβ3(pE)-42 peptides

The Aβ3(pE)-42 peptide was ordered from AnaSpec (Cat. No. AS-29907).

2.1.3 Plant substances

The plant substances were kindly selected by Prof. Dr. L. Wessjohann and Dr. Katrin Franke from the Natural Product Library of the Leibniz-Institute of Plant Biochemistry, Dept. Bioorganic Chemistry, Halle (Saale). The substances were dissolved in dimethyl sulfoxide (DMSO, >99%, Duchefa Biochemie) at a concentration of 10 mM. For stimulation of astrocytes, the substances were further diluted in water with 0.5% DMSO to a concentration of 1 mM and subsequently applied to the culture medium resulting in a concentration of 5 µM or 50 µM.

2.1.4 Buffers and solutions

Table 1: Buffers and solutions

Name	**Composition**	**Application**
TRIS buffered saline (TBS)	25 mM Tris-HCl, 150 mM NaCl, pH 7.4	Washing buffer
TBS-T	TBS+ 0.1% (v/v) Twien-20, pH 7.4	Washing buffer
TBS-A	TBS+ 0.02% NaN3 pH 7.4	Primary antibody dilution for WB

Permeabilization buffer	PBS, 0.25% Triton-X-100, pH 7.4	Permeabilization buffer for ICC
ICC blocking buffer	2% glycine, 2% BSA, 0.2% gelatin and 50 mM NH_4Cl	Blocking buffer for ICC
10x phospho-buffered saline (PBS) 100mM	1.4 M NaCl, 83 mM Na_2HPO_4, 17mM, NaH_2PO_4, pH 7.4	ICC washing buffer
Mowiol	10% mowiol, 25% glycerol, 100mM, Tris-HCl, pH 8.5, 2.5%, 1,4-Diazabicyclo-[2.2.2]-octane	Mounting reagent
4x SDS sample buffer	250 mM Tris-HCl, pH 6.8, 1% (w/v) SDS, 40% (v/v) glycerol, 20% (v/v) mercaptoethanol, 0.004 % bromophenol Blue	SDS-PAGE sample buffer
Electrophoresis buffer	192 mM glycine, 0.1 % (w/v) SDS, 25 mM Tris-base, pH 8.3	SDS-PAGE
Blotting buffer	192 mM glycine, 0.2 % (w/v) SDS, 20% (v/v) methanol, 25 mM Tris-base, pH 8.3	Western Blot
Stacking Polyacrylamide gel (5%) calculated for 5 gels	2.5ml 0.5M Tris-HCl pH 6.8, 1.6ml 30% acrylamide, 3.25ml dH_2O, 100µl 10% SDS, 100µl 0.2M EDTA, 50µl phenol red, 57µl 10% APS, 7µl TEMED	SDS-PAGE gel
Running polyacrylamide gel (20%) calculated for 5 gels	2.85ml 1.5M Tris-HCl pH 8.8, 6.75ml 40% acrylamide, 0.58ml dH_2O, 3ml 87% glycerol, 132µl 10% SDS, 132µl 0.2M EDTA, 20µl bromophenol blue, 57µl 10% APS, 9µl TEMED	SDS-PAGE gel
Running polyacrylamide gel (5%) calculated for 5 gels	2.85ml 1.5M Tris-HCl pH 8.8, 1.69ml 40% acrylamide, 7.89ml dH_2O, 0.75ml 87% glycerol, 132µl, 10% SDS, 132µl 0.2M EDTA, 48µl 10% APS, 9µl TEMED	SDS-PAGE gel

2.1.5 Common media

Table 2: Common media

Name	**Composition / Supplier**	**Application**
Dulbecco's Modified Eagle Medium (DMEM+)	1% (v/v) Penicillin/Streptomycin 100x, 10% (v/v) fetal calf serum, 0.8 mM L-glutamine in DMEM (all from Gibco)	Culturing of primary astrocytes and HEK293T cells
Neurobasal medium (NB)	Gibco/Life Technologies, Darmstadt, Germany	Aβ3(pE)-42 oligomers preparation

NB+	NB with 0.8mM glutamine, 1% Penicillin/Streptomycin 100x, 2% B27 serum	Culturing of rat hippocampal neurons
Ringer solution	NaCl 124mM, KCl 4.83mM, MgSO4x7H2O 1.3mM, CaCl2x2H2O 1.97mM, KH2PO4 1.21mM, NaHCO3 25.6mM, 1mL/L phenol red, pH 7.4	Culturing of primary astrocytes (glucose-deprived medium)
Artificial cerebrospinal fluid (aCSF)	110mM NaCl, 2.5mM KCl, 2.5mM CaCl2, 1.5mM MgSO4, 1.24mM KH2PO4, 10mM glucose, 27.4mM NaHCO3, pH 7.3	Acute slices for electrophysiology

2.1.6 Pharmacological reagents

Table 3: List of pharmacological reagents

Name	**Used concentration**	**Supplier**	**Application**
4',6-Diamidin-2-phenylindol (DAPI)	1:1000 in PBS	Sigma-Aldrich	Fluorescent dye to stain DNA
Propidium iodide	2 µL/mL	Sigma Aldrich (P4864)	Apoptosis assay
1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP)	-	Sigma Aldrich (105228-5G)	Aβ3(pE)-42 preparation
Dimethylsulfoxide (DMSO)	0.5%, 1%, 5%	ITW Reagents (A3672)	Solvent for plant extracts, apoptosis inducer
Lipopolysaccharide (LPS)	100 ng/mL	Sigma Aldrich (L6529)	Stimulation of astrocytes
Rat Interferon gamma (IFNγ)	100ng/mL	Pepro Tech (400-20-100UG)	Stimulation of astrocytes
Transforming Growth Factor β1 (TGF-β1)	10ng/mL	R&D Systems (240-B-002)	Stimulation of astrocytes

2.1.7 Primary antibodies

Table 4: Primary antibodies

Name	**Supplier**	**Species**	**Dilution / Application**
Anti-Aβ3(pE)-42	Synaptic Systems (218003)	Rabbit	1:500 ICC, WB
Anti-GFAP	Sigma Aldrich (G-9269)	Rabbit	1:500 ICC
Anti-GFAP	Synaptic Systems (173-004)	Guinea Pig	1:500 ICC

Anti-MAP2	Sigma Aldrich (M-4403)	Mouse	1:500 ICC
Anti- NFkB p65	Cell Signaling (8242)	Rabbit	1:100 ICC
Anti-NLRP3	Invitrogen (SC06-23)	Rabbit	1:100 ICC
Anti-pCREB (Ser 133)	Cell Signaling (9198)	Rabbit	1:500 ICC
Anti-STAT1	Cell Signaling (14994S)	Rabbit	1:500 ICC
Anti-Vimentin V9	Sigma Aldrich (V-6630)	Mouse	1:100-1:200 ICC

2.1.8 Secondary antibodies

Table 5: Secondary antibodies

Name	**Supplier**	**Species**	**Dilution / Application**
Anti-Rabbit Alexa Fluor 488	Invitrogen / Thermo Fisher Scientific	Goat or donkey	1:500 ICC
Anti-Rabbit Alexa Fluor 568	Invitrogen / Thermo Fisher Scientific	Goat or donkey	1:500 ICC
Anti-Rabbit-HRP	Dianova	Goat	1:10 000 WB
Anti-Guinea Pig Alexa Fluor 488	Invitrogen / Thermo Fisher Scientific	Goat or donkey	1:500 ICC
Anti-Guinea Pig Alexa Fluor 568	Invitrogen / Thermo Fisher Scientific	Goat or donkey	1:500 ICC
Anti-Guinea Pig Abberior STAR 635p	Abberior	Goat	1:500 ICC
Anti-Mouse Alexa Flour 488	Invitrogen / Thermo Fisher Scientific	Goat or donkey	1:500 ICC
Anti-Mouse Alexa Fluor 568	Invitrogen / Thermo Fisher Scientific	Goat or donkey	1:500 ICC
Anti-Mouse ATTO 594N	Sigma Aldrich	Goat	1:200 ICC (STED)
Anti-Mouse Abberior STAR 635p	Abberior	Goat	1:200 ICC (STED)

2.2 Methods

2.2.1 Preparation of oligomeric Aβ3(pE)-42 peptides

Aβ3(pE)-42 oligomers were prepared following previously described protocols [20, 19]. Aβ3(pE)-42 was dissolved in 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) to a concentration of 0.5mg/L, aliquoted and stored at -80°C. Two days before each experiment, HFIP was evaporated for 24 hours at room temperature under the hood. 0.1M NaOH was added to dissolve the peptide, followed by dilution in Neurobasal medium (NB) and neutralization with 0.1M HCl to a final concentration of 5µM. Finally, oligomers were formed during 24 hours incubation at 37°C.

2.2.2 Immunocytochemistry

Following each experimental design, the cells were fixed in 4% paraformaldehyde (PFA) and 4% sucrose for 10 minutes at room temperature and washed three times in PBS, five minutes each. Next, the coverslips were transferred into a dark humidified chamber, where the cells were incubated with permeabilization buffer for 10 minutes, making intracellular proteins accessible. Following repeated washing steps in PBS, unspecific bounds were blocked by incubating the cells with blocking buffer for 45 minutes. Subsequently, the cells were incubated with primary antibodies diluted in blocking buffer overnight at 4°C. The next day, following three 20-minutes washing steps in PBS, the cells were incubated with suitable fluorescent-coupled secondary antibodies diluted 1:500 in blocking buffer for 90 minutes at room temperature and washed three times in PBS, this time 30 minutes each. If needed, DAPI was applied for 10 minutes to stain cell nuclei. The cells were rinsed with PBS and finally mounted on ethanol-cleaned glasses with Mowiol mounting buffer and stored in the dark at 4°C.

2.2.3 Aβ3(pE)-42 peptide characterization

2.2.3.1 SDS-Page and Western blot

For verification of Aβ3(pE)-42 oligomerization, a standard western blot protocol was applied [80]. 20 µL of the prepared Aβ3(pE)-42 solution was solubilized in 6 µL 4X SDS-sample buffer and loaded onto a 5-20% gradient polyacrylamide gel, which was set under a constant electric

field of 12 mA for 1.5 hours. The samples were then transferred to a nitrocellulose membrane during 1.5 hours of blotting at a current of 200 mA and a voltage between 60 and 80 V in a blotting chamber supplied by Hofer Scientific Instruments. Following blocking for 45 minutes in 5% non-fat milk at room temperature, the membrane was incubated with the primary antibody (Rabbit-Anti-Aβ3(pE)-42, 1:500) diluted in TBS-A at 4°C on a shaker overnight. The next day, after several washing steps with TBS and TBS-T, the membrane was incubated with the secondary antibody (Anti-Rabbit-HRP, 1:10 000) for 1.5 hours at room temperature. Following 4 more washing steps in TBS and TBS-T, images of the bands were acquired using ECL solution (Luminata, Merck Millipore, Darmstadt, Germany) and Intas ECL Chemocam imager (Licor, Cambridge, UK).

2.2.3.2 Acute hippocampal slices preparation and long-term potentiation (LTP) recording

Long-term potentiation (LTP) recordings were performed in collaboration with Dr. PingAn Yuanxiang. Hippocampal slices from male C57/B6J mice were prepared according to previously described protocols (Behnisch et al., 2011; Yuanxiang et al., 2014). Mouse brains were sliced into 350 µm thick slices using a Vibratome (Leica VT1000ST, Nussloch, Germany) and incubated with 500nM Aβ3(pE)-42 oligomers in carbogenated (CO_2 5%, O_2 95%) aCSF solution at room temperature for 2 hours. The slices were transferred to a recording chamber (at 31±1°C), where field excitatory postsynaptic potentials (fEPSPs) were evoked by stimulation of CA1 Schaffer-collateral fibers with stainless electrodes. fEPSPs were recorded with aCSF filled glass capillary microelectrodes (3-5 MΩ), amplified by an Extracellular Amplifier (EXT-02B, NPI electronic, Germany) and digitized at a sampling frequency of 5 kHz by Digidata 1401plus AD/DA converter (CED, England). Stimulation strength was adjusted to 20% - 30% (LTP) of the maximum fEPSP-slope values. A single stimulus with 0.1 ms width was applied every 30 seconds (at 0.0333 Hz) and was averaged every 3 minutes. Following a 30 minutes stable baseline recording, 3 trains of high frequency stimuli (0.2 ms width) with 5 minutes inter-train interval were used for LTP-induction, each train lasted for 1 second at 100 Hz.

2.2.3.3 Mixed hippocampal cultures and pCREB staining

Rat hippocampi were dissected from embryonic 18-day old Sprague Dawley rats and primary neuronal cultures were prepared following a previously described protocol [81]. Cells were seeded in poly-D-lysine coated 18 mm coverslips in 12 well plates at a density of 15 000 cells per coverslip. Neurons were plated in 1 mL DMEM+, which was changed to Neurobasal medium (NB+) after 24 hours. Cultures were kept in the incubator at 37°C, 5% CO2 and 95% humidity. On day in vitro (DIV) 18 the cultures were incubated with 1µM Aβ3(pE)-42 oligomers for 3 days, fixed with 4% PFA and 4% sucrose and stained with pCREB-, Aβ3(pE)-42-, MAP2- and GFAP primary antibodies (for immunocytochemistry protocol see 2.2.2). Additionally, DAPI was used to co-stain the nuclei. Images were acquired using the inverse Leica TCS STED-SP8 3X microscope in the confocal mode at 63X magnification and analyzed with ImageJ software. Nuclear pCREB intensities were measured within region of interest defined by DAPI with the help of the ImageJ drawing tool. Data were normalized to the control group and statistically analyzed using a two-tailed, unpaired t-test.

2.2.4 Primary astrocyte cell culture

2.2.4.1 Isolation of primary astrocytes

Astrocytes were isolated from P0 rat forebrain by technical assistant Isabel Herbert. After trypsinization and several washing steps in HBSS-, cells were dissociated and plated in DMEM+. After 1 week, when the culture reached confluency, the plate was shaken to remove microglia. For splitting, the cultures were washed with HBSS- and trypsinized for 5 minutes at 37°C. After 3 weeks the cells were aliquoted and stored at -80°C.

2.2.4.2 Thawing of astrocyte cryo-vials

Cells were slowly thawed in a 37°C water bath, suspended in 9 ml of pre-warmed DMEM+ and plated in a 1:4 or 1:3 dilution in DMEM+ in 12 or 24 well plates with poly-D-lysine coated coverslips, according to the respective experimental design.

2.2.4.3 Stimulation of primary astrocytes

According to each experimental design, astrocytes were plated in a 1:3 or 1:4 dilution in DMEM+, which was changed to DMEM+, serum-free medium (DMEM) or serum- and glucose-

free medium (Ringer solution with 10mM mannitol) on the second or third day after plating. A specific amount of Aβ3(pE)-42 oligomers (0.1 µM, 0.5 µM or 1 µM; 30 minutes, 16 hours, 24 hours, 48 hours, 3 days or 5 days of incubation), IFNγ (100ng/mL; 15, 30 or 60 minutes), TGF-β1 (10ng/mL; 5 days) or LPS (100ng/mL; 24 or 48 hours) was added to the cells, depending on the experimental design. Afterwards, the cells were fixed in 4% PFA and 4% sucrose and stained with corresponding antibodies. Details are stated in the respective figure legends.

2.2.5 Apoptosis assays

2.2.5.1 Live and dead cell assay – Live cell imaging

Astrocytes were plated in a 1:4 dilution in DMEM+ in 12 well plates with poly-D-lysine coated coverslips. After 48 hours, the medium was exchanged and the cells were incubated with 0.5% DMSO and 0.5µM or 1µM Aβ3(pE)-42 oligomers for another 24 hours. The control group was incubated with 0.5% DMSO only; as positive control, cells were incubated with 5% DMSO for 2 hours. The staining was performed following abcam protocol (abcam ab115347) using a green dye to label living cells and a red dye for dead cells. The cells were incubated with the dying solutions for 10 minutes at 37°C and then immediately analyzed under fluorescence microscopy in live cell imaging in GFP (excitation at 488nm) and RFP (excitation at 568nm) channels. 10 – 27 fields of view (FOVs) were acquired from at least 4 different coverslips per condition and image were analyzed in ImageJ software. For quantification of apoptotic cells, an automated threshold ("Triangle") was applied, resulting binary images were quantified and number and relative area of particles were calculated. Statistical analysis was done using a Kruskal-Wallis test with Dunn's test for multiple comparisons.

2.2.5.2 Propidium Iodide assay – Fixed cells

Astrocytes were plated in a 1:4 dilution in DMEM+ in 12 well plates with poly-D-lysine coated coverslips. After 48 hours, the medium was changed and the cells were incubated with 0.5% DMSO and 1µM Aβ3(pE)-42 oligomers for 3 or 5 days. The control group was incubated with 0.5% DMSO only and for a positive control, cells were incubated with 5% DMSO for 1 hour. The cells were incubated with propidium iodide at a concentration of 2 µL/mL for 1 hour, fixed in 4% PFA and 4% sucrose and stained with GFAP antibodies. Images were acquired under fluorescence microscopy.

2.2.5.3 Apoptosis assay – Fixed cells

Cultivation of astrocytes as well as concentration and duration of DMSO and Aβ3(pE)-42 incubation was done corresponding to 2.2.5.2. Cells were fixed in 4% PFA and 4% sucrose and stained with an apoptosis dye containing ApopxinGreen Indicator, labelling apoptotic cells upon binding to phosphatidyl-serine residues, following the abcam protocol (abcam ab176749). Images were acquired with a fluorescence microscope.

2.2.6 Organotypic hippocampal slice cultures

Organotypic hippocampal slices from rat were prepared by Dr. Katarzyna Grochowska. Experiments were performed with cultures at DIV8. 500 nM Aβ3(pE)-42 were applied for 24 hours. Slices were stained, with a modified permeabilization step – 0.3% TX-100 for 1 hour at room temperature. The astrocytes were labelled with GFAP antibodies detected with anti-guinea pig-AlexaFluor 488 and subsequently counterstained with DAPI. The images were acquired under confocal microscopy, 10 images from z-optical plane with a step size of 0.5 µm for each FOV. The maximum fluorescence intensity was quantified using ImageJ software excluding the pyramidal layer region defined by DAPI.

2.2.7 TBA2.1 mice

TBA2.1 mice were housed in groups of up to four in individually ventilated cages (IVCs, Green line system, Tecniplast, Lugano, Switzerland) under controlled environmental conditions (22 ± 2°C, 55% ± 10% humidity, 12-hour light/ dark cycle, with lights on at 06:00). Animals had free access to food and water. Mice used in the experiments were 6–8 months old. Mice were anaesthetized with isoflurane (Baxter Deutschland GmbH, Unterschleißheim, Germany) and then perfused with 0.9% NaCl followed by fixation with 4% PFA in PBS. Briefly, slices were incubated in blocking buffer followed by primary antibody diluted in blocking buffer. The slices were counterstained with DAPI. Entorhinal cortices of both hemispheres from 2 mice per group with 2 brain slices from each animal were imaged under a confocal microscope with 20x magnification. Maximum projections of the scans, comprised of 5 z-stacks (0.39 µm z-step), were created using ImageJ software for subsequent analysis. The FOV was defined by positioning a square frame of 387 x 387 µm² on the entorhinal cortex. The number of positive cells for GFAP was counted and normalized to the total DAPI count in the respective FOV.

2.2.8 HEK-293T cell culture

Human embryonic kidney-293T cells (HEK-293T) were cultured in DMEM+, in a Heraeus incubator at 37°C, 5% CO2 and 95% humidity. Maintenance and passaging were done by technical assistants. The cells were plated in 12 or 24 well plates with poly-D-lysine coated coverslips and incubated with 0.5 µM or 1 µM Aβ3(pE)-42 oligomers after 10 hours. Following 16 hours of incubation, cells were fixed in 4% PFA and 4% sucrose and stained with vimentin, Aβ3(pE)-42, STAT1 and NLRP3 antibodies and co-stained with DAPI. Images were acquired under fluorescence microscopy.

2.2.9 Screening assay

For the screening assay, primary astrocytes were plated in 24-well plates with poly-D-lysine coated coverslips in a 1:3 dilution in DMEM+. After 24 hours the medium was exchanged to serum-free DMEM containing 1% Penicillin/Streptomycin. 32 hours following medium exchange, astrocytes were treated with the plant natural products at a concentration of 5 µM or 50 µM and 0.5µM Aβ3(pE)-42 for 16 hours. The cells were fixed in 4% PFA and 4% sucrose and stained with GFAP and Aβ3(pE)-42 antibodies (see 2.2.2 for immunocytochemistry protocol). Each condition was performed in duplicates. As controls, two coverslips were incubated with 0.5µM Aβ3(pE)-42 only or vehicle control (0.5% DMSO).

Images were acquired from five randomly chosen FOVs per coverslip from two independent experiments under fluorescence microscopy, resulting in 20 FOVs per condition. For quantification of intracellular Aβ3(pE)-42 uptake, ImageJ software was used to create a macro to automatize image analysis. Fixed threshold values were applied to both 16-bit channels (GFAP threshold at 135, Aβ3(pE)-42 at 1000), resulting binary images were quantified and intracellular area, number, and average size of Aβ3(pE)-42 deposits were calculated. Aβ3(pE)-42 area and number of deposits were normalized to the respective GFAP area. All plotted values were normalized to the corresponding control. Outliers were removed using ROUT method, a test combining robust regression and outlier removal, with Q=1%. Since extracellular Aβ3(pE)-42 deposits were not observed, all signal from the Aβ3(pE)-42 channel was considered intracellular Aβ3(pE)-42. All experimental steps were performed blinded to the plant substances.

2.2.10 Nuclear pCREB and synaptic staining, imaging, and quantification

Nuclear pCREB and synaptic density experiments were performed by Dr. Katarzyna Grochowska. On DIV 16-18 mixed hippocampal cultures were incubated with 50 µM quercetin and / or 500 nM Aβ3(pE)-42 oligomers for 2 (synaptic density assay) or 3 days (CREB shutoff assay), fixed with 4% PFA and 4% sucrose and stained according to the protocol described above with antibodies detecting pCREB and MAP2, detected with secondary antibodies anti-mouse-AlexaFluor 488 and anti-rabbit-AlexaFluor 568. Next, the samples were washed and co-stained with DAPI. For synaptic staining, the samples were stained with anti-PSD95 minibody, anti-synaptophysin 1, and anti-MAP2 detected with anti-guinea pig-AlexaFluor 568, anti-rabbit-AlexaFluor 488 and anti-mouse-AlexaFluor 647. Images were acquired with an inverse Leica TCS STED-SP8 3X microscope (Leica-Microsystems, Germany) equipped with pulsed White Light Laser (WLL) and diode 405 nm for excitation with 63x (Leica) objective lens along the z-axis with 300 nm z-step in a 512 × 512-pixel formats at 8-bit image depth at 400 Hz laser frequency. Images were analyzed with ImageJ. Nuclear pCREB intensities were measured within region of interest defined by DAPI. Synapses (overlap or opposing pre- and post-synaptic signal) were counted along defined dendritic stretches.

2.2.11 Image acquisition

2.2.11.1 Fluorescence microscopy

Unless otherwise stated, all images were acquired using Zeiss Axio Imager A2 fluorescence microscope (Zeiss, Jena, Germany) with the 20x objective Plan-Apochromat (20x/0,8 M27, a=0,55mm, Item no.: 420650-9901-000), Cool Snap EZ Monochrome camera (Photometrics) and VisiView Imaging software (Visitron Systems GmbH). 16-bit images were acquired using the 20x objective with an excitation time of 200ms, binning of 1 and image size of 1392 x 1040 pixels (pixel size of 0.1031 x 0.1031 µm²).

2.2.11.2 Confocal laser scan microscopy

Stainings for pCREB and some vimentin stainings were acquired using the inverse Leica TCS STED-SP8 3X microscope (Leica Microsystems, Wetzlar, Germany) in the confocal mode with the 63x objective with immersion oil. Images were scanned at a speed of 400Hz and 1024 x

1024 pixels image size (Pixel size: 0.0902 x 0.0902 µm²), with laser lines at 405nm, 488nm, 568nm and 635 nm excitation wavelength.

2.2.11.3 Stimulated Emission Depletion (STED) microscopy

For GFAP and vimentin colocalization experiments, STED imaging was performed with the help of the inverse Leica TCS STED-SP8 3X microscope with STED module (Leica Microsystems, Wetzlar, Germany). Images were acquired with a 100x objective and a zoom of 5, a speed of 600Hz and an image size of 1024 x 1024 pixels (Pixel size: 0.0227x0.0227 µm²). As STED secondary antibodies, anti-Gp Abberior-STAR 635p and anti-Ms ATTO 594N were applied.

2.2.12 Statistics

Statistical analyses were performed using GraphPad Prism software (GraphPad software version 8.3.0 and 9.4.1, Inc., La Jolla, USA). Data were represented as mean ± s.e.m. Shapiro-Wilk test was used to assess Gaussian distribution of the data. Student's t-test, one-way ANOVA followed by Tukey's Multiple Comparison Test, Mann-Whitney-U test or Kruskal-Wallis test were used as appropriate and as stated in the figure legends.

3. Results

3.1 Establishing an *in vitro* system for astrocyte cultures

3.1.1 Determination of the ideal culture conditions

Aβ accumulation, oligomerization and glial uptake with subsequent chronic neuroinflammation are central aspects of AD pathogenesis [6, 82, 19, 56, 83]. In order to establish a screening system that identifies plant substances with the ability to influence reactive astrogliosis caused by Aβ3(pE)-42, I first aimed to set up a suitable culturing system, confirm Aβ3(pE)-42 uptake by astrocytes and, in a second step, find appropriate measures for an efficient screen of numerous plant natural products.

Initially, primary rat astrocytes were plated in different densities in DMEM+ to establish an appropriate cell culture system in our laboratory. Dilutions of 1:1, 1.25:1, 1.5:1 and 1.75:1 (astrocyte solution : DMEM+) were too dense to identify single GFAP-positive cells via fluorescence microscopy ***(Fig. 4A-D)***. Dilutions of 1:3 and 1:4 showed reduced cell density and were therefore set as the standard for all following experiments, since they allowed for single-cell imaging while maintaining cell-to-cell contacts for intercellular signaling and exchange of metabolites ***(Fig. 4E, F)***.

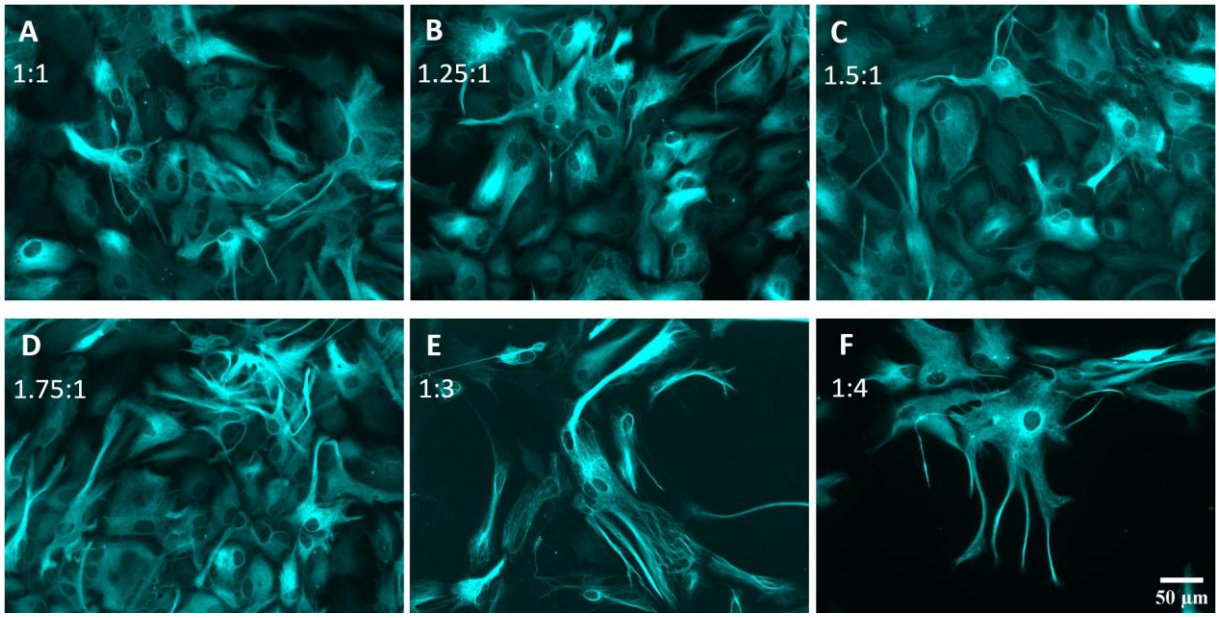

Figure 4: Testing different dilutions of cells in DMEM+ medium to identify isolated GFAP positive astrocytes. *Astrocytes were plated in DMEM+ in different dilutions of (**A**) 1:1, (**B**) 1.25:1, (**C**) 1.5:1, (**D**) 1.75:1, (**E**) 1:3 and (**F**) 1:4, followed by fixation and GFAP immunostaining. Dilutions (**A - D**) were too dense to identify single cells in fluorescence microscopy, whereas (**E and F**) showed an appropriate cell density for the experimental setup. Scale bar is 50 µm. DMEM = Dulbecco's Modified Eagle's Medium; GFAP = glial fibrillary acidic protein.*

3.1.2 Testing the effects of different DMSO concentrations on astrocyte viability

DMSO is an amphiphilic substance and can thus be used as solvent for lipophilic substances, e.g., plant substances, in water. To test, whether DMSO itself already elicits an effect on astrocyte morphology and viability, the cells were incubated with different concentrations of DMSO deriving from either the lab in Magdeburg or Halle. Compared to a control group, incubation with 0.5% DMSO did not cause visible alterations in cell morphology, whereas 1% DMSO already resulted in a few modified cell bodies with small hyperintense fragments. In the 5% DMSO group, fragmented nuclei and shrunk cell bodies were visible in all FOVs, most likely as a sign for apoptotic cell death. The results were concordant for both sources of DMSO ***(Fig. 5)***. We decided to perform all experiments with 0.5% DMSO from the Magdeburg lab.

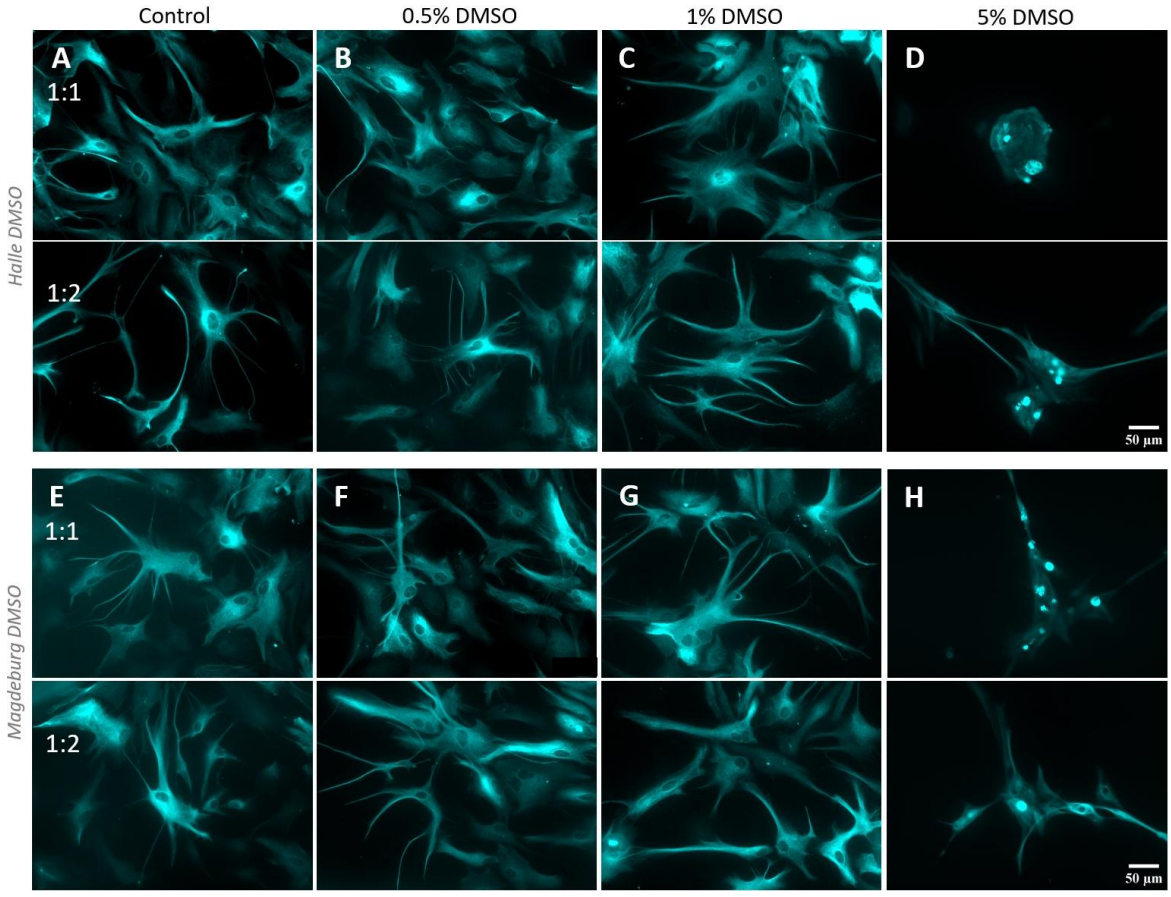

Figure 5: Treating astrocyte cultures with different concentrations of DMSO showed no alterations for the 0.5% dilution. *Astrocytes were plated in DMEM+ and incubated with concentrations of 0.5% (**B and F**), 1% (**C and G**) or 5% (**D and H**) DMSO from Halle (**A-D**) or Magdeburg (**E-H**) for 24h and compared to a control group without any DMSO (**A and E**). In GFAP immunostaining and fluorescence microscopy the 0.5% dilutions showed no major morphological alterations. In the 1% DMSO dilution there were few modified cells visible, whereas in the 5% groups almost all cells showed fragmented nuclei and cell shrinkage. Scale bar is 50µm. DMSO = dimethyl sulfoxide; DMEM = Dulbecco's Modified Eagle's Medium; GFAP = glial fibrillary acidic protein.*

3.1.3 Testing different cell culture media

To establish appropriate cell culture conditions the most suitable medium had to be determined. For that purpose, astrocytes were cultivated in medium with or without 10% fetal calf serum. In fluorescence microscopy astrocytes kept with serum appeared broader with more expanded somata ***(Fig. 6A)***, whereas astrocytes kept in serum-free medium appeared more delicate with thinner and longer processes and exhibited a more star-shaped phenotype ***(Fig. 6B)***. As cultivation in serum-free medium has been suggested to be a more physiological environment for primary astrocyte cultures, we decided to follow this approach. [84, 39, 85]. In later experiments, we also tested media with less or no glucose to obtain more reactive astrocytes through glucose deprivation, but did not observe notable alterations regarding morphology or expression of astrocyte reactivity markers (see *3.2.6*).

3.1.4 Astrocyte cultures are free from microglia

Astrocyte-specific GFAP and microglia-specific IBA1 immunostainings were performed to rule out microglia contamination in our cell-culture system, as this might occur during the isolation process of primary rat astrocytes. Staining with corresponding antibodies showed no signal for IBA1, confirming that no microglia were apparent in the cultures ***(Fig. 6C)***.

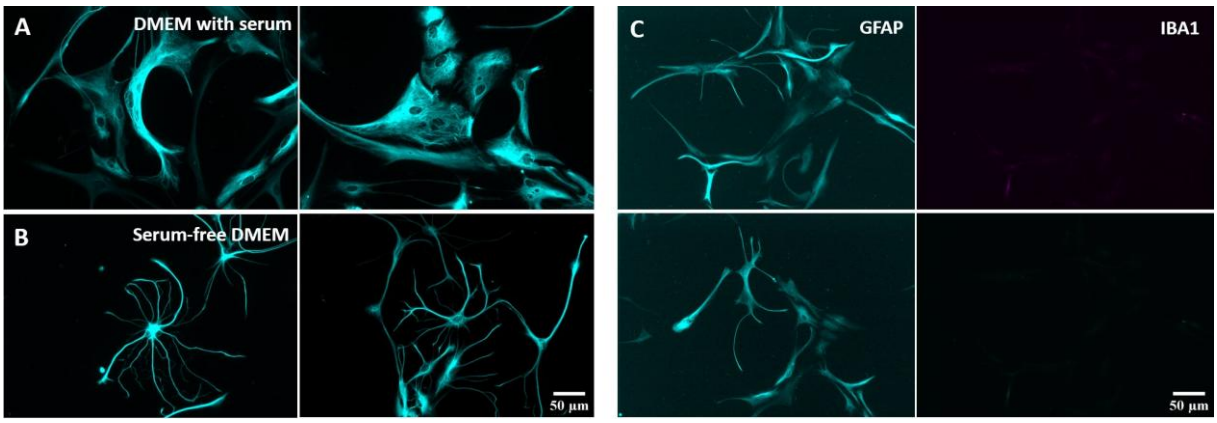

Figure 6: Astrocytes develop different morphology when kept in serum-free medium and cultures are free from microglia. *The astrocyte medium was changed to either serum-free DMEM or DMEM+ containing 10% fetal bovine serum on day 3 after plating. (**A**) Cultivation in DMEM+ and subsequent GFAP immunostaining revealed a flat and broad phenotype with expanded cell bodies, whereas (**B**) astrocytes kept in serum free medium developed fine processes and smaller somata. (**C**) Fluorescence microscopy showed GFAP positive cells, but no signal in the IBA1 channel, thus confirming that astrocyte cultures are free from microglia. Scale bar is 50 µm. DMEM = Dulbecco's Modified Eagle's Medium; GFAP = glial fibrillary acidic protein; IBA1 = ionized calcium-binding adapter molecule 1.*

3.2 The effect of Aβ3(pE)-42 oligomers on primary rat astrocyte cultures

3.2.1 Characterization of Aβ3(pE)-42 oligomers

Soluble oligomeric Aβ species are known to be among the most neurotoxic Aβ aggregates and cause early synaptic dysfunction and neuronal damage in AD [86, 87, 6]. In this study, n-terminally modified, pyro-glutamylated amyloid-β oligomers (Aβ3(pE)-42) were produced and characterized before usage.

SDS-PAGE followed by western blot and antibody detection of oligomers was performed to confirm the broad distribution of different oligomer sizes in the mixture. Protein sizes ranged from low-molecular-weight di-, tri-, tetra- and pentamers (8, 12, 16 and 20 kDa) over midrange-molecular-weight oligomers such as decamers or dodecamers (40 – 48 kDa) to high-molecular-weight oligomers (70 - 200 kDa) ***(Fig. 7A)***. Especially the identified di- and trimers are known to be highly abundant in AD brains and elicit severe neurotoxicity [88].

It is well established in the field, that neuronal extrasynaptic NMDAR signaling induced by oligomeric Aβ species can lead to dephosphorylation of the pro-survival transcription factor cAMP response element-binding protein (CREB), termed CREB-shutoff, which in turn causes early synaptic dysfunction and synapse loss [89, 90]; [91]. In fact, it has been shown that CREB-shutoff mediated by Aβ3(pE)-42 relies mainly on astrocytic Aβ3(pE)-42 uptake and subsequent release of pro-inflammatory cytokine TNFα [19]. Respectively, our experiments showed lower pCREB intensities in mixed hippocampal cultures containing neurons and glial cells following incubation with Aβ3(pE)-42 oligomers ***(Fig. 7B, C)***. Previous studies revealed that astroglial uptake of soluble oligomeric Aβ3(pE)-42 leads to secretion of pro-inflammatory cytokines, which in turn induce an impairment of late-phase long-term potentiation (LTP) in acute hippocampal slices [19, 92]. We therefore decided to test whether our oligomer preparation is functional in terms of synaptotoxicity. Acute brain slices were incubated with Aβ3(pE)-42 oligomers, which indeed resulted in an impairment in late-phase LTP as compared to control slices, while basal synaptic transmission remained unaffected ***(Fig. 7D, E)***.

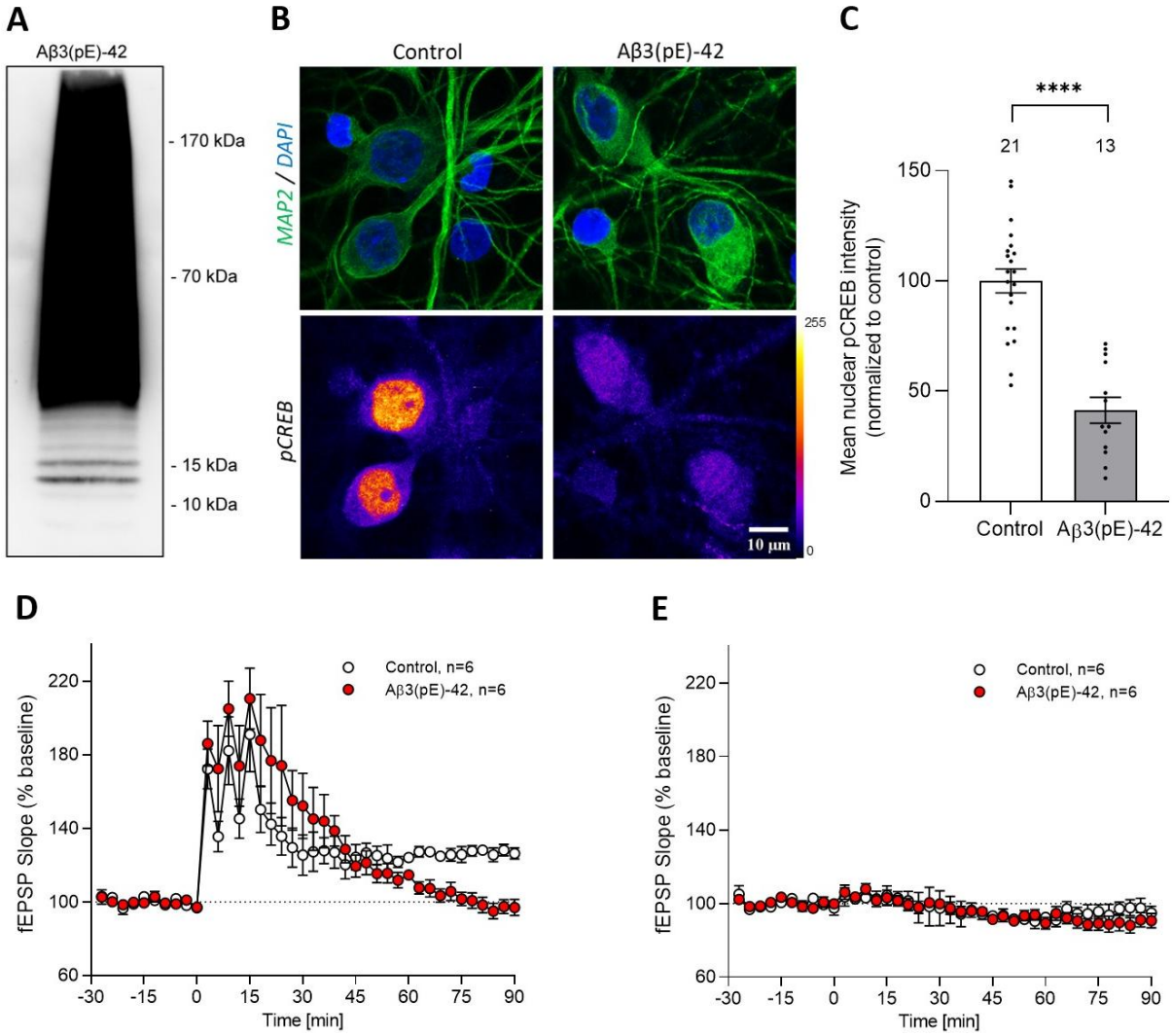

Figure 7: Characterization of oligomeric Aβ3(pE)-42 preparations revealed broad distribution of different oligomer sizes in an SDS-PAGE, CREB shut-off in mixed neuronal cultures and late-phase LTP impairment in acute hippocampal slices. *(**A**) Western blotting and antibody detection of Aβ3(pE)-42 oligomers revealed protein sizes from di- and trimeric oligomers (8 and 12 kDa) up to larger oligomeric protein complexes (>170 kDa). (**B**) Representative confocal images of DIV18 neurons treated with 1 µM Aβ3(pE)-42 oligomers for 72h. Neurons were stained with MAP2 and pCREB antibodies and co-stained with DAPI. Scale bar is 10 µm. Lookup table indicates the pixel intensities from 0 to 255. (**C**) Mean pCREB fluorescence intensity within nuclear region (defined by DAPI) normalized to control. N= 13-21 nuclei. Statistical analysis was done using an unpaired t-test, data are shown as mean ± s.e.m. ****p < 0.0001. (**D**) LTP recordings revealed that Aβ3(pE)-42 causes impairment of late phase LTP as compared to control measurements. (**E**) Basal synaptic transmission is not affected by bath application of Aβ3(pE)-42 oligomers compared to a control group. N=6 slices per group from at least 2 mice. Data are represented as mean ± s.e.m. Aβ3(pE)-42 = pyro-glutamylated amyloid-β; SDS-PAGE = sodium dodecyl sulfate–polyacrylamide gel electrophoresis, CREB = cAMP response element-binding protein; LTP = long-term potentiation*

3.2.2 Astrocytes take up Aβ3(pE)-42 oligomers

In contrast to Aβ1-42, Aβ3(pE)-42 has been shown to be taken up by astrocytes, inducing reactive astrogliosis and subsequent release of the pro-inflammatory cytokine TNFα [19]. To confirm astrocytic Aβ3(pE)-42 uptake under the established experimental conditions, the cultures were incubated with 0.5 µM or 1 µM Aβ3(pE)-42 oligomers for 24 hours. Following immunostaining with corresponding antibodies, fluorescence microscopy showed colocalization of the GFAP and Aβ3(pE)-42 signals, suggesting oligomer uptake by astrocytes *in vitro* ***(Fig. 8)***. In confocal microscopy the same results were observed, proving that astrocytes do indeed take up oligomers instead of merely binding them on the outside of their cellular membranes ***(Fig. 11A, B)***.

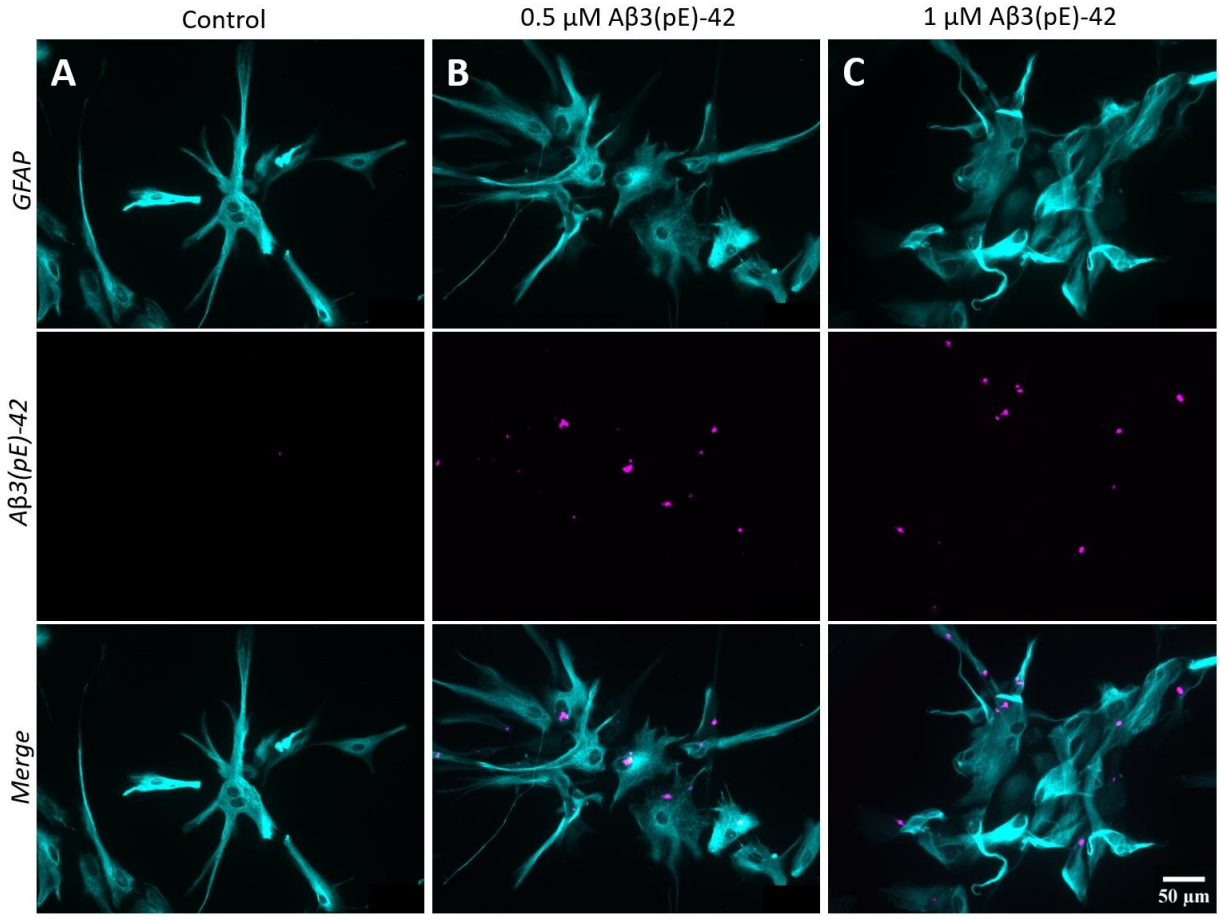

Figure 8: Immunostaining of astrocyte cultures suggests Aβ3(pE)-42 oligomers uptake by astrocytes in vitro. *(**A-C**) Representative fluorescence microscopy images of astrocytes treated with (**A**) no (**B**) 0.5 µM or (**C**) 1µM Aβ3(pE)-42 oligomers for 24 hours. Cells were stained with GFAP and Aβ3(pE)-42 antibodies. Scale bar is 50 µm. Aβ3(pE)-42 = pyro-glutamylated amyloid-β; GFAP = glial fibrillary acidic protein.*

3.2.3 Incubating primary astrocytes with Aβ3(pE)-42 oligomers does not induce significant morphological alterations in GFAP immunostainings

One marker for reactive astrogliosis is the proliferation of intermediate filaments such as GFAP or vimentin [93, 56, 61]. To evaluate, whether GFAP upregulation, quantified by an increase in GFAP-positive area, might be a suitable readout parameter for establishing a screening assay, primary astrocytes were incubated with 0.5 µM or 1 µM Aβ3(pE)-42 oligomers for 24 hours. As an estimation of astrocyte morphology, GFAP positive area was measured manually with a drawing tool in ImageJ software and compared to a control group. A trend towards enlarged GFAP-positive areas was visible, but nevertheless no significant increase could be observed ***(Fig. 9)***. Accordingly, incubating astrocyte cultures with 1 µM Aβ3(pE)-42 for three and five days did not induce visible morphological alterations ***(Fig. 10)***.

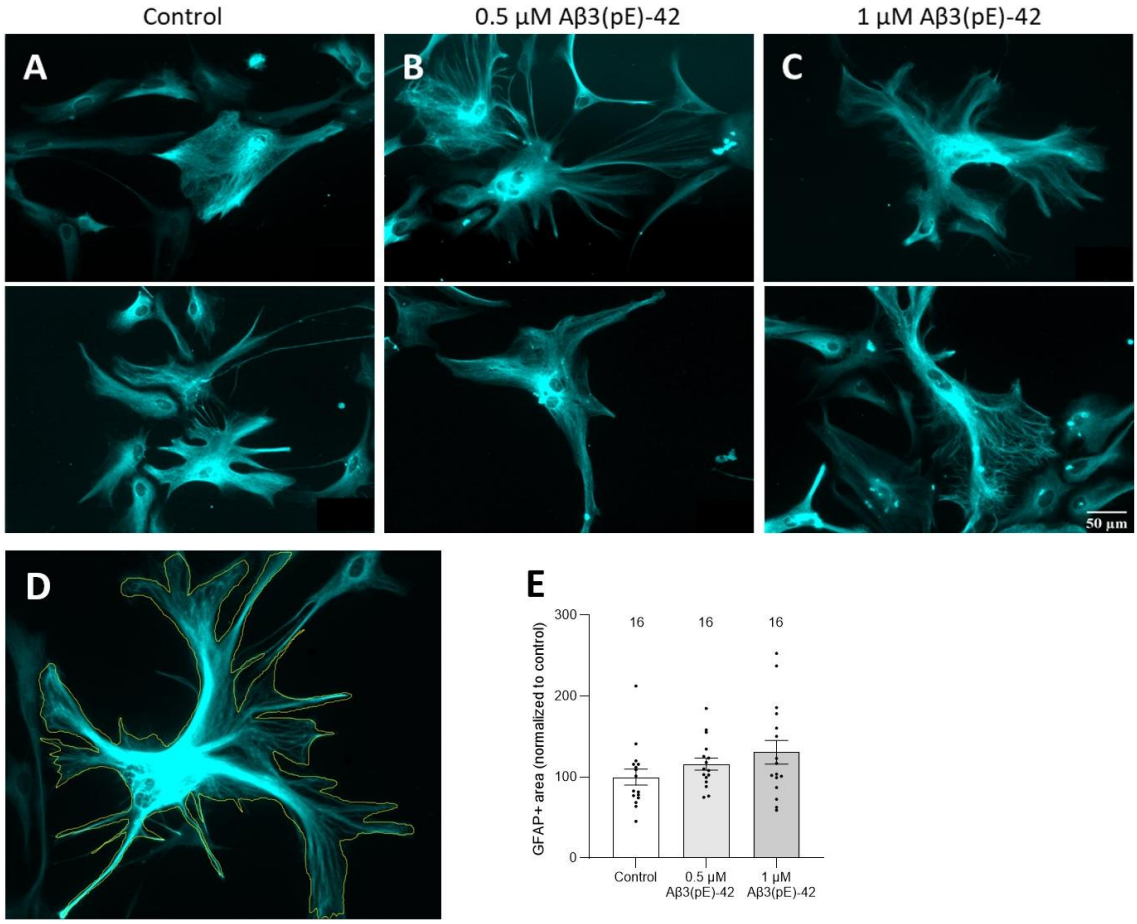

Figure 9: Incubating astrocytes with Aβ3(pE)-42 oligomers shows no significant morphological alterations. *(**A – C**) Exemplary images of astrocytes incubated with (**B**) 0.5 µM or (**C**) 1 µM Aβ3(pE)-42 oligomers for 24 hours and compared to (**A**) a control group without any treatment. Cells were stained with GFAP antibodies. (**D, E**) Image analysis revealed no significant differences in total GFAP positive area. Values were normalized to the control and data show mean ± s.e.m. N = 16 randomly chosen single cells per group. Statistical analysis was done using Welch's and Brown-Forsythe ANOVA with Dunnett's multiple comparisons test. Scale bar is 50 µm. Aβ3(pE)-42 = pyro-glutamylated amyloid-β; GFAP = glial fibrillary acidic protein.*

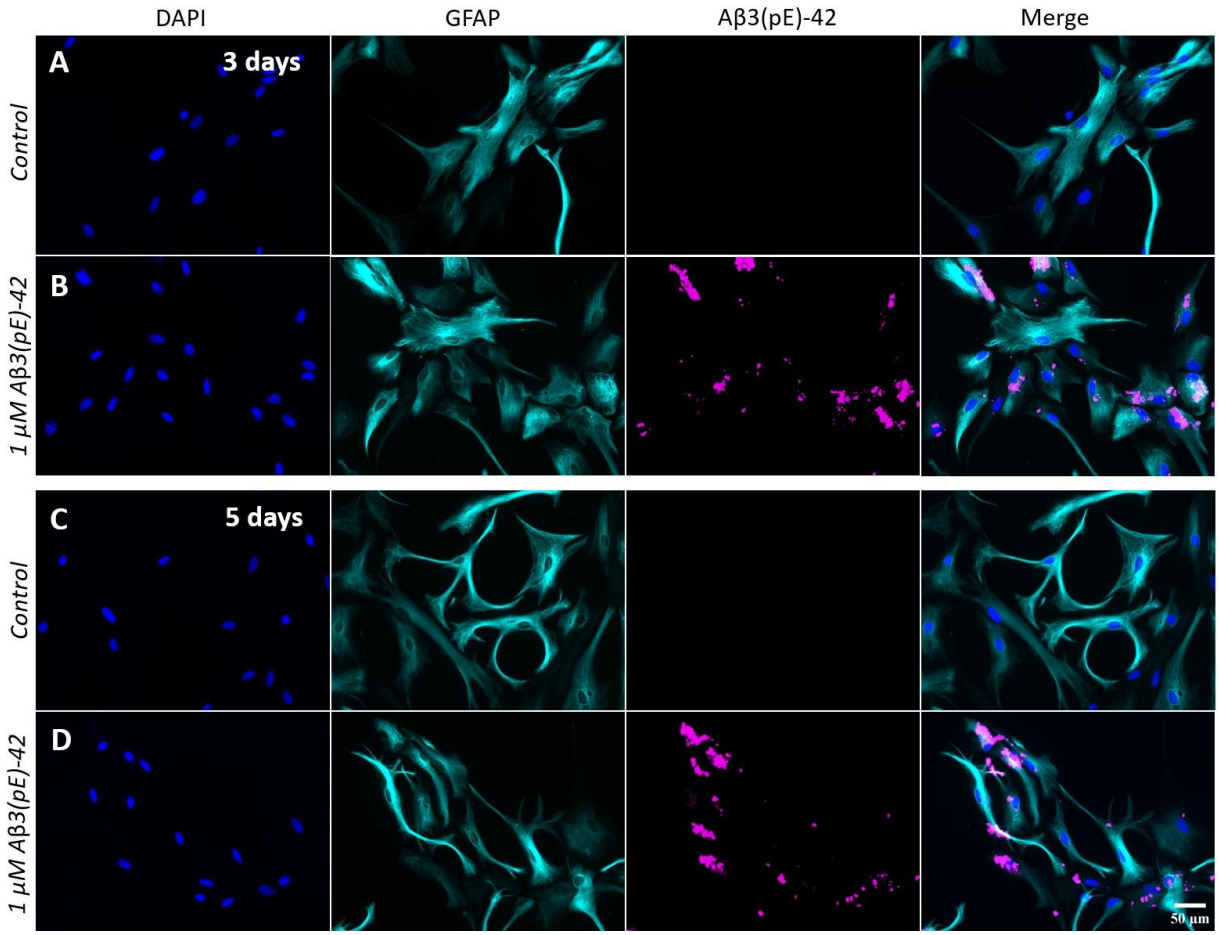

Figure 10: Incubating astrocyte cultures with Aβ3(pE)-42 oligomers for 3 and 5 days does not show morphological alterations. *(**A, B**) Following 3 or (**C, D**) 5 days of incubation with 1µM Aβ3(pE)-42 oligomers no major morphological alterations were visible compared to the control group. Astrocytes were stained with GFAP and Aβ3(pE)-42 antibodies and co-stained with DAPI. Image acquisition was done using fluorescence microscopy. Scale bar is 50µm. Aβ3(pE)-42 = pyro-glutamylated amyloid-β; GFAP = glial fibrillary acidic protein.*

3.2.4 The intermediate filaments GFAP and vimentin colocalize as evidenced by STED microscopy

GFAP and vimentin are two intermediate filaments known to be upregulated in reactive astrocytes and are consequently serving as a marker for reactive astrogliosis. In order to elucidate, whether vimentin might be a more suitable marker than GFAP, we incubated primary astrocytes with Aβ3(pE)-42 oligomers and visualized the cells with GFAP and vimentin antibodies. Unfortunately, similar to GFAP, confocal microscopy did not show any visible alterations regarding vimentin-positive astrocyte morphology ***(Fig. 11A, B)***, which is why we also ruled out vimentin as a possible read-out parameter for the screening assay. Additionally, we performed STED microscopy to show GFAP – vimentin colocalization and therefore confirm depiction of the whole cytoskeleton ***(Fig. 11C)***.

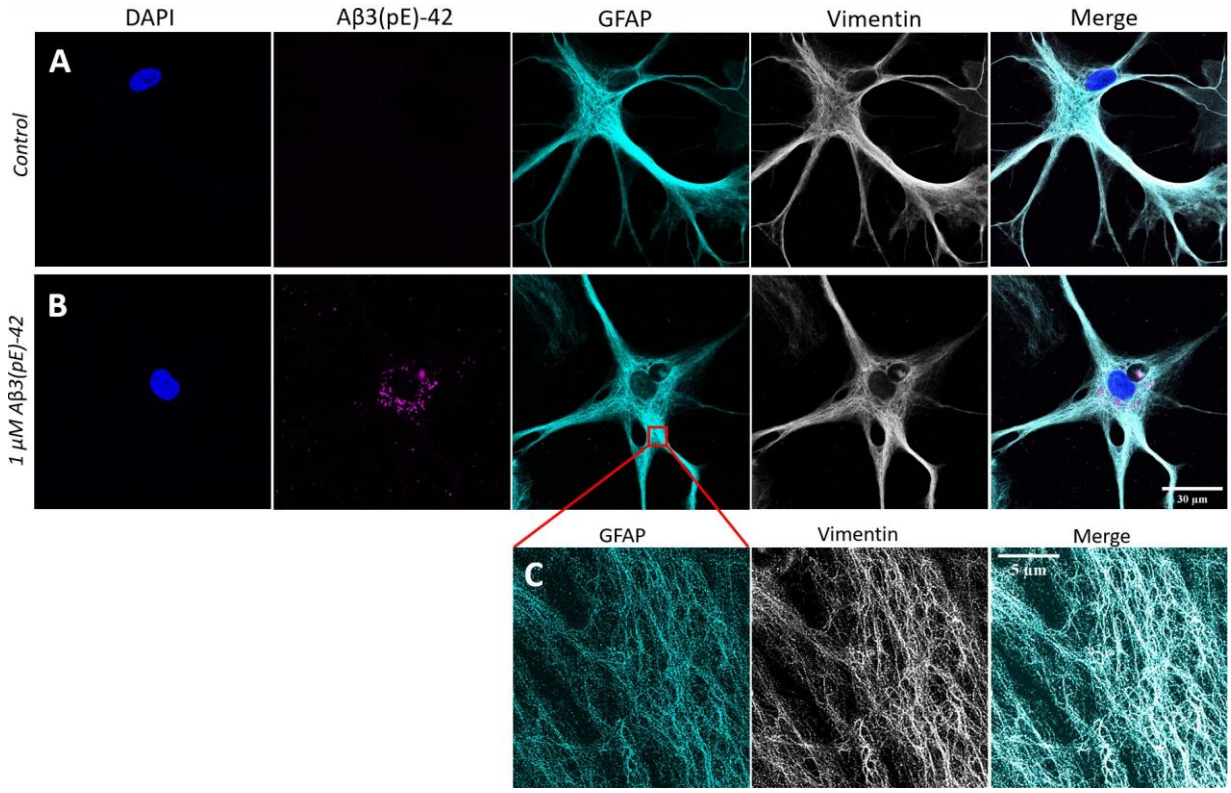

Figure 11: Confocal and STED microscopy show Aβ3(pE)-42 oligomer uptake by astrocytes and GFAP - vimentin colocalization. *(**A, B**) Representative confocal images reveal colocalization of Aβ3(pE)-42, GFAP and vimentin signals in primary astrocyte cultures that were incubated with 1 µM Aβ3(pE)-42 oligomers for 5 days and compared to a control group without Aβ treatment, thus suggesting oligomer uptake by astrocytes in vitro. Scale bar is 30 µm. (**C**) Merging of channels in STED microscopy confirms colocalization of the two intermediate filaments GFAP and vimentin in astrocytes. Scale bar is 5 µm. Aβ3(pE)-42 = pyro-glutamylated amyloid-β; STED = Stimulated emission depletion; GFAP = glial fibrillary acidic protein.*

3.2.5 Incubating astrocytes with Aβ3(pE)-42 oligomers does not induce apoptosis

As a possible screening parameter and to rule out massive cell death following Aβ stimulation, we next assessed whether Aβ3(pE)-42 oligomers induce apoptosis in primary astrocyte cultures. For that purpose, living cells were marked with a green dye, that is turned into a fluorescent state by intracellular esterases and dead cells were marked with a membrane-impermeable red dye labelling DNA fragments with high affinity. Neither number of dead cells, nor the area covered by dead cells showed a significant increase after incubating the cultures with 0.5 µM or 1 µM Aβ3(pE)-42 for 24 hours compared to the control group in live-cell microscopy ***(Fig. 12A – C, E, F)***. 5% DMSO was used as a positive control ***(Fig. 12D)***.

Propidium iodide (PI) is a chemical used to detect apoptosis, as it is also a membrane impermeable and fluorescent DNA-intercalating agent. Treating astrocyte cultures with 2

µL/mL PI solution following Aβ3(pE)-42 incubation did not show any signal in the respective channel in fluorescence microscopy as well ***(Fig. 13A-D)***.

Another measure of apoptotic cell death is binding of Apopxin Green to phosphatidyl-serine residues, that were flipped to the outside of the cells during apoptosis. Again, also with this technique there were no apoptotic astrocytes visible following incubation with Aβ3(pE)-42 oligomers compared to the positive control ***(Fig. 13E-H)***.

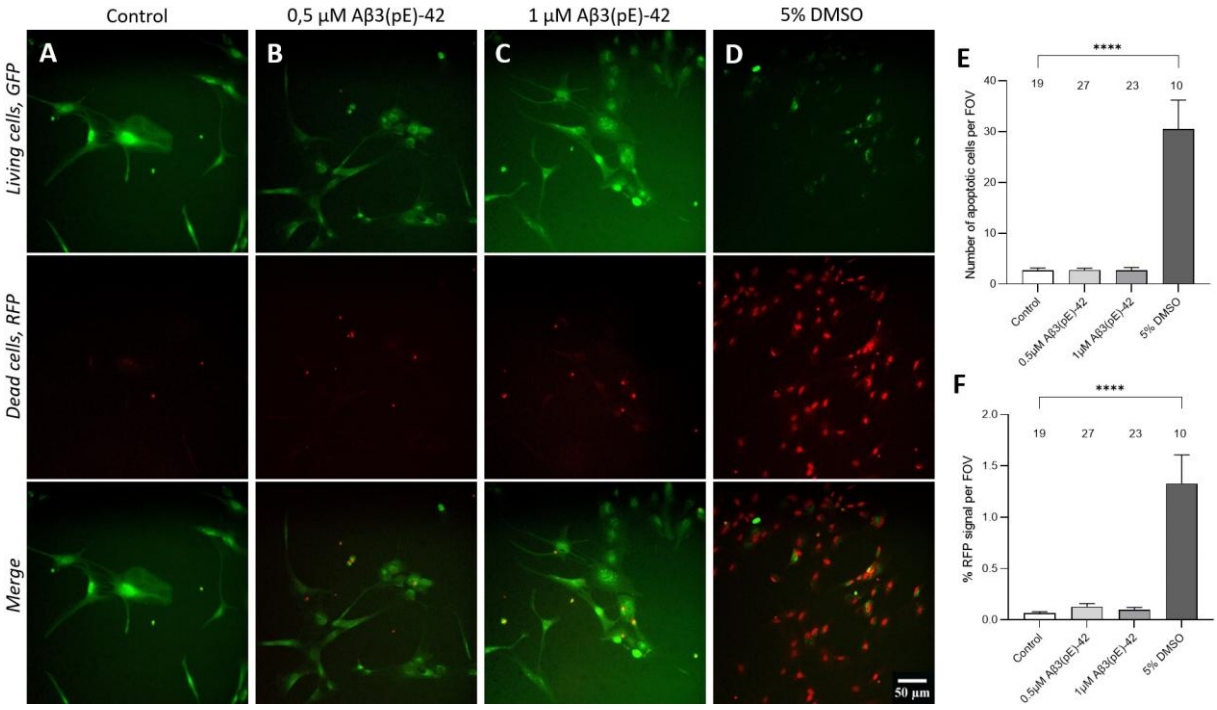

Figure 12: Incubating astrocytes with Aβ3(pE)-42 oligomers does not cause apoptotic cell death in a live and dead cell assay. *(**A – F**) Live-cell imaging of astrocytes following incubation with (**B**) 0.5 µM or (**C**) 1µM Aβ3(pE)-42 oligomers for 24h shows no significant increase of apoptotic cells. (**A – D**) The cultures were incubated with live and dead cell dye for 10 minutes and compared to a control group with no Aβ3(pE)-42 treatment. Living cells are marked in green (GFP) and dead cells in red (RFP). (**D**) 5% DMSO incubation for 2 hours was used as positive control. Scale bar is 50 µm. (**E – F**) Number and % area of RFP-positive cells. Statistical analysis was done using a Kruskal-Wallis test with Dunn's test for multiple comparisons. 10 - 27 FOVs were acquired from at least 4 different coverslips. ****p < 0.0001; Aβ3(pE)-42 = pyro-glutamylated amyloid-β; DMSO = dimethyl sulfoxide.*

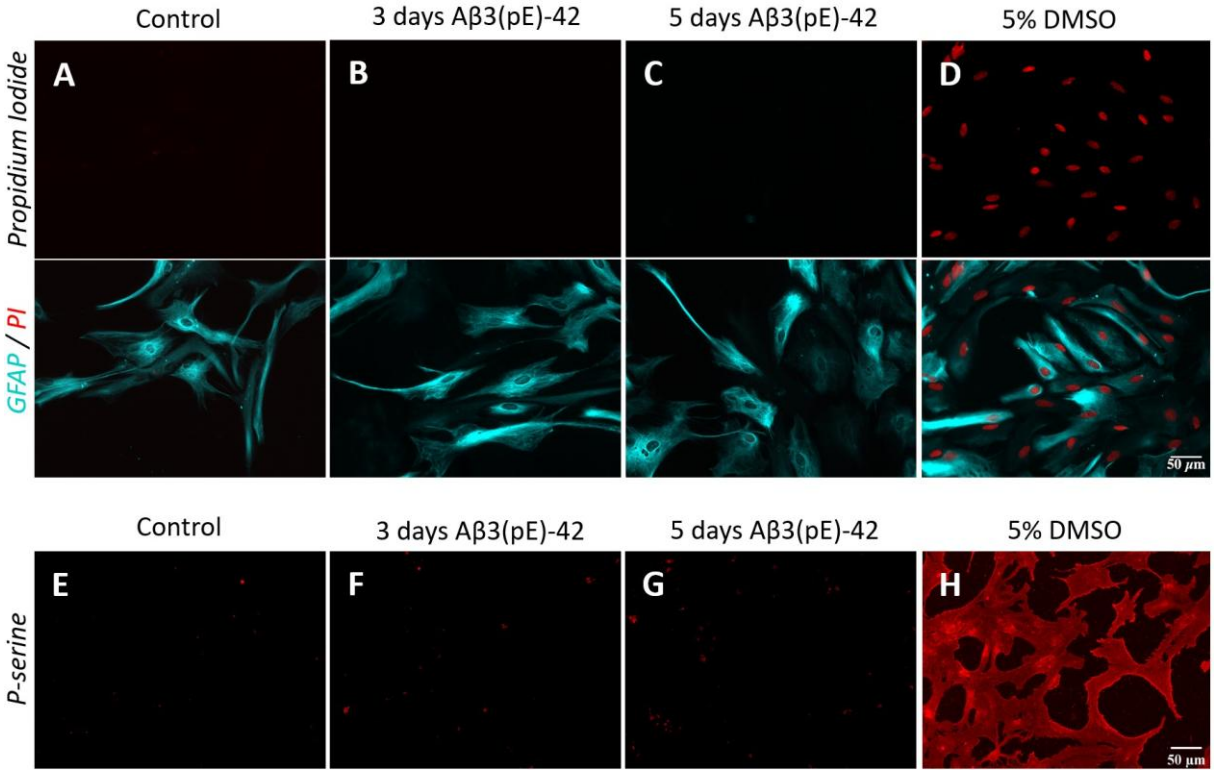

Figure 13: Propidium iodide and an apoptosis assay based on the detection of p-serine residues do not reveal apoptotic cell death in astrocyte cultures following 1µM Aß3(pE)-42 incubation for 3 and 5 days. *(**A – H**) Representative fluorescence microscopy images of astrocytes incubated with 1 µM Aß3(pE)-42 oligomers show no increase in apoptotic cell death. (**A – D**) Cells were treated with 2 µL/mL of propidium iodide or (**E – H**) Apopxin Green and compared to a control group without any Aß3(pE)-42. (**D, H**) 5% DMSO incubation for 1h was used as a positive control. Scale bar is 50µm. Aß3(pE)-42 = pyro-glutamylated amyloid-ß; GFAP = glial fibrillary acidic protein; DMSO = dimethyl sulfoxide; PI = propidium iodide.*

3.2.6 Incubating astrocytes with Aβ3(pE)-42 oligomers does not induce changes in intracellular STAT1, NLRP3 and NF-κB-p65 intensity

The transcription factor Signal transducer and activator of transcription 1 (STAT1) and the inflammasome component NOD-like receptor family pyrin domain containing 3 (NLRP3) are two intracellular proteins, that are known to react to inflammatory stimuli by promoting cell viability and activating pro-inflammatory cytokines. However, following stimulation of astrocytes with Aβ3(pE)-42, STAT1 and NLRP3 immunostainings did not show any significant differences in signal intensities among the groups ***(Fig. 14C - H)***. Consistent with previous results, GFAP-positive cells showed no morphological alterations in fluorescence microscopy following incubation with Aβ3(pE)-42 oligomers ***(Fig. 14A, B)***. The same findings were made in further experiments, where the cells were cultivated in glucose-free medium in order to obtain more reactive astrocytes through glucose deprivation. Morphology as well as STAT1

and NLRP3 intensities did not change significantly upon Aβ3(pE)-42 stimulation as compared to the control group ***(Fig. 15)***.

In line with previous experiments, the transcription factor Nuclear factor-kappa B (NF-κB), subunit p65 was assessed following astrocyte stimulation with Aβ3(pE)-42 oligomers, as it has been shown to be a marker for cell activation and inflammation and to be upregulated in neurons and glia in AD [72, 93, 94, 60]. In fluorescence microscopy, no differences in nuclear p65 intensities were visible after incubating astrocyte cultures with Aβ3(pE)-42 oligomers in comparison to the control group ***(Fig. 16)***.

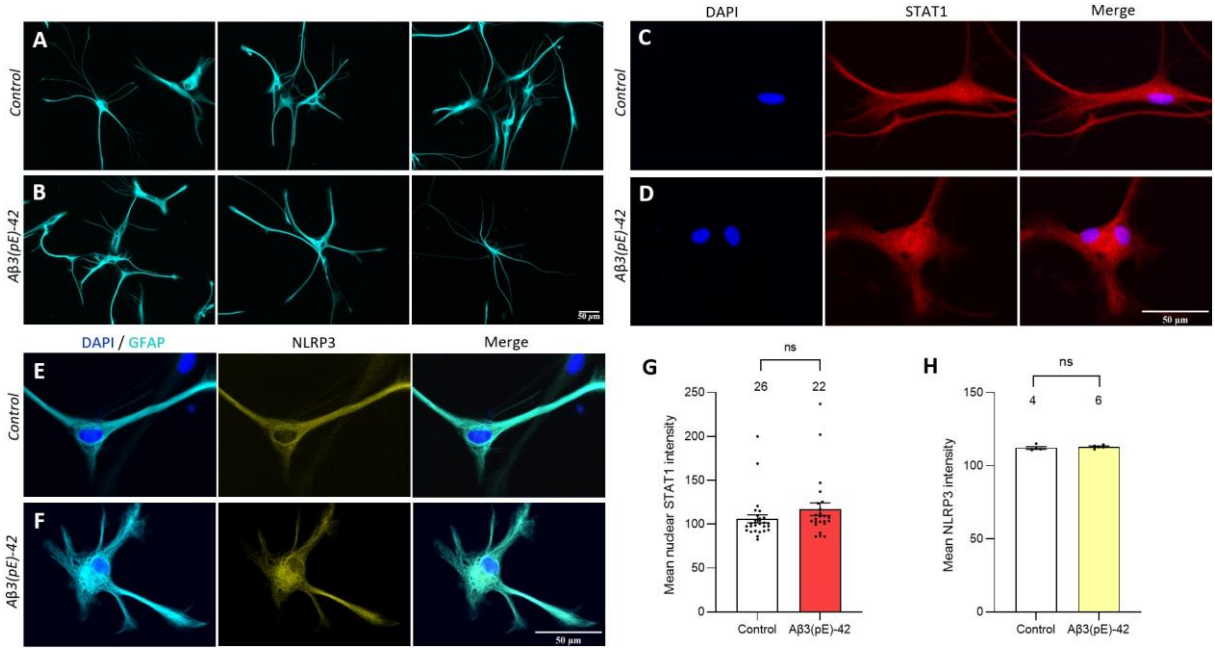

Figure 14: Incubating astrocytes with 1 µM Aβ3(pE)-42 oligomers shows no major alterations in morphology as well as mean nuclear STAT1 and NLRP3 intensity. *(**A - F**) Representative images of astrocytes stimulated with 1µM Aβ3(pE)-42 oligomers for 5 days. (**A, B**) Fluorescence microscopy revealed no morphological alterations following Aβ3(pE)-42 incubation. Cells were stained with GFAP antibodies. (**C, D**) Cells were stained with STAT1 antibodies and co-stained with DAPI. (**E, F**) Cells were stained with NLRP3 and GFAP antibodies and co-stained with DAPI. (**G**) Mean nuclear STAT1 intensity (defined by DAPI) normalized to the control. N = 22 – 26 nuclei from 2 independent experiments. (**H**) Mean NLRP3 intensity per FOV; n = 4 – 6 FOVs. Statistical analysis was done using an unpaired t-test, data are shown as mean ± s.e.m. Scale bar is 50 µm. Aβ3(pE)-42 = pyro-glutamylated amyloid-β; STAT1 = Signal transducer and activator of transcription 1; NLRP3 = NOD-like receptor family pyrin domain containing 3; GFAP = glial fibrillary acidic protein.*

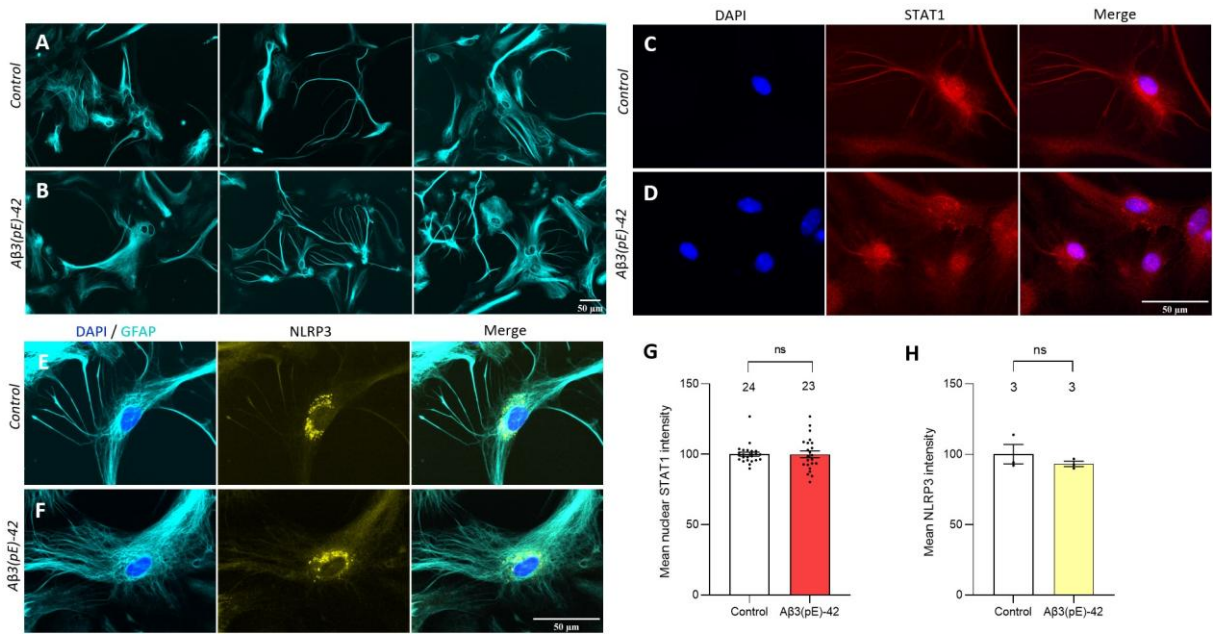

Figure 15: Incubating astrocytes with 1 µM Aβ3(pE)-42 oligomers in glucose-free medium shows no alterations in morphology, mean nuclear STAT1 intensity and NLRP3 intensity. *(**A - F**) Representative images of astrocytes stimulated with 1µM Aβ3(pE)-42 oligomers in glucose-free medium for 5 days. (**A, B**) No morphological alterations were visible following Aβ3(pE)-42 incubation. Cells were stained with GFAP antibodies. (**C, D**) Cells were stained with STAT1 antibodies and co-stained with DAPI. (**E, F**) Cells were stained with NLRP3 and GFAP antibodies, co-stained with DAPI and analyzed with confocal microscopy. (**G**) Mean nuclear STAT1 intensity (defined by DAPI) normalized to the control. N corresponds to 23 – 24 nuclei from 2 independent experiments. (**H**) Mean NLRP3 intensity per FOV; n = 3 FOVs. Statistical analysis was done using an unpaired t-test, data are shown as mean ± s.e.m. Scale bar is 50 µm. Aβ3(pE)-42 = pyro-glutamylated amyloid-β; STAT1 = Signal transducer and activator of transcription 1; NLRP3 = NOD-like receptor family pyrin domain containing 3; GFAP = glial fibrillary acidic protein.*

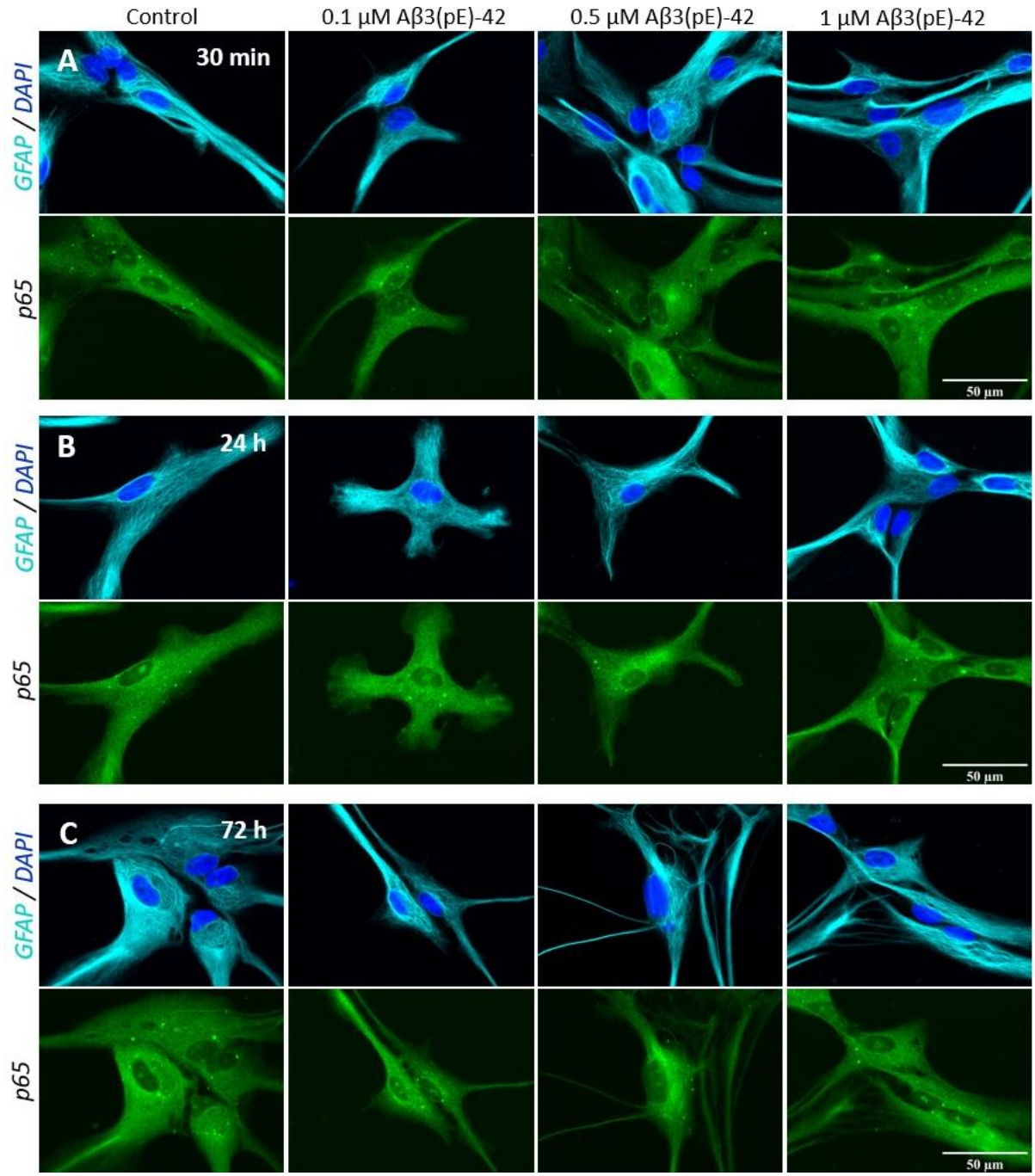

Figure 16: Incubating astrocytes with Aβ3(pE)-42 oligomers does not result in nuclear enrichment of NF-κB subunit p65. *Incubating astrocytes with 0.1µM, 0.5µM or 1µM Aβ oligomers for (**A**) 30 min, (**B**) 24h or (**C**) 72h did not show an enhancement in nuclear p65 intensity in fluorescence microscopy. Astrocytes were stained with NF-κB-p65 antibodies and co-stained with DAPI. Scale bar is 50 µm. Aβ3(pE)-42 = pyro-glutamylated amyloid-β; NF-κB = Nuclear factor-kappa B.*

3.3 Stimulation of primary astrocytes with the pro-inflammatory IFNγ, TGF-β1 and LPS induces alterations in astrocyte morphology and reactivity markers

The highly pro-inflammatory glycoprotein interferon-γ (IFNγ) and the cytokine transforming growth factor β1 (TGFβ1) are known to stimulate astrocytosis and GFAP upregulation and to affect glia cell differentiation, astrocyte-neuron signaling and immune functions *in vivo*. Moreover, their signaling is altered in the brain in neurodegenerative diseases and in aging [95, 96]. Correspondingly, stimulation of astrocytes with 100ng/mL IFNγ revealed significantly increased nuclear STAT1 intensities compared to the control group ***(Fig. 17A, B)***. Incubating the cells with 10 ng/mL TGFβ1 recombinant protein according to a protocol from [97] showed alterations in astrocyte morphology. In fluorescence microscopy the astrocytes appeared in a flat, widely-spread phenotype with visible GFAP positive fibers in the cell soma, compared to the delicate and highly branched cells from the control group ***(Fig. 17C, D)***. Consequently, proper reactivity of primary astrocytes to pro-inflammatory stimuli was verified.

The highly pro-inflammatory agent lipopolysaccharide (LPS), which is originally obtained from the outer layer of gram-negative bacterial walls, was used to stimulate the astrocyte cultures as well. GFAP immunostaining showed morphological alterations in terms of wider and flatter cell somata in the LPS group ***(Fig 18A, B)***. STAT1 stainings revealed significantly higher nuclear STAT1 intensities in the LPS group compared to the control group in fluorescence microscopy ***(Fig. 18C, D, G)***. Additionally, cytosomal NLRP3 intensities increased significantly after incubation with LPS ***(Fig 18E, F, H)***. This experiment, like the previous ones with IFNγ and TGFβ1, ensured astrocyte reactivity under the established *in vitro* conditions and confirmed the functionality of the applied antibodies.

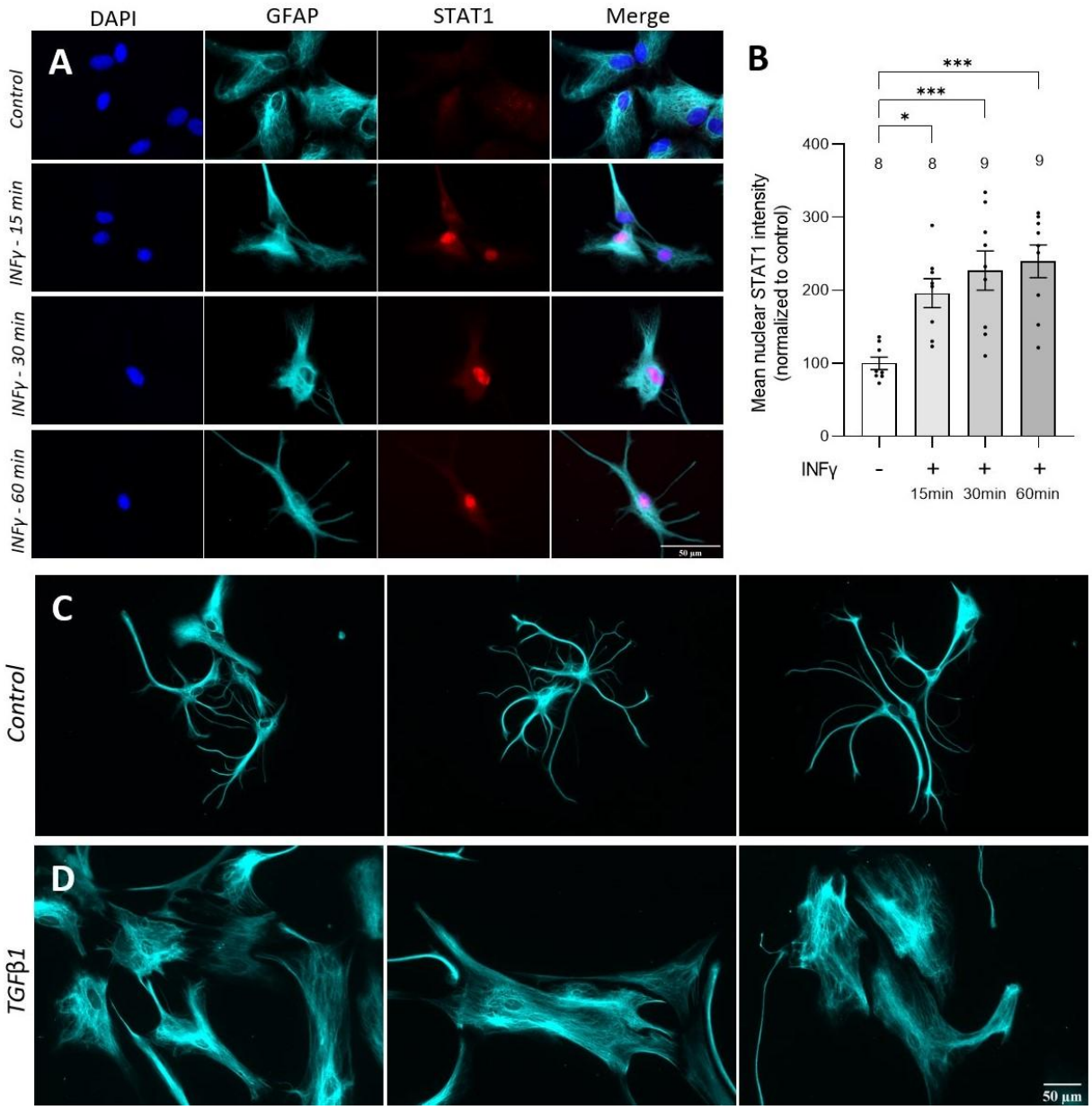

Figure 17: IFNγ treatment increases nuclear STAT1 intensities and TGF-β1 alters astrocyte morphology. *(**A, B**) Incubating astrocytes with 100ng/mL IFNγ for 15, 30 and 60 minutes significantly increased nuclear STAT1 intensities. (**A**) Representative fluorescence microscopy images of astrocytes stained with STAT1 and GFAP antibodies and co-stained with DAPI. (**B**) Mean nuclear STAT1 intensities were normalized and compared to a control group. N refers to number of analyzed FOVs. Statistical analysis was done using a one-way ANOVA and Dunnett's multiple comparisons test and shows mean $\pm$ s.e.m. *p < 0.05; ***p < 0.001. (**C, D**) Incubating astrocytes with 10ng/mL TGF-β1 for 5 days revealed drastic morphological changes with a phenotype of flat, widely-spread cells with visible GFAP positive fibers in the cell soma, compared to the thin and highly branched cells from the control group without any TGFβ treatment. Cells were stained with GFAP antibodies. Scale bar is 50 µm. IFNγ = Interferon γ; TGF-β1 = transforming growth factor β1; STAT1 = Signal transducer and activator of transcription 1; GFAP = glial fibrillary acidic protein.*

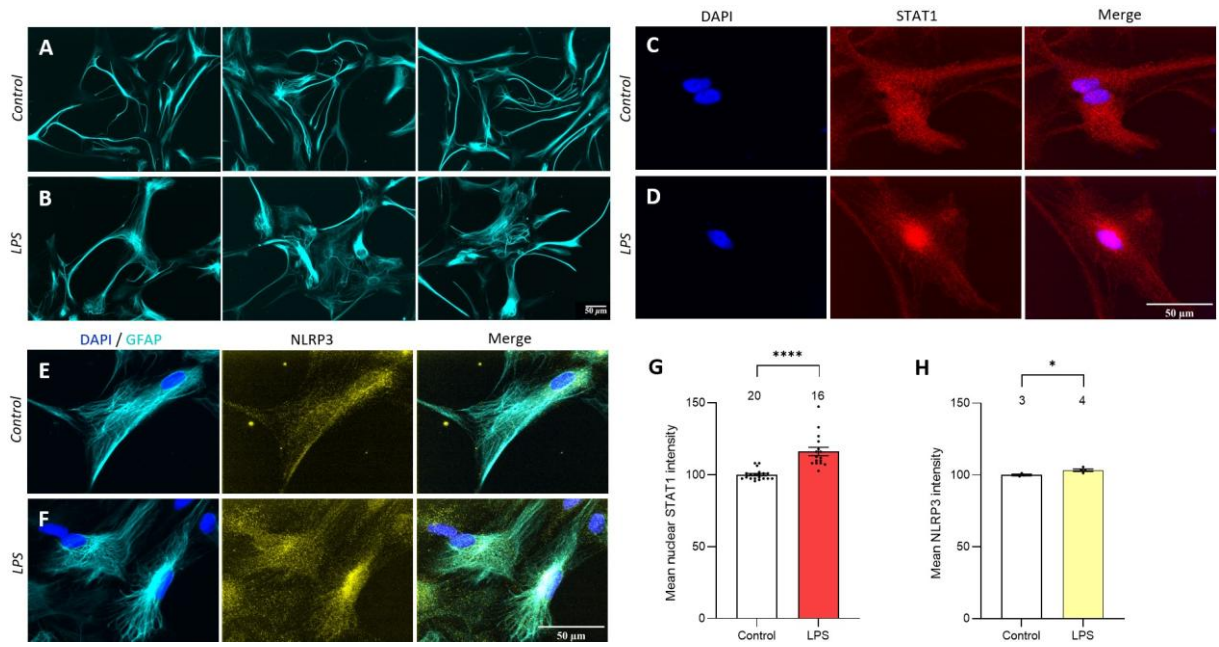

Figure 18: Incubating astrocytes with LPS leads to morphological alterations and increased STAT1 and NLRP3 intensities. *(**A - F**) Representative images of astrocytes stimulated with 100ng/mL LPS in serum- and glucose-free medium for 48h. (**A, B**) Astrocytes exhibited a flatter and broader phenotype compared to the control group with thinner and more branched cell bodies following LPS stimulation. Cells were stained with GFAP antibodies. (**C, D**) Cells were stained with STAT1 antibodies and co-stained with DAPI. (**E, F**) Cells were stained with NLRP3 and GFAP antibodies and co-stained with DAPI. (**G**) Mean nuclear STAT1 intensity (defined by DAPI) normalized to the control. N = 16 – 20 nuclei. (**H**) Maximum NLRP3 intensity per FOV; n = 3 - 4 FOVs. Statistical analysis was done using an unpaired t-test, data are shown as mean ± s.e.m. Scale bar is 50 µm. ∗p < 0.05; ∗∗∗∗p < 0.0001; LPS = Lipopolysaccharide STAT1 = Signal transducer and activator of transcription 1; NLRP3 = NOD-like receptor family pyrin domain containing 3; GFAP = glial fibrillary acidic protein.*

3.4 The effect of Aβ3(pE)-42 on astrocytes in organotypic hippocampal slice cultures and TBA2.1 mouse brain slices

In another experiential setting showed that, opposed to primary astrocyte cultures, Aβ3(pE)-42 has an effect on astrocytic GFAP expression in organotypic hippocampal slice cultures (OHSC) and TBA2.1 mouse brain slices. Organotypic hippocampal slices from rat brains were prepared by Dr. Katarzyna Grochowska and the experiments were performed with cultures at DIV8. Following incubation with 500 nM Aβ3(pE)-42 for 24h, confocal microscopy revealed significantly higher GFAP expression compared to the control group without any Aβ treatment ***(Fig. 19A, B)***. TBA2.1 mice express high amounts of Aβ3(pE)-42 and show profound AD pathology and neuroinflammation especially in the homozygous genotype [13]. Correspondingly, we were able to show significantly higher GFAP levels in the TBA2.1 group compared to wildtype mice ***(Fig. 19C, D)***.

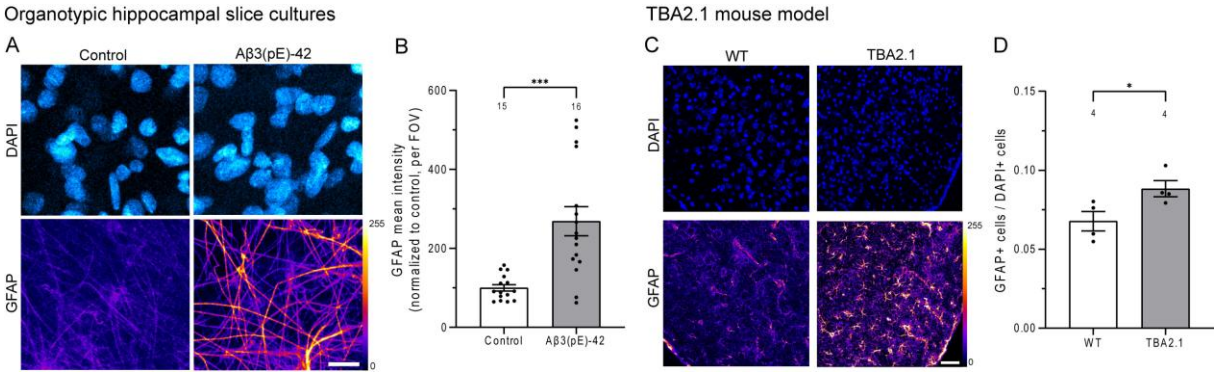

Figure 19: Aβ3(pE)-42 induced GFAP upregulation in organotypic hippocampal slice cultures (OHSC) and brain slices from TBA2.1 mice. *(**A**) Representative confocal images of DIV8 OHSC treated with 500 nM Aβ3(pE)-42 oligomers for 24h. OHSC were stained with antibodies detecting GFAP and co-stained with DAPI as nuclear marker. Scale bar is 15 µm. (**B**) Mean GFAP fluorescence intensity normalized to control. N=15-16 FOVs from at least 5 slices per group from 2 independent cultures. (**C**) Representative confocal images showing the entorhinal cortex of wildtype (WT) and TBA2.1 mice with an increased Aβ3(pE)-42 load. The slices were stained with GFAP antibodies and co-stained with DAPI. Scale bar is 50 µm. Lookup table indicates the pixel intensities from 0 to 255. (**D**) Number of GFAP positive cells per DAPI positive cells. N=4 corresponds to 4 animals per group with each data point showing the mean of 2 - 4 FOVs. *p<0.05, ***p<0.001 by unpaired t-test. Data are presented as mean ± s.e.m. Aβ3(pE)-42 = pyro-glutamylated amyloid-β; GFAP = glial fibrillary acidic protein.*

3.5 Uptake of Aβ3(pE)-42 oligomers in HEK-293T cells

We next asked whether Aβ3(pE)-42 uptake in astrocytes is a general feature of adherent non-neuronal cells. Therefore, we aimed to evaluate whether HEK-293T cells also take up Aβ3(pE)-42 oligomers *in vitro*. In fluorescence microscopy, Aβ3(pE)-42 signals colocalized with the microfilament marker vimentin and were absent in the extracellular space. Based on these findings, we concluded intracellular oligomer uptake by HEK-293T cells ***(Fig. 20A)***. Against our expectations, incubating HEK-293T cells with Aβ3(pE)-42 oligomers did not induce visible changes in fluorescence intensities of the inflammasome marker NLRP3 and the activation marker STAT1 compared to a control group ***(Fig. 20B, C)***.

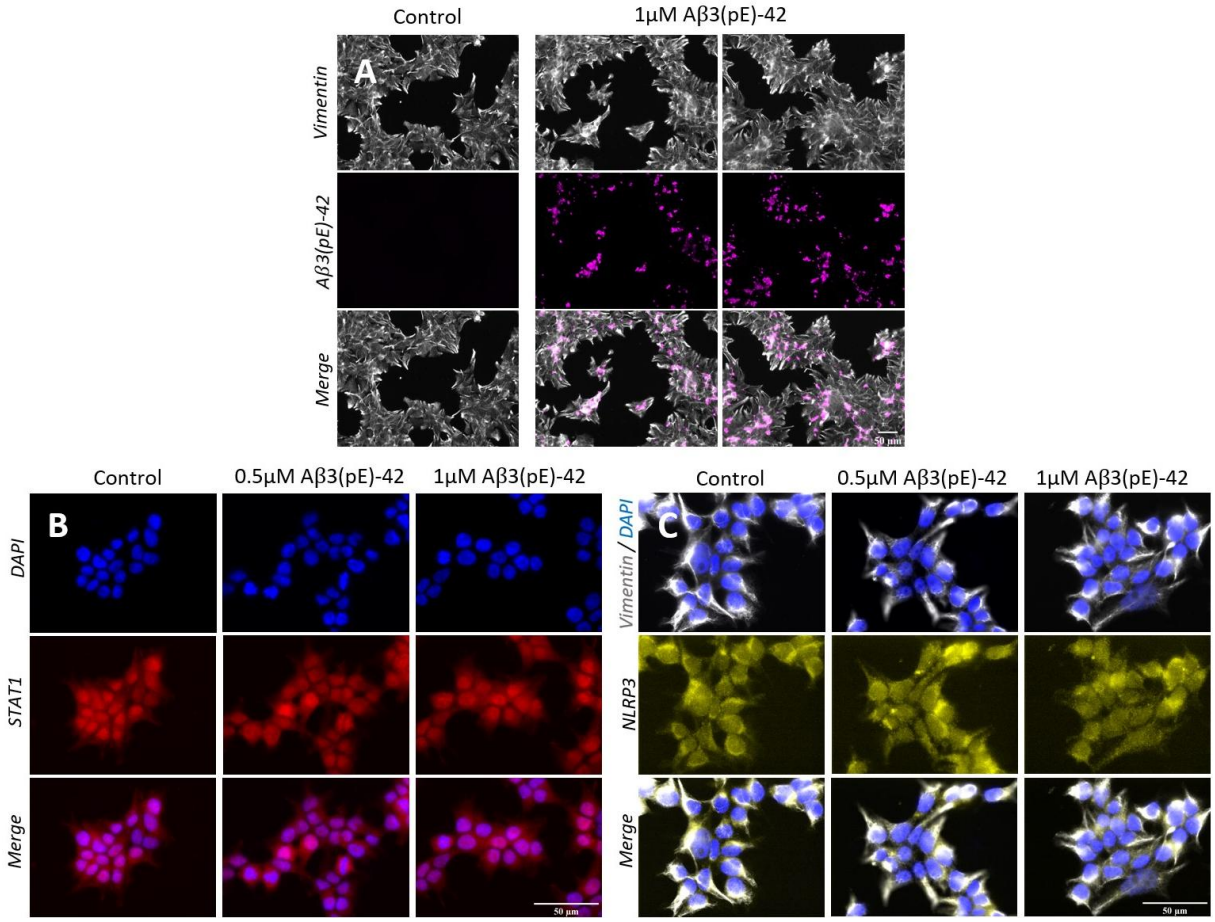

Figure 20: HEK-293T cells take up Aβ3(pE)-42 oligomers, but do not exhibit changes in nuclear STAT1 and cytosomal NLRP3 intensity upon Aβ3(pE)-42 stimulation. *(**A**) Incubating HEK-293T cells with 1µM Aβ3(pE)-42 showed oligomer uptake by HEK cells in vitro. Cells were stained with vimentin and Aβ3(pE)-42 antibodies. (**B, C**) HEK-293T cells showed no alterations in (**B**) nuclear STAT1 intensity and (**C**) cytosomal NLRP3 intensity following incubation with 0.5µM or 1µM Aβ3(pE)-42 oligomers. Cells were stained with vimentin, STAT1 and NLRP3 antibodies and co-stained with DAPI. Scale bar is 50 µm. Aβ3(pE)-42 = pyro-glutamylated amyloid-β; HEK-293T cells = Human Embryonic Kidney-cells; STAT1 = Signal transducer and activator of transcription 1; NLRP3 = NOD-like receptor family pyrin domain containing 3.*

3.6 Screen of plant-based natural products on glial Aβ3(pE)-42 uptake and downstream effects

3.6.1 Establishment of the screening assay

Following the establishment of suitable culturing conditions, including the ideal plating density of astrocytes, the most appropriate medium composition, glial Aβ3(pE)-42 uptake and astrocyte viability, reactivity and reaction to Aβ3(pE)-42 under numerous different culturing conditions *in vitro*, a screening assay to identify plant substances with the ability to influence reactive astrogliosis was implemented in the second part of this thesis. Since previously tested readout-parameters did not show conclusive results, we decided to search for substances that

directly target the uptake of Aβ3(pE)-42 oligomers in astroglia. Therefore, I took advantage of a Natural Product library from the Leibniz Institute of Plant Biochemistry in Halle, Saale.

Initially, 17 substances were pre-selected based on the following criteria: 1) availability/amounts accessible; 2) chemical stability; 3) small molecule, ideally obeying Lipinski rules; 4) natural product or simple derivative, preferentially from plant or mushroom; 5) derived from a plant with reported Anti-Alzheimer or similar neurological activity, including such data from previous research; or from a compound class reported beneficial to neuroinflammation; or compounds with structural similarity to such with reported activity in mental health [98–104]. As the substances were pre-selected by researchers from Halle, their identity remained unknown for the performance of the experiments.

The tested natural products comprised a wide range of compound classes, including flavonoids, triterpenoids, anthraquinones, coumarins and diverse phenolics. Primary astrocytes were cultivated under standardized conditions and 0.5 µM Aβ3(pE)-42 together with the plant substance (5 µM or 50 µM) were applied to the cell culture medium (***Fig. 21; Fig. 22***). Uptake of Aβ3(pE)-42 was monitored following fixation and immunostaining with corresponding antibodies (***Fig. 21A, B***). Due to several washing steps, no extracellular Aβ3(pE)-42 deposits were observed. Quantitation of Aβ3(pE)-42 immunofluorescence was done double-blind with the GFAP mask as ROI (***Fig. 21B***). Therefore, three parameters were introduced to more precisely describe intracellular deposition of Aβ3(pE)-42 oligomers, namely overall intracellular Aβ area, which is the sum of the bidimensional surface of Aβ3(pE)-42, the number of deposits as well as the mean size, which corresponds with the average area per deposit. Hence, intracellular area of Aβ3(pE)-42 presents the best approximation for total glial Aβ3(pE)-42 load and thus uptake, while number and size rather describe morphological features of the deposits.

Out of 17 screened substances 5 significantly reduced the uptake of oligomeric Aβ3(pE)-42 to astrocytes (Epicatechin – 50 µM $p = 0.0015$; Quercetin – 50 µM $p = 0.0003$; Rutin – 5 µM $p = 0.02$; Resveratrol – 50 µM $p = 0.0009$; Apigenin – 5 µM $p = 0.04$; *highlighted in green*), while the others showed no major effect on Aβ3(pE)-42 internalization compared to the control group (***Fig. 22A***). Correspondingly, these 5 substances also decreased the number of intracellular Aβ3(pE)-42 deposits (***Fig. 22B, 23A***), while for 7 other plant substances, a reduced number was compensated by larger intracellular deposits (***Fig. 22C, 23A*** – *indicated in red*),

thus keeping total Aβ3(pE)-42 uptake unaltered. While most substances yielded stronger effects at 50 µM, some proved more efficient at 5 µM concentration, which can be accounted for by different dose-response curves or additional, detrimental activities at very high concentrations. Total GFAP area did not differ significantly from the control in any condition, confirming that the tested plant substances did not affect cell growth and proliferation as well as GFAP expression in primary astrocyte cultures ***(Fig. 23B).***

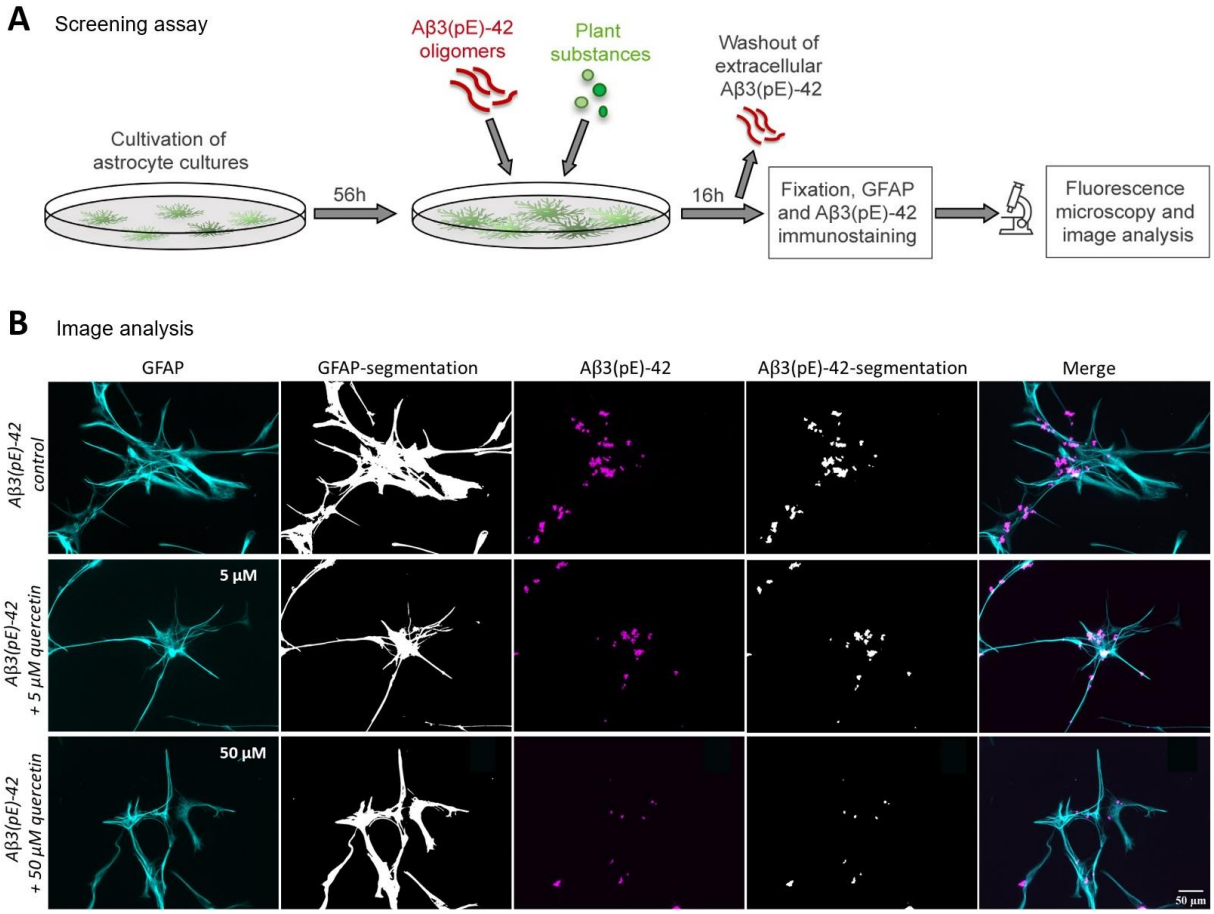

Figure 21: Workflow and image segmentation for a screening assay of plant based natural products on primary astrocyte cultures. *(**A**) Schematic workflow of the screening assay. (**B**) Representative fluorescence and segmented binary images for image analysis with ImageJ software. Astrocyte cultures were simultaneously incubated with 0.5 µM Aβ3(pE)-42 oligomers and 5 µM or 50 µM plant substances for 16 hours, fixed and stained with GFAP and Aβ3(pE)-42 antibodies. Fixed threshold values were applied to 16-bit fluorescence images and resulting binary images were quantified regarding intracellular Aβ3(pE)-42 deposition. The control group was incubated with 0.5 µM Aβ3(pE)-42 oligomers only. Scale bar is 50 µm. Aβ3(pE)-42 = pyro-glutamylated amyloid-β; GFAP = glial fibrillary acidic protein.*

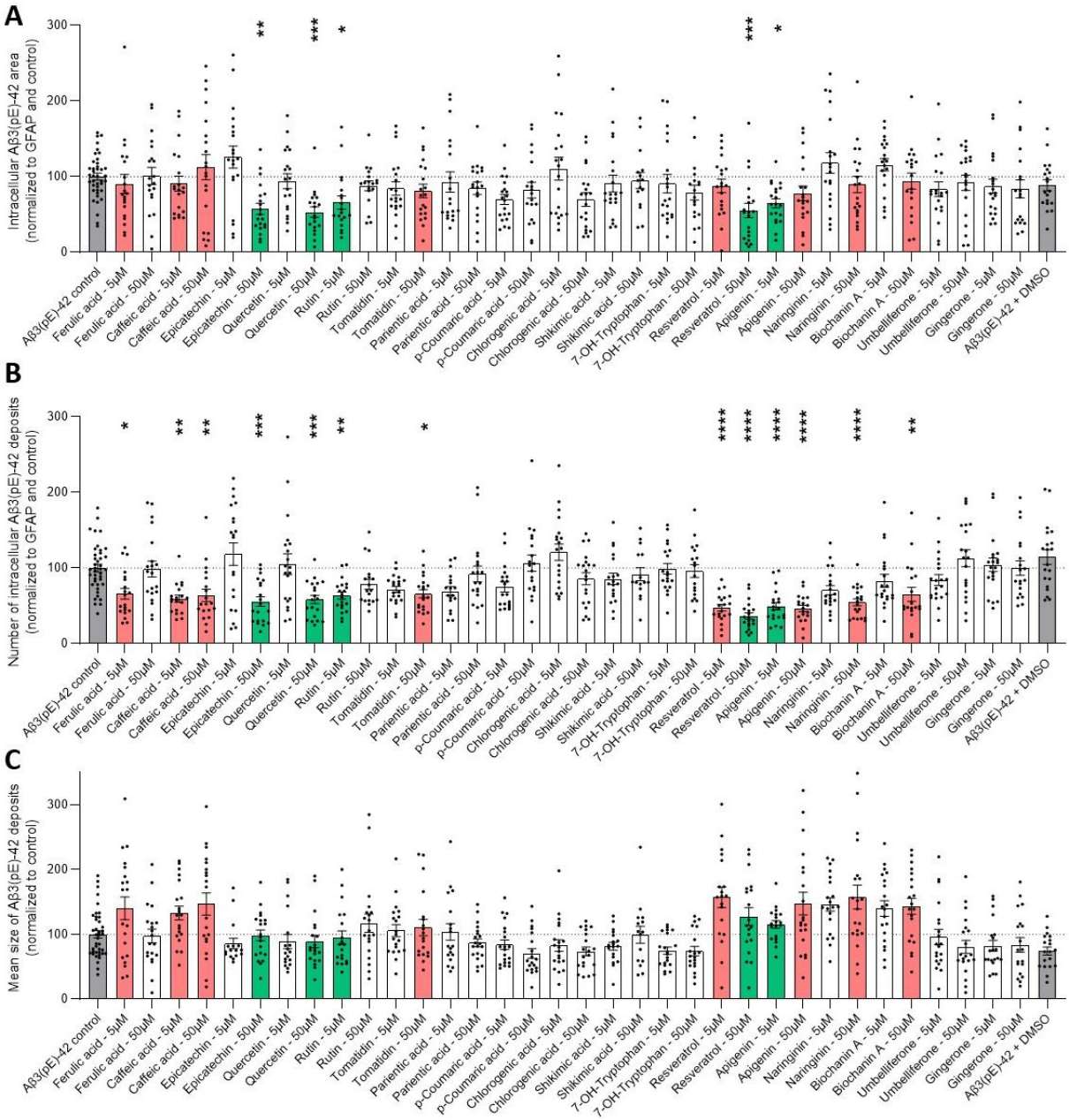

Figure 22: Plant substances alter uptake and morphology of astrocytic, intracellular Aβ3(pE)-42 deposits in a screening assay. *(**A-C**) Plant natural products lead to altered (**A**) intracellular area, (**B**) number and (**C**) size of intracellular Aβ3(pE)-42 deposits. Plant substances highlighted in green reduce intracellular Aβ3(pE)-42 area and number of deposits, while red substances only alter number of deposits, but not total intracellular Aβ3(pE)-42 area. 20 FOVs per condition from two independent experiments were analyzed. Data were normalized to the corresponding control and for (**A, B**) also to GFAP area per FOV. Control represents incubation with Aβ3(pE)-42 only; Aβ3(pE)-42 plus vehicle control for plant substances (DMSO) showed no effect (both in grey). Statistical analysis was done using a Kruskal-Wallis test followed by Dunn's multiple comparisons test. Data are presented as mean ± s.e.m. ∗p < 0.05; ∗∗p < 0.01; ∗∗∗p < 0.001; ∗∗∗∗p < 0.0001; Aβ3(pE)-42 = pyro-glutamylated amyloid-β; GFAP = glial fibrillary acidic protein; 7-OH-Tryptophan = 7-Hydroxy-Tryptophan; Biochanin A = 5,7-Dihydroxy -4'-methoxy-isoflavone.*

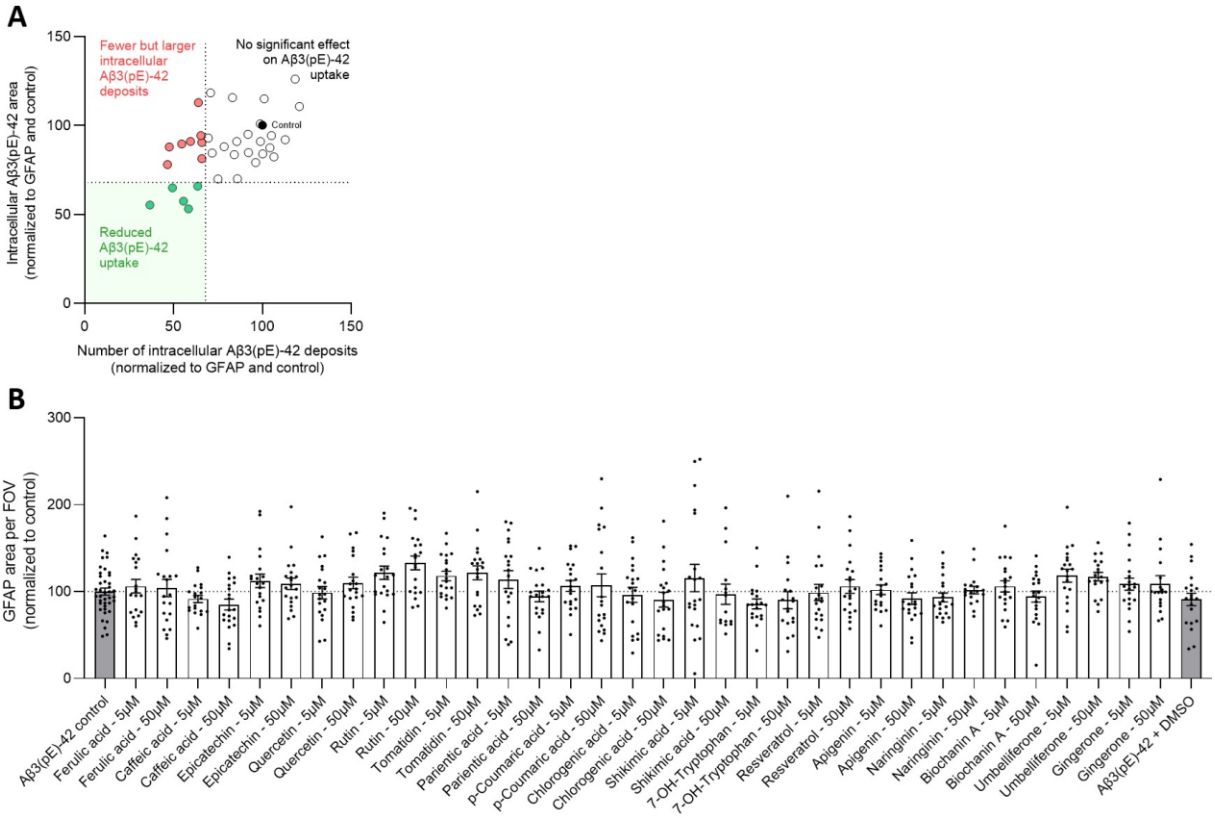

Figure 23: Plant substances alter uptake and morphology of intracellular Aβ3(pE)-42 deposits but not total GFAP area in primary astrocyte cultures. *(**A**) Number of Aβ3(pE)-42 deposits versus intracellular Aβ3(pE)-42 area was plotted for all tested plant substances. The same plant substances like in figure 18 are highlighted in green and red. Green plant substances significantly reduce Aβ3(pE)-42 uptake to astrocytes, while red substances lead to fewer but therefore larger deposits. (**B**) Total GFAP area shows no significant differences compared to the control in any condition. 20 FOVs per condition from two independent experiments were analyzed and normalized to the control. Control represents incubation with Aβ3(pE)-42 only. Statistical analysis was done using a Kruskal-Wallis test followed by Dunn's multiple comparisons test. Data are presented as mean ± s.e.m. Aβ3(pE)-42 = pyro-glutamylated amyloid-β; GFAP = glial fibrillary acidic protein; 7-OH-Tryptophan = 7-Hydroxy-Tryptophan; Biochanin A = 5,7-Dihydroxy -4'-methoxy-isoflavone.*

3.6.2 The effect of quercetin on reactive astrogliosis and synaptic dysfunction

The strongest effect on astrocytic Aβ3(pE)-42 uptake was observed for the two plant substances quercetin and resveratrol (***Fig. 22A***). While protective effects of both substances were previously recognized in AD [64, 68], the literature search revealed that only for quercetin it was demonstrated that it mediates the reduction of Aβ secretion in mammalian cells and protects age-associated neurodegeneration in vivo [105]. Hence, we selected quercetin for more detailed studies, aiming to determine whether our screening reveals a novel, protective function of this substance.

Quercetin is a polyphenolic compound found in a variety of plants. It has many documented biological effects and a prominent anti-inflammatory action in a wide range of assays [64, 68], including senolytic effects in senescent tissue [106]. A hallmark of amyloid-pathology in AD is decreased phosphorylation of the neuronal pro-survival transcription factor CREB, leading to its transcriptional inactivation (CREB shutoff) and the activation of pro-apoptotic signaling [91]. We therefore assessed CREB shutoff in mixed hippocampal cultures (***Fig. 24A, B***). As reported previously, application of Aβ3(pE)-42 resulted in robust neuronal CREB shutoff [19]. Remarkably, however, co-application of quercetin to the medium attenuated CREB shutoff (***Fig. 24A, B***). Thus, quercetin appears to be effective to reduce measures of early neuronal dysfunction in primary cell cultures. The dephosphorylation of CREB is closely associated with Aβ-induced early synaptic dysfunction and synapse loss, which can be observed relatively early in AD. This phenomenon has been described in *in vitro* AD models using mixed hippocampal cell cultures [19]. We followed up on this observation and quantified synapse density in hippocampal primary neurons at DIV16-18 (***Fig. 24C, D***). A single synapse was defined as overlapping or opposing immunosignals of Synaptophysin-1 and PSD95 as pre-and postsynaptic markers respectively (***Fig. 24C, D***). In accord to a previous study, we found clear synapse loss in response to Aβ3(pE)-42 application, which has been shown to be induced by astroglial release of TNFα [19]. Notably, co-application of quercetin to mixed hippocampal cultures significantly reduced the observed synapse loss (***Fig. 24C, D***).

To test whether the protective effect of quercetin is associated with glial activation, we employed organotypic hippocampal slices. Consistent with GFAP being a common marker of reactive astrogliosis, we found that application of Aβ3(pE)-42 clearly increased GFAP-immunoreactivity in these slices (***Fig. 25A, B***). However, when quercetin was co-administered, the Aβ3(pE)-42-induced increase in GFAP-positive reactive astroglia was no longer detectable (***Fig. 25A, B***). The same effect was observed for intermediate filament vimentin, another marker of reactive astrogliosis (***Fig. 25C, D***).

Aβ3(pE)-42-induced glia activation leads to an impairment in synaptic function [19]. Therefore, to see whether quercetin can rescue synaptic impairment, we next evaluated LTP as a cellular model of learning and memory in acute hippocampal slices. As shown above, following the administration of oligomeric Aβ3(pE)-42, field recordings revealed a severe impairment of late phase LTP (***Fig. 7, Fig. 26A, B***). However, we observed a stunning rescue of

late-phase LTP in slices co-treated with quercetin (***Fig. 26C-F***). The substance as such had no effect on the fEPSP during the time of recording (***Fig. 26C-F***). Interestingly, we found that quercetin was also effective in preventing uptake of Aβ3(pE)-42 to HEK293T cells (***Fig. 27A-D***), pointing to a general role in preventing cellular accumulation of this plant metabolite. The experiments from ***Fig. 24 and 25*** were performed by Dr. Katarzyna Grochowska, LTP experiments (***Fig. 26***) were performed by Dr. PingAn Yuanxiang.

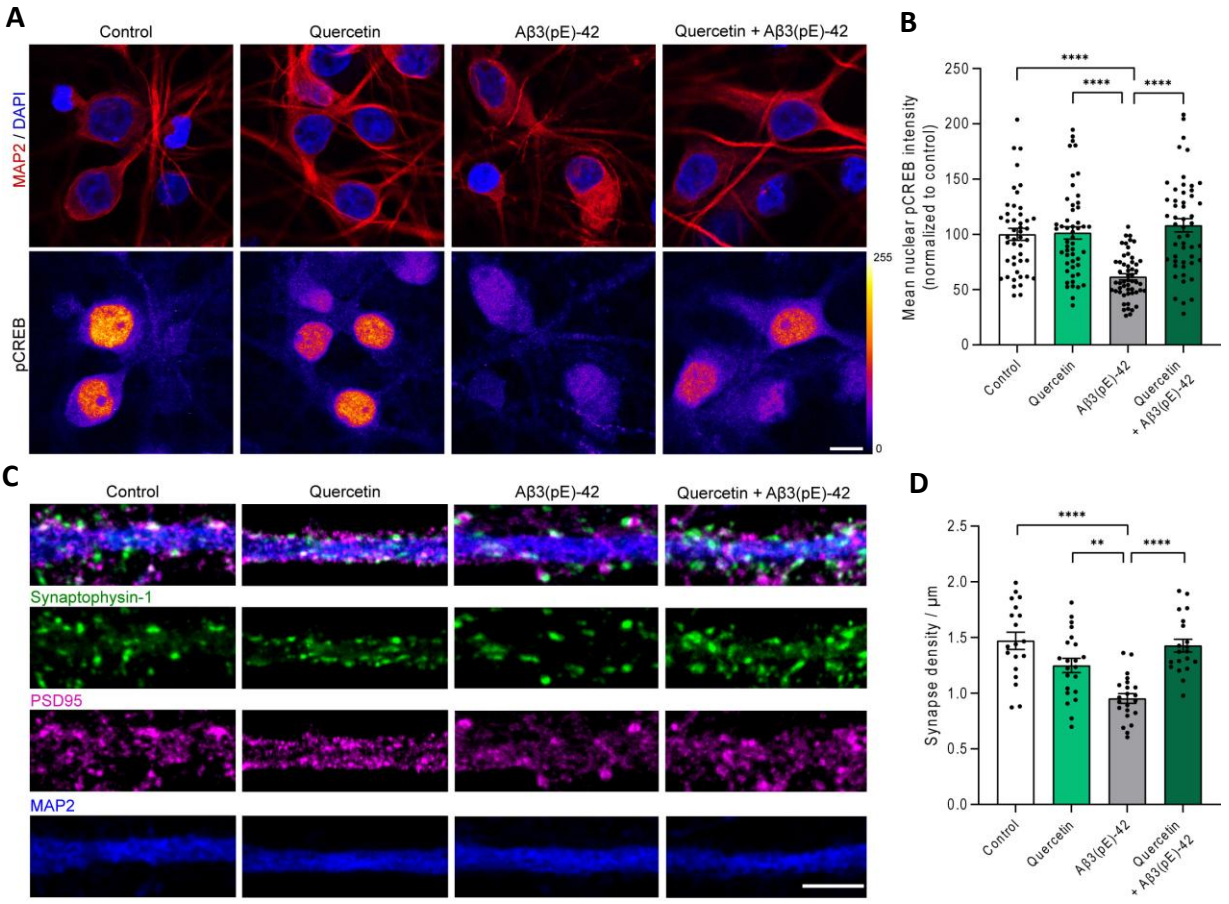

Figure 24: Quercetin rescues Aϐ3(pE)-42-induced CREB shutoff and loss of synapse density in mixed hippocampal cultures. *(**A, B**) Co-application of quercetin prevents Aϐ3(pE)-42-induced loss in CREB phosphorylation. (**A**) Representative confocal images of DIV18 mixed cultures treated with 50 µM quercetin, 500 nM Aϐ3(pE)-42 oligomers or 50 µM quercetin and 500 nM Aϐ3(pE)-42 oligomers for 72h. Neurons were stained with MAP2 and pCREB antibodies and co-stained with DAPI. Scale bar is 10 µm. (**B**) Mean pCREB fluorescence intensity within nuclear region (defined by DAPI) normalized to control. N= 46-53 nuclei from 2 independent cell cultures. (**C, D**) Co-application of quercetin prevents Aϐ3(pE)-42-induced synapse loss. (**C**) Representative images of DIV18 mixed cultures treated with 50 µM quercetin, 500 nM Aϐ3(pE)-42 oligomers or 50 µM quercetin and 500 nM Aϐ3(pE)-42 oligomers for 48h. Neurons were stained with Synaptophysin-1, PSD95, and MAP2 antibodies. Individual synapse was defined as overlapping or opposing Synaptophysin-1 and PSD95 immunosignal. Scale bar is 5 µm. (**D**) Mean number of synapses per 1 µm. N=19-22 dendritic segments from at least 2 independent cultures. (**B, D**) **p<0.01, ****p<0.0001 by two-way ANOVA with posthoc Tukey test. Data are presented as mean ± s.e.m. Aϐ3(pE)-42 = pyro-glutamylated amyloid-ϐ; CREB = cAMP response element binding protein.*

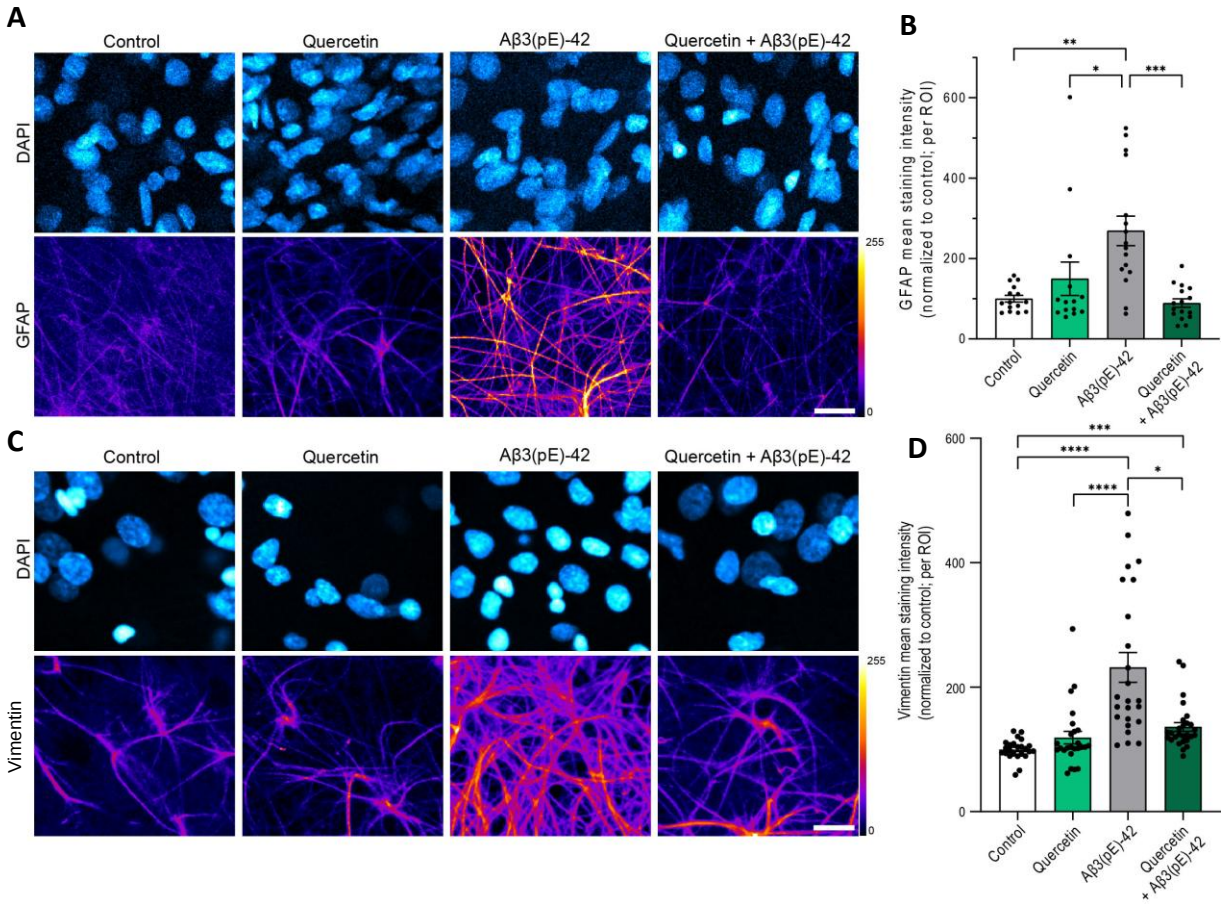

Figure 25: Quercetin prevents upregulation of GFAP and vimentin in astrocytes. *(**A-D**) Co-application of quercetin prevents Aβ3(pE)-42-induced increased (**A, B**) GFAP or (**C, D**) vimentin immunoreactivity in organotypic hippocampal slices (OHSC). (**A**) Representative confocal images of DIV8 OHSC treated with 50 µM quercetin, 500 nM Aβ3(pE)-42 oligomers or 50 µM quercetin and 500 nM Aβ3(pE)-42 oligomers for 24h. OHSC were stained with antibody detecting GFAP and co-stained with DAPI (nuclear marker). Scale bar is 15 µm. Lookup table indicates the pixel intensities from 0 to 255. (**B**) Mean GFAP fluorescence intensity normalized to control. N=14-16 ROIs from at least 5 slices per group from 2 independent cultures. (**C**) Representative confocal images of DIV8-15 OHSC treated with 50 µM quercetin, 500 nM Aβ3(pE)-42 oligomers or 50 µM quercetin and 500 nM Aβ3(pE)-42 oligomers for 24h. OHSC were stained with antibody detecting vimentin and co-stained with DAPI (nuclear marker). Scale bar is 15 µm. Lookup table indicates the pixel intensities from 0 to 255. (**D**) Mean vimentin fluorescence intensity normalized to control. N=24-27 ROIs from at least 3 slices per group from 2 independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by Kruskal-Wallis test followed by Dunn's multiple comparisons test. Data are presented as mean ± s.e.m. Aβ3(pE)-42 = pyro-glutamylated amyloid-β; GFAP = glial fibrillary acidic protein.*

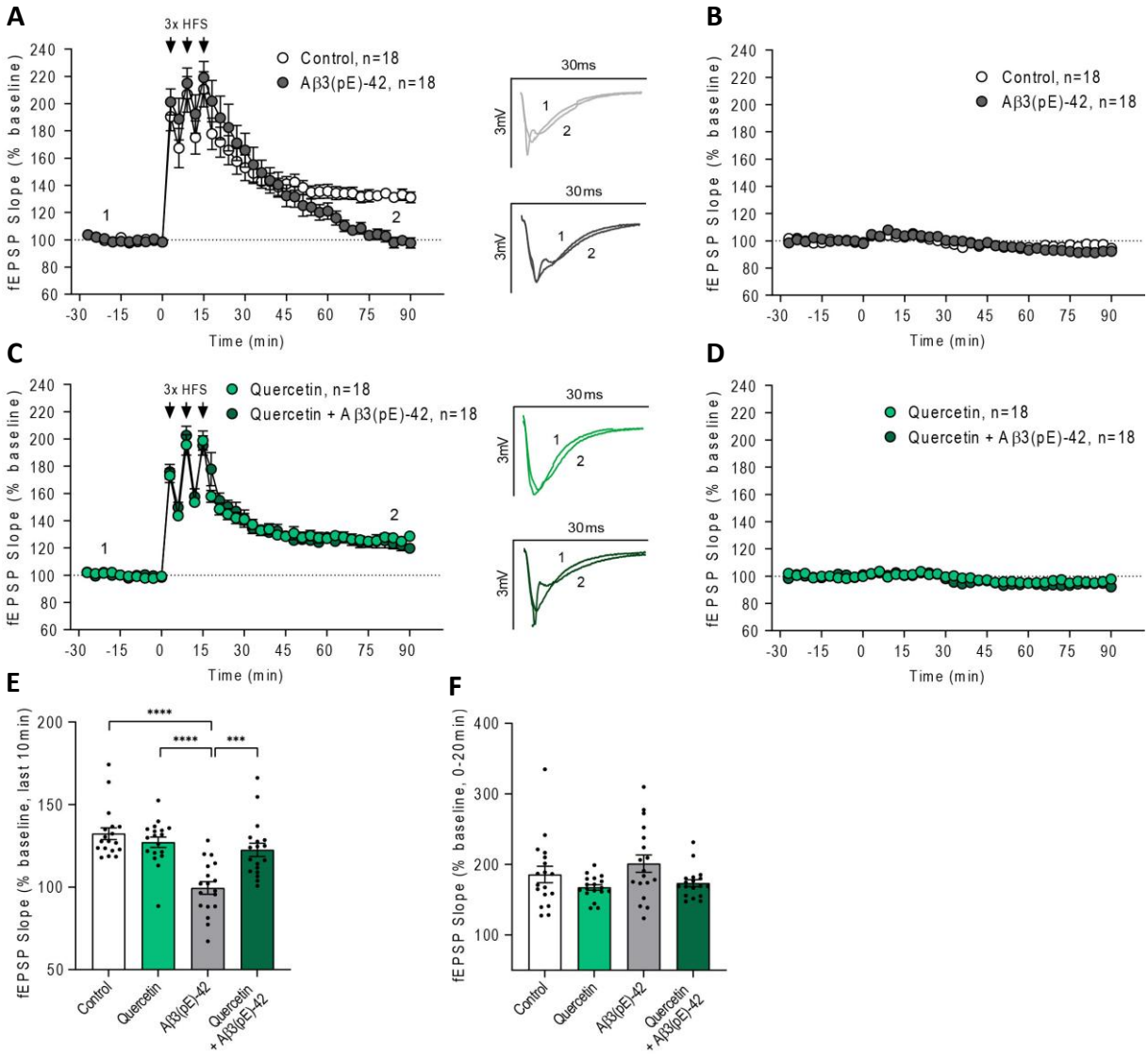

Figure 26: Quercetin rescues Aβ3(pE)-42-induced late LTP impairment. ***(A-D)*** *LTP recordings revealed that* ***(A)*** *2h pre-treatment with Aβ3(pE)-42 oligomers causes impairment of late phase LTP, which is rescued by application of* ***(C)*** *quercetin, compared to control measurements. Accordingly, to the experimental group, the oligomers and quercetin were present in the bath during recordings. Insets show representative fEPSPs analog traces at indicated time points: 1 = baseline, 2 = late LTP.* ***(B, D)*** *Basal synaptic transmission is not affected by bath application of* ***(B)*** *Aβ3(pE)-42 oligomers compared to a control and* ***(D)*** *by quercetin applied alone or with Aβ3(pE)-42 oligomers.* ***(E, F)*** *Dot plot representing* ***(E)*** *Aβ3(pE)-42 induced late phase LTP rescue by quercetin, while* ***(F)*** *early LTP and induction phase are not affected. N = 18 slices per group from at least 6 mice.* ***(E, F)*** ****p<0.001. ****p<0.0001 by two-way ANOVA with Tukey's multiple comparison test. Data are presented as mean ± s.e.m.*

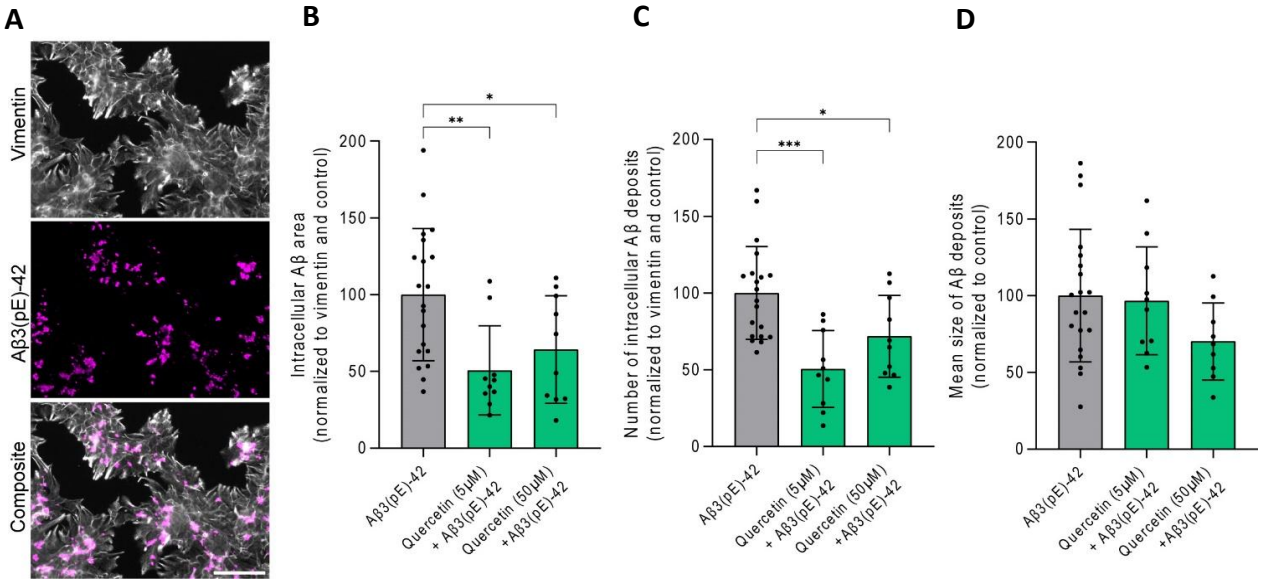

Figure 27: Quercetin reduces uptake and number of intracellular Aβ3(pE)-42 deposits in HEK-293T cell cultures. *(**A**) Representative fluorescence microscopy images of HEK-293T cells treated for 10h with Aβ3(pE)-42 and stained with antibodies detecting Aβ3(pE)-42 and vimentin. Scale bar is 100 µm. (**B-D**) Treatment with both 5 µM and 50 µM quercetin reduces (**B**) area and (**C**) number but not (**D**) mean size of intracellular Aβ3(pE)-42 deposits. N = 10 FOVs. *p<0.05, **p<0.01, ***p<0.001 by one-way ANOVA followed by Dunnett's multiple comparison test. Data are presented as mean ± s.e.m. Aβ3(pE)-42 = pyro-glutamylated amyloid-β; HEK-293T = human embryonal kidney cells.*

4. Discussion

AD is a devastating neurodegenerative disease with yet insufficiently understood pathogenesis and ineffective therapeutic strategies. Among others, two major hallmarks of the disease include deposition of Aβ and chronic low-level neuroinflammation. A specifically toxic PTM of Aβ, Aβ3(pE)-42, has been shown to be a major part of Aβ deposits specific to human AD brains while being absent in normal aging. Aβ3(pE)-42 is taken up by astrocytes, which in turn release pro-inflammatory cytokines such as TNFα, driving neuroinflammation, synaptic dysfunction and neurodegeneration [19]. Therefore, one intriguing therapeutical approach, that comes to mind is the inhibition of astroglial Aβ3(pE)-42 uptake, which could reduce advancing neuroinflammation and thereby slow progressive cognitive decline.

Plant substances are an essential component of a healthy diet and have come into greater medical focus in the past decades. They comprise numerous beneficial properties and have been shown to be useful in a number of diseases, such as heart disease, inflammatory diseases, cancer or neurodegenerative diseases in animal and human studies [64–67]. However, the exact molecular mechanisms underlying these positive effects are largely unknown and need yet to be studied. Therefore, one aim of this thesis was to develop a screening assay to evaluate, whether certain plant substances from a Natural Product library show protective properties on primary astrocytes upon exposure to Aβ3(pE)-42.

4.1 Establishment of culturing system and screening assay

4.1.1 Finding the ideal culturing conditions – serum proteins and glucose

This thesis comprises two major parts. The first part focuses on establishing a suitable culturing system to study primary astrocytes and their responses to different stimuli *in vitro*. The primary aim here was to identify optimal culturing conditions that induce a reactive phenotype in astrocytes upon stimulation with Aβ3(pE)-42, enabling the selection of an appropriate readout parameter for the intended screening assay.

Initially, I experimented with different cell culture media. Several studies underline the notion, that primary astrocytes should be cultivated and grown in serum-free medium. Upon exposure to blood serum, astrocytes developed altered gene expression profiles with

unphysiological phenotypes, since they are typically separated from serum proteins by the blood-brain-barrier *in vivo* [39, 84, 40, 85]. In fact, Foo and colleagues found out, that over 350 genes and up to 28 intracellular pathways were induced and modified in astrocyte cultures with exposure to serum proteins, most of which did not revert back after serum removal [84]. Even though gene expression was not quantified, I did observe astrocytes with altered morphological features that were kept in medium containing 10% fetal-calf-serum as compared to serum-free medium (*see 3.1.3*). Consequently, primary astrocytes were seeded and grown in serum-free medium.

Once stimulated with Aβ3(pE)-42, the astrocytes showed no measurable alterations regarding morphology and GFAP expression as well as activation markers like NF-κB, NLRP3 and STAT1 (*see 3.2.6*). I therefore implemented an additional modification to simulate stressed astrocytes: specifically, the removal of glucose from the culture medium. This adjustment aimed to provoke a more reactive astrocytic phenotype upon stimulation with Aβ3(pE)-42. Previous work with glucose deprivation in primary astrocyte cultures demonstrated reactive astrogliosis as seen e.g., by GFAP+ hypertrophic cells or upregulated proliferation markers like Proliferating Cell Nuclear Antigen [107]. Unfortunately, we were not able to observe any alterations regarding morphology, GFAP levels, nuclear STAT1 or cytosomal NLRP3 intensity in our cultures following Aβ3(pE)-42 stimulation in medium deprived of glucose (*see 3.2.6*). Astrocytes serve critical functions as glucose sensors and energy reservoirs in the brain, as they are the only CNS cells with the ability to build and store glycogen and in turn release glucose for neuronal nutritional support [108]. Since the astrocytes in our experiments were cultivated and grown in medium containing glucose, which was then removed simultaneously to adding Aβ3(pE)-42, it is imaginable that they still had sufficient glycogen reserves, which were transformed into lactate once cellular energy demands increased upon glucose deprivation. Concordantly, a meta-analysis of astrocyte markers in human post-mortem AD tissue revealed increased levels of glycogenolysis-associated key enzymes, indicating elevated mobilization of astrocytic energy reserves [109]. Additionally, the medium used for cultivation contained 0.8 mM L- glutamine, another metabolic substrate for the cells.

4.1.2 Finding suitable readout-parameters for the screening assay – GFAP and vimentin

After deciding to perform the experiments in serum-free medium containing glucose, I next aimed to determine suitable readout parameters that would be reproducible as well as cost- and time-efficient for a high throughput of substances.

GFAP, an intermediate filament protein of the astrocytic cytoskeleton, is widely used as a marker for reactive astrocytes. In neurodegenerative diseases and aging, astrocytes typically exhibit altered gene expression profiles, including increased GFAP gene expression and protein levels, leading to astrocytic growth and hypertrophic cellular changes, a process referred to as reactive astrogliosis [32–35, 110, 111]. Therefore, countless studies use GFAP immunostainings with subsequent quantitation of GFAP area, fluorescence intensity or branching architecture as a marker for reactive astrogliosis. Hence, it was naturally our first approach to measure GFAP upregulation following astrocyte stimulation with Aβ3(pE)-42. GFAP immunostainings were quantified regarding size and overall changes in astrocyte morphology in multiple different experimental settings. Unfortunately, no morphological alterations could be observed (see *3.2.3 and 3.2.6*).

Several possible explanations come to mind. First, it must be pointed out that GFAP as a "pan-astrocyte marker" for reactive astrogliosis has been discussed controversially among researchers over the past years. While increased GFAP content and morphological appearance were the only readouts of reactive astrogliosis in the last century, single cell analyses, genetic engineering and novel molecular targets opened new possibilities in understanding the molecular underpinnings of astrocyte reactivity. A recent collective review by some of the leading experts in the field emphasizes that the morphological characteristics of astrocytes do not always correspond with their functional phenotypes [32]. The authors developed a basic consensus, emphasizing the sensitive and very distinct reactive states of astrocytes in response to different stimuli *in vitro* as compared to disease-related phenotypes *in vivo* and therefore contradicting the existence of one “prototypical reactive astrocyte”. Furthermore, they underline the notion, that loss and gain of functions as well as up- and downregulation of reactivity-related genes may not happen simultaneously and thus not be detectable in all cells at once. The authors phrase a set of recommendations, including the combination of molecular and functional readouts to classify reactive astrocytes as well as the overall

awareness for regional, temporal, subject or sexual heterogeneity in gene expression profiles and protein levels in reactive astrogliosis.

As highlighted also by Escartin et al., one reason for the lack of astrocytic morphological alterations in response to Aβ3(pE)-42 stimulation in our experimental setting might be the use of a primary culturing system. Under physiological conditions astrocytes constantly communicate with other CNS cells and might therefore need stimuli from other cell types to elicit their full reactive phenotype. Several authors confirm the sensitive bi-directional crosstalk especially between astrocytes and microglia, involving autocrine feedback through the release of signaling molecules in response to neurodegenerative diseases or CNS injury [112, 55, 113]. Lian et al. demonstrated that astrocyte-microglia crosstalk involving complement activation, specifically through Aβ-induced astrocytic secretion of complement factor C3, dynamically regulates microglial phagocytosis of Aβ in an AD mouse model [113]. Another study demonstrated the induction of reactive astrogliosis not by LPS itself, but by LPS conditioned microglia medium, containing Il-1α, TNFα and C1q, in primary astrocyte cultures [40]. Our group previously showed GFAP upregulation upon Aβ3(pE)-42 stimulation in organotypic slice cultures and in a transgenic AD mouse model expressing high levels of Aβ3(pE)-42 (mouse line TBA2.1) (see *3.4*), but were here unable to replicate these findings in primary astrocyte cultures, which we confirmed are free from microglia (see *3.1.4*). Therefore, the lack of interaction with other cell types represents a plausible explanation.

Nevertheless, we made a well-considered decision working with this particular culturing system. When studying basic cellular functions, it is essential to consider whether to prioritize the analysis of complex cellular interactions, which mimic physiological conditions, or focus on understanding the distinct roles of individual cell types in specific pathophysiologies. Additionally, studying single cells provides insights into underlying cause-and-effect relationships, which can be difficult to distinguish when observing multiple cell types in complex interactions. In this study, we were particularly interested in the role of astrocytes in the context of Aβ3(pE)-42 ingestion and possible therapeutic interventions specifically targeting astrocytes, which is why we stayed committed to this approach.

Another explanation for the missing astrocytic GFAP upregulation or morphological alterations might be the fact that cultured astrocytes already exhibit a baseline activity and thus show a reactive phenotype to begin with. If external stressors had already induced

reactive astrogliosis, application of small amounts of Aβ3(pE)-42 would most likely not induce any further changes. Due to physical stress during the initial preparation and cultivation processes, e.g., shaking to remove microglia or freezing, thawing and pipetting steps, astrocyte reactivity *in vitro* might in fact differ immensely from *in vivo* conditions and astrocytes might in this scenario not be susceptible to further activation and GFAP upregulation [27]. Moreover, the traditional two-dimensional culturing system for astrocytes, as it is also used in this study, limits morphological complexity and proliferation possibilities and has been shown to cause an undesired astrocytic baseline activity, which is why studies with three-dimensional culturing environments or brain organoids hold promising possibilities for future *in vitro* research regarding astrocyte reactivity [54].

Regardless of the culturing system, origin and developmental stage of the astrocytes might play an important role as well. It was for example shown, that primary astrocyte cultures originating from different or even the same brain region exhibit very diverse molecular and functional phenotypes as well as highly varying amounts of GFAP within one culture. While e.g. white matter astrocytes express high GFAP levels, astrocytes deriving from grey matter elicit very low levels of GFAP protein [114]. Additionally, GFAP expression in rat cortices has been shown to be linked to brain development and increases gradually from embryonal to adult stages [115, 116, 32] and different intracellular pathways, that repress or activate GFAP expression as astrocytes begin to differentiate have been described for different developmental stages [117]. Since the primary astrocytes cultured for this project were derived from neonatal rat forebrains, the particular age and brain region might affect GFAP expression levels and therefore also account for the observed results. Alternatively, it might be an interesting approach to study the response of astrocytes isolated from aged mice to Aβ3(pE)-42 oligomers, as this represents a more physiological scenario.

Another important point is the fact that anti-GFAP antibodies, that are commonly used for astrocyte labelling in immunohisto- or cytochemistry, do not label the whole astrocytic skeleton. GFAP antibodies mainly stain the perinuclear region as well as the main branches. Recent studies support this concept, as they were able to visualize the entire cellular morphology of astrocytes that were manually dye-filled with lucifer yellow or that expressed enhanced green fluorescent protein under control of the mouse or human GFAP promoter

compared to astrocytes that were stained with conventional GFAP antibodies (***Fig. 28***) [118]; [35].

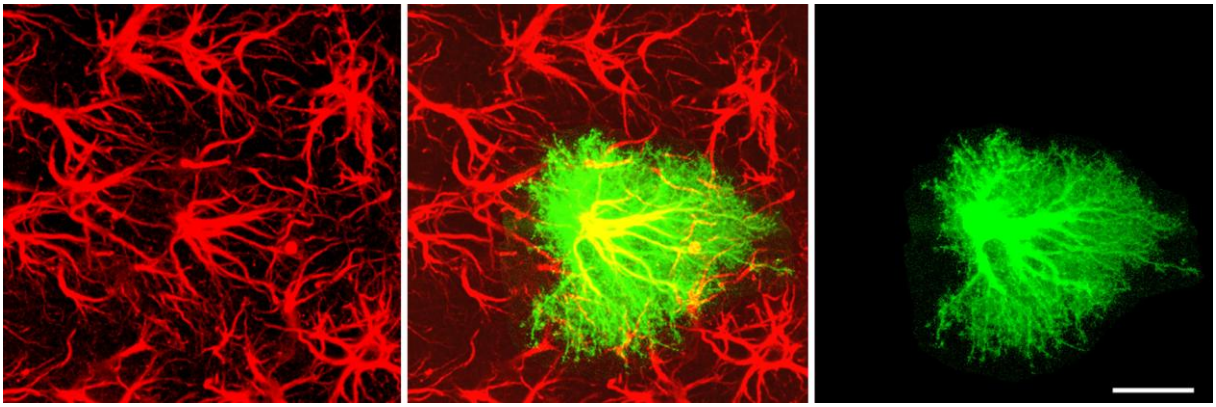

Figure 28: Differences in astrocyte size and morphology in mouse brain slices visualized with GFAP antibody (red) and manual dye-injection with lucifer yellow (green). *Different labelling techniques reveal how conventional anti-GFAP antibodies do not visualize the whole cell body but rather the main branches of the intermediate filament skeleton, while intracellular infusion with a special dye (lucifer yellow) reveals the whole expansion of the cell as well as its morphological complexity. Scale bar is 20µm. Adapted from Wilhelmsson et al., 2004. GFAP = glial fibrillary acidic protein*

Consequently, the quantification methods applied in this study, such as measurement of total GFAP+ area or estimation of GFAP morphology by eye, may not have been the most advantageous approaches. Quantification of process elongation, loss of primary or secondary branching, sholl analysis or use of higher resolution microscopy might have been more sensitive approaches. However, these parameters conflict with the efficiency of the targeted screening method, since higher resolution microscopy or different image analysis techniques might have been more time consuming and therefore not applicable for testing the desired number of plant substances.

Taken together, these findings suggest that GFAP expression *in vitro* varies strongly depending on culturing environment, physical stress, astrocyte origin, developmental stage or GFAP visualization technique, some of which may explain the missing evidence of astrocyte reactivity and proliferation witnessed by GFAP upregulation in our experiments. And, as mentioned earlier, recent discussions in the field have highlighted the fact that GFAP may not be the most suitable marker for reactive astrogliosis, but that there are more appropriate markers for activation, some of which I examined next.

Vimentin is another intermediate filament, which is upregulated upon astrocyte stimulation and is therefore another well-known marker for reactive astrogliosis [39]. As the most

common co-oligomerization partner of GFAP, both proteins form major parts of the astrocytic cytoskeleton. GFAP and vimentin double knockout mice have been studied extensively and showed reduced astrocyte reactivity with decreased branching and proliferation rates as well as gene profiles of non-reactive astrocytes, underlining their joint role in reactive astrogliosis. [115, 54]. Additionally, a meta-analysis of human post-mortem tissue described upregulated vimentin levels in astrocytes associated with AD [109]. I therefore asked if vimentin might be upregulated upon Aβ3(pE)-42 stimulation in primary astrocytes. I observed GFAP – vimentin colocalization in STED microscopy following incubation with Aβ3(pE)-42 oligomers, supporting the notion that they are present as co-polymers in the astrocytic skeleton (see *3.2.4*). Similar to GFAP, vimentin structures throughout the cells showed no visible morphological changes, possibly for similar reasons discussed above, which is why vimentin immunostainings were excluded as possible readout parameter for the screening assay as well.

4.1.3 STAT1, NLRP3 and NF-κB as readout parameters

Once astrocytes incorporate Aβ oligomers, numerous intracellular pathways are induced, gene expression profiles shift and astrocytes transform into a reactive phenotype. [113, 19, 61, 60, 54]. Important key regulatory proteins in those intracellular pathways are for example NF-κB, NLRP3 and STAT1, which is why I next evaluated the qualities of these markers as potential readout parameters for our screening assay.

NF-κB is a family of transcription factors expressed in a variety of cells, including astrocytes. Some of its multiple functions include the regulation of intracellular signaling pathways associated with cell differentiation, cell growth, apoptosis and stress but also the expression of genes involved in the inflammatory response. Upon stimulation, NF-κB is translocated to the nucleus, were it initiates the transcription of several pro-inflammatory genes, such as cyclooxygenase 2, TNF-α or interleukin-1. NF-κB signaling has been reported to contribute to AD pathology, especially since research confirmed upregulation of NF-κB subunit p65 in neurons and glia cells in close proximity to amyloid plaques, where its enhanced activity might be associated with increased pro-inflammatory signaling [60, 94, 113]. Hence, I also measured nuclear intensities of NF-κB subunit p65 following stimulation with Aβ3(pE)-42 oligomers for different concentrations and time points. Unfortunately, I was not able to observe increased nuclear p65 intensities, which would suggest nuclear translocation as expected (see *3.2.6*).

The cytosolic protein NLRP3 is part of the inflammasome, which is a multimeric protein complex and belongs to the innate immune system. Activation by a wide range of inflammatory stimuli leads to cleavage of inactive pro-inflammatory cytokines. The NLRP3 inflammasome is believed to play a major role in the neuroinflammatory response of glial cells to Aβ proteins in AD [25, 41]. Nevertheless, I was not able to detect increased NLRP3 intensities following Aβ stimulation of primary astrocyte cultures in our experiments (see *3.2.6*). Even though the role of astrocytes in the disease course of AD is non-negotiable, involvement of the NLRP3 pathway is discussed controversially. While some studies confirmed IL-1β upregulation in astrocytes in close proximity to Aβ plaques and thereby hypothesized NLRP3 dependency of astrocytes inflammatory response in transgenic mice and human brain tissue, no such results could be shown for primary astrocyte cultures [119, 120]. Since several studies confirmed the involvement of NLRP3 in the Aβ-mediated inflammatory responses of microglia [121, 25], it is imaginable, that astrocytes rely on the interplay with microglia or neurons, for activation of the NLRP3 pathway following Aβ stimulation. Thus, astrocyte-microglia co-cultures might present a possible culturing system to test this hypothesis more thoroughly. Additionally, NLRP3 activation in glial cells seems to occur in an NF-κB dependent manner, which is why another possible explanation of the lacking NLRP3 activation is the above-described missing NF-κB translocation to the nucleus.

STAT1 is another transcription factor that, upon activation by different cytokines and growth factors, is phosphorylated and translocated to the nucleus, where it regulates the expression of genes related to cell growth, differentiation, apoptosis, and immune functions. Interferon gamma (IFNγ) is one of the most potent activators of STAT1, which I confirmed by measuring increased nuclear STAT1 intensities following IFNγ treatment of primary astrocytes (see *3.3*). Some studies also suggest STAT1 activation through Aβ induced signaling in the CNS [122, 123], which is why I also tested STAT1 as a potential readout parameter for our screening assay. Unfortunately, there was no significant increase in nuclear STAT1 intensities following astrocytic Aβ3(pE)-42 stimulation (see *3.2.6*). Possible reasons are numerous, especially since the exact molecular pathways induced in Aβ stimulated astrocytes are still not completely understood and even though there are further interesting molecules to be tested, at some point we had to reach a conclusion and develop a new approach for the screening assay.

We also raised the question if the missing upregulation of the tested activation markers was related to cellular dysregulation or dysfunction. For this purpose, astrocyte reactivity was confirmed by stimulating the cells with the highly pro-inflammatory LPS derived from gram-negative bacterial walls, the pro-inflammatory cytokine INFγ and the injury-related growth factor TGF-β1, all of which have been shown to potently induce inflammatory responses in various cell types, including glia [96, 95, 124] Correspondingly, I was able to show morphological alterations as well as GFAP, NLRP3 and STAT1 upregulation in astrocytes treated with LPS, IFNγ or TGF-β1 (see *3.3*), thus confirming astrocyte reactivity under the established *in vitro* conditions as well as the functionality of the applied antibodies.

4.1.4 Astrocytic Aβ3(pE)-42 uptake as readout parameter

Since Aβ3(pE)-42 incubation did not induce reactive astrogliosis in terms of upregulated intermediate filaments GFAP and vimentin, or activation markers NLRP3, NF-kB and STAT1, I next evaluated astrocytic Aβ3(pE)-42 uptake as a potential readout parameter for the screening assay.

Although astrocytes are known to endocytose Aβ, the relationship between Aβ uptake, astrogliosis, and disease onset and progression is not sufficiently understood. However, current research has shown that an aberrant astrocytic endolysosomal system leads to impaired Aβ-clearance and progression of AD [125]. Moreover, aberrant intracellular accumulation of Aβ3(pE)-42 has been shown to induce astrocyte reactivity and subsequent pro-inflammatory signaling [19], which eventually results in decreased astrocytic clearance of Aβ and thus contributes to early synaptic dysfunction in AD [6]. Based on this, we decided to investigate if pre-selected plant substances might prevent the uptake of Aβ3(pE)-42 to astrocytes. After I confirmed Aβ3(pE)-42 uptake by primary astrocytes under different experimental conditions (see *3.2.2, 3.2.3 and 3.2.4*), I also showed Aβ3(pE)-42 uptake by HEK-293T cells (see *3.5*), indicating a general uptake mechanism for different cell types besides glia. As the measurement of astrocytic Aβ3(pE)-42 uptake is a very straightforward approach that can be performed under fluorescence microscopy, requires the use of only two different antibodies and can be analyzed with a fairly simple and automatable quantification protocol with conventional image analysis software and is therefore time- and cost-efficient for numerous plant substances, we decided to pursue this approach for our screening assay.

4.1.5 The special role of Aβ3(pE)-42

The n-terminally truncated Aβ3(pE)-42 is among the predominant posttranslationally modified Aβ species and has been reported as a main component of Aβ deposits associated with human AD, but absent in normal aging. Besides generating highly toxic co-oligomers with conventional Aβ1-42 resulting in increased neuronal loss and reactive gliosis in a transgenic mouse model, Aβ3(pE)-42 is predisposed to aggregation and the formation of fibrillar species. Additionally, Aβ3(pE)-42 is not taken up by neurons and does not associate with neuronal membranes [19]. Its neurotoxic effects are a consequence of astrocytic uptake, subsequent astroglial activation and release of synaptotoxic, pro-inflammatory TNFα [20, 18, 13, 21]. Therefore, n-terminally modified Aβ species represent ideal therapeutic targets to combat the onset and progression of Alzheimer's disease.

However, besides its clinical relevance, it needs to be noted that most studies on molecular fundamentals and therapeutic strategies regarding AD focus on the most common Aβ species Aβ1-40 and Aβ1-42. Although, some papers highlight the specific role of Aβ3(pE)-42 on astrocyte reactivity [19, 22, 13, 17, 21], most papers cited in this thesis focus on the effects of full-length Aβ. Thus, we can only speculate whether Aβ3(pE)-42 activates similar intracellular cascades as do Aβ1-42 and Aβ1-40. While GFAP upregulation in organotypic brain slices and mixed cultures has been described as a consequence of astrocytic Aβ3(pE)-42 internalization, markers such as STAT1, NLRP3 or NF-kB can only be assumed to be similarly upregulated. Next to the above-mentioned explanations, this might be another reason we did not observe the expected upregulation of the tested activation markers. However, the uptake of Aβ3(pE)-42 by astrocytes was not only confirmed in this study, but has been previously highlighted in contrast to full-length Aβ [19] and therefore presents a particularly suitable readout parameter for the screening assay.

4.2 Plant substances

4.2.1 Plant secondary metabolites present a potential therapeutic approach in AD

Although substantial research is currently performed in this field, there are still no effective therapeutic options for the treatment of AD. Few therapeutic approaches particularly target

glial Aβ uptake and the subsequent release of pro-inflammatory cytokines causing neurodegeneration and synaptic dysfunction in the disease cascade. Plants and their extracts might be the oldest pharmaceuticals known and contain numerous health-promoting secondary metabolites like polyphenols (including flavonoids and non-flavonoids), carotenoids, mono- and triterpenes, phytoestrogens and more.

In the second part of this thesis several plant substances were screened with regard to their abilities to interfere with glial Aβ3(pE)-42 uptake, potentially affecting chronic low-level neuroinflammation caused by astrocytic cytokine release as it occurs in human AD brains. In fact, out of 17 pre-selected compounds from a Natural Product library from the Leibniz Institute of Plant Biochemistry, we identified 5 substances that significantly reduced Aβ3(pE)-42 uptake in primary astrocytes. These 5 substances include 4 flavonoids (quercetin, epicatechin, rutin and apigenin) and one substance from the stilbene group (resveratrol), all of which belong to the class of polyphenols (***Fig 29****;* see *3.6*).

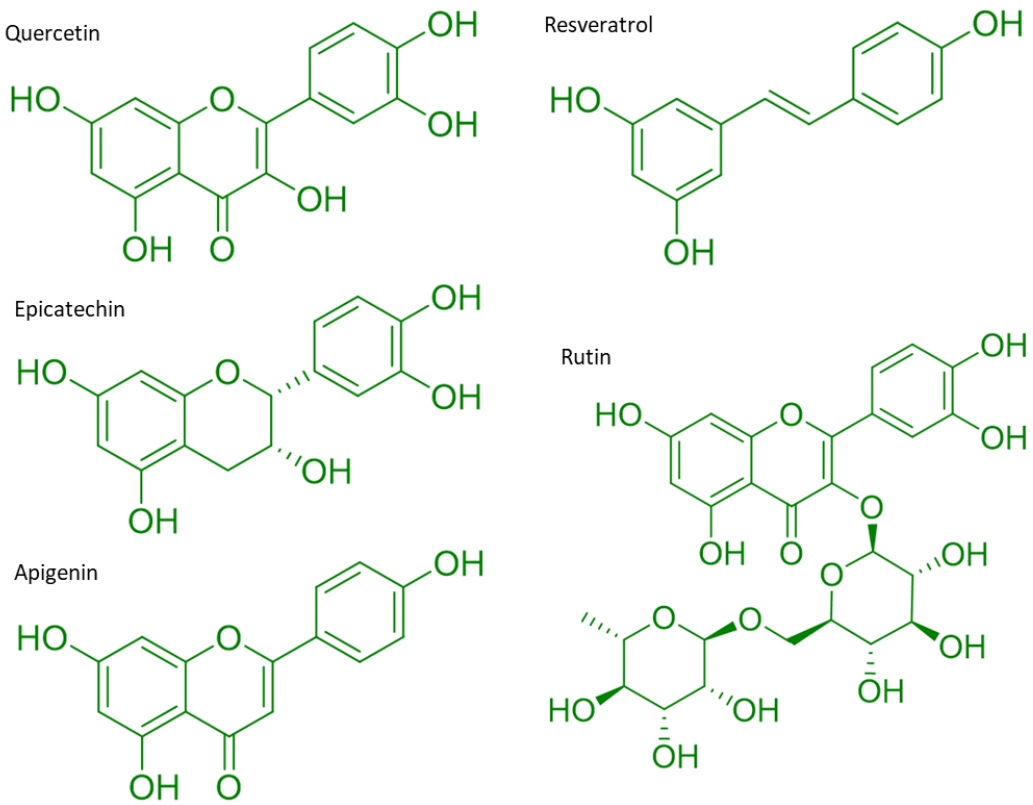

Figure 29: Structures of plant substances that significantly reduced the uptake of oligomeric Aβ3(pE)-42 to astrocytes. *Aβ3(pE)-42 = pyro-glutamylated amyloid-β*

Plant substances, particularly polyphenols, are extensively researched for their anti-inflammatory, antioxidant and antiproliferative properties. Their ability to combat oxidative stress and reduce neuroinflammation is believed to contribute significantly to their beneficial effects in managing neurodegenerative diseases and cognitive decline and they are often well tolerated medical alternatives by the general public [64–67]. Studies on their bioavailability and ability to cross the blood-brain-barrier (BBB) are controversial. However, they all agree, that their permeation through the intestinal walls and BBB seems to be dependent on the degree of lipophilicity, implying that intestinal and hepatic biotransformation are important steps to improve availability *in vivo* [64, 77, 78].

Recent large cohort studies confirmed the benefits of dietary flavonoid intake in AD research. Higher flavonol consumption was for example linked to slower cognitive decline across multiple domains affected in AD and reduced risks of AD and related dementias. Animal studies injecting flavonoids into aged AD mice showed improvements in histological markers and cognitive function, highlighting the potential of plant secondary metabolites in AD and neurodegeneration research [67, 66, 79].

However, the involved cell types as well as the exact molecular pathways underlying these effects are controversial and can often only be speculated. Given their phenolic hydroxyl groups, polyphenols undeniably exert some of their beneficial effects associated with aging, cardiovascular diseases and cancer by scavenging free radicals and thereby reducing harmful oxidative stress [64, 67] (***Fig. 29***). Yet, besides simply preventing oxidation, polyphenols have been reported to protect neurons from damaging pro-inflammatory cytokines or neurotoxins, such as Aβ, and thereby suppress neuroinflammation and promote memory, learning and cognitive functions in a much more complex manner than originally expected. They have for example been shown to influence various intracellular pathways and key regulatory molecules such as NF-κB, CREB, extracellular signal-regulated kinases, phosphatidylinositol-3 kinase or janus kinases, consequently promoting the upregulation of neuroprotective genes or suppressing the release of pro-inflammatory cytokines [77, 78].

However, in this work, I highlight another possible route of action by which polyphenols might exert their beneficial properties, namely by hindering Aβ3(pE)-42 uptake to astrocytes. Previous studies have shown the special role of Aβ3(pE)-42 in inducing reactive astrogliosis and the subsequent release of the pro-inflammatory cytokine TNFα, ultimately causing

neuronal damage and impaired neuronal signaling [19]. Thus, by preventing astrocytic Aβ3(pE)-42 uptake, a reduction in these deleterious effects could be hypothesized. And in fact, we confirmed that the polyphenol quercetin, one of the five identified substances, protects against synaptic loss and CREB-shutoff in mixed hippocampal cultures and LTP impairment in acute mouse hippocampal slices, all of which are associated with AD pathogenesis and typically impaired upon Aβ3(pE)-42 administration (see *3.6.2) [126]*.

4.2.2 Blocking Aβ uptake – possible underlying mechanisms

The mechanisms underlying the reduced astrocytic accumulation of Aβ3(pE)-42 following incubation with plant substances are to a large extent unclear. One possibility that comes to mind, is the interference of polyphenols with Aβ3(pE)-42 endocytosis, which is in our opinion the most plausible scenario. Concordantly, our group showed that dynasore, a potent endocytosis inhibitor, significantly reduced intracellular Aβ3(pE)-42 accumulation in primary astrocytes. Additionally, we showed prominent Aβ3(pE)-42 localization to the astrocytic endolysosomal compartment via TMR-dextran, an endolysosomal fluorescent marker, thus suggesting Aβ3(pE)-42 uptake to astrocytes via endocytosis [126]. Importantly, we were also able to show that the polyphenol quercetin does selectively hinder quercetin endocytosis, but does not alter the overall endocytosis rate, indicating that quercetin selectively prevents Aβ3(pE)-42 endocytosis [126]. A plausible explanation is binding of quercetin to Aβ3(pE)-42 oligomers, thus preventing their attachment to astrocytic endocytic receptors. This is supported by evidence showing quercetin can bind to fibrillar α-synuclein and Aβ [127] and as Aβ3(pE)-42 shows increased hydrophobicity, binding to hydrophobic quercetin might be facilitated in a similar manner. Additionally, quercetin blocked Aβ3(pE)-42 uptake in HEK293T cells, suggesting a general relevance of this mechanism (see *3.6.2*). The essential noncovalent interactions between the OH groups and phenolic rings in flavonoids with β-sheet structures common to all amyloid proteins further support this binding mechanism.

Notably, a consequence of this direct interaction with polyphenols seems to be the inhibition of oligomer and fibril formation and thus Aβ aggregation [128, 75]. However, the prevention of oligomerization cannot account for the observed effect on Aβ3(pE)-42 accumulation in astrocytes, since we have worked with preformed oligomers, whose protein sizes were validated in the beginning (see *3.2.1*).

Nonetheless, alternative scenarios can be derived from published work. Another explanation for the observed effects involves the regulation of gene expression and function, namely of Apolipoprotein E (ApoE). ApoE is a cholesterol carrier, supporting lipid transport and injury repair in the brain. The APOE-ε4 allele is a genetic risk factor for late onset AD (LOAD), possibly via its effects on the accumulation and clearance of Aβ. ApoE in the CNS is mainly produced by astrocytes and astrocyte-derived ApoE is pivotal for clearance of oligomeric Aβ [125, 129]. Previous research identified the polyphenol quercetin to increase ApoE levels most likely by inhibiting ApoE degradation in astrocyte cultures [130]. While this mechanism seems to be notable with regard to long-term treatment of Aβ pathologies, we here observed a rather rapid effect occurring long before the regulation of gene expression. Thus, acutely hindered uptake of Aβ3(pE)-42 to astrocytes following incubation with polyphenols might be the more likely scenario. Concordantly, other before-mentioned long-term effects of polyphenols, that affect gene expression regulation are equally unlikely to cause the observed acute effects on Aβ3(pE)-42 accumulation in astrocytes [78, 77, 64]. ***Figure 30*** summarizes possible mechanisms of how plant polyphenols might affect Aβ3(pE)-42 uptake to astrocytes.

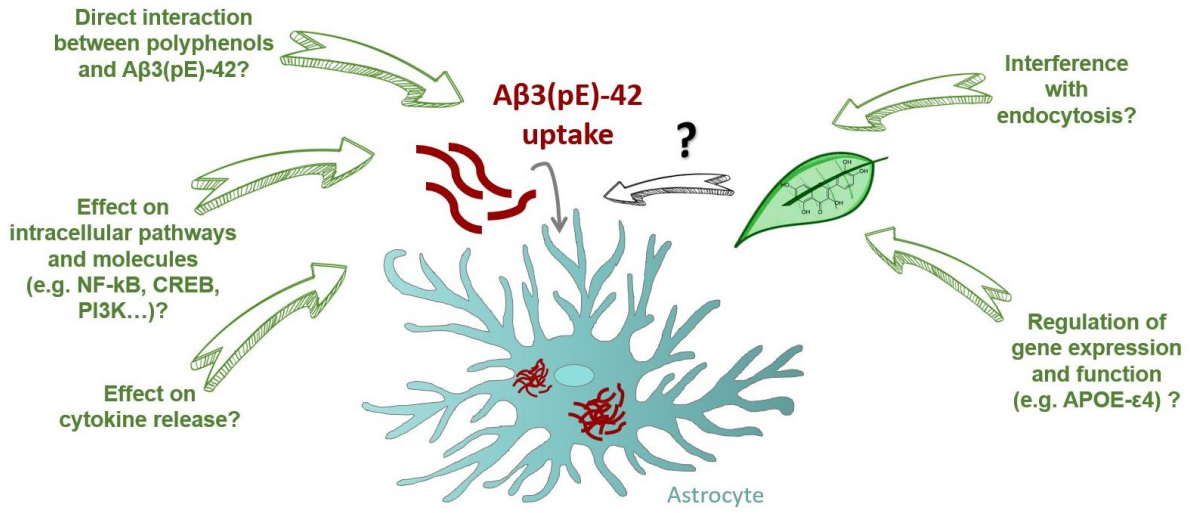

Figure 30: Potential mechanisms underlying a reduced astrocytic Aβ3(pE)-42 uptake following incubation with plant metabolites. *The scheme illustrates how plant substances might affect Aβ3(pE)-42 uptake to astrocytes. These include, but are not limited to, interference with endocytosis, direct extracellular interaction between plant substances and Aβ3(pE)-42 oligomers, effects of plants on intracellular pathways, cytokine production or regulation of gene expression profiles. Aβ3(pE)-42 = pyro-glutamylated amyloid-β; NF-κB = Nuclear factor-kappa B; CREB = cAMP response element-binding protein; PI3K = phosphatidylinositol-3 kinase; APOE-ε4 = Apolipoprotein E allele ε4.*

4.2.3 Conclusions and future perspectives

In this work we propose a novel therapeutic approach to limit astrocytic Aβ uptake and thereby reactive astrogliosis and resulting neuroinflammation. Admittedly, this raises the question of whether the inhibition of reactive astrogliosis as well as the disruption of Aβ clearance might also have a downside.

Astrocyte reactivity has long been regarded as passive and homogenous and overall negative event, but with many new lines of evidence turned out to be a very diverse, heterogenous and purposeful component of the CNS immune response, comprising many essential and beneficial functions, particularly in terms of neuronal protection and repair and regulation of CNS inflammation [32, 39, 8, 43]. In its basis, reactive astrogliosis has the function to protect neural tissue, maintain tissue homeostasis and preserve neurological functions following CNS insults and disease, similar to immune cells in other types of tissue. One of the key functions of reactive astrocytes is the release of cytokines to attract further inflammatory and immune cells. Furthermore, astrocytes are known to form scar-like tissue, restricting the spread of inflammation [43]. A genetically targeted ablation mouse model lacking reactive astrocytes demonstrated increased spread of inflammation, increased neuronal degeneration, failure of BBB repair and reduced recovery of neurological functions following CNS injury [44]. Accordingly, astrocyte loss-of-function studies with ablation of different effector molecules or transcriptional regulators associated with reactive astrogliosis have shown exacerbated tissue injury with increase in dystrophic neurites and exacerbated amyloid load and tauopathy in AD mouse models [131, 132]. In AD pathogenesis, astrocytes play a crucial role as they localize near senile plaques and have been shown to phagocytize Aβ deposits. [27–30].

As mentioned above, reactive astrogliosis is not a phenomenon that can be viewed in “black and white”. Besides its beneficial functions, there is also proof of the detrimental effects of reactive astrogliosis, especially in the context of neurodegenerative diseases. Astrocytes produce various pro-inflammatory cytokines, many of which have been shown to be upregulated in human AD brain samples and in transgenic AD mouse models [133, 134, 43] and attenuation of pro-inflammatory astrocyte signaling is known to improve neurological outcome in molecular deletion studies in brains of transgenic AD mice [43]. Additionally, astrocytes seem to play an important role in the intracellular accumulation of hyperphosphorylated tau, another important hallmark of AD pathogenesis. A study by

Garwood et al. confirmed, that reactive astrocytes are essential for the Aβ-induced tau phosphorylation in primary neurons, which was inhibited by an anti-inflammatory agent only in the presence of astrocytes [61]. Furthermore, it has been suggested that reactive astrocytes themselves may contribute to the amyloid burden in the CNS, as they express increased levels of the necessary proteins for Aβ production, such as APP, β-secretase (BACE1) and γ-secretase *in vitro* as well as in human and mouse AD brains [57].

Several studies propose the notion, that astrocyte reactivity can occur in two different states, on the one hand the regular inflammatory short-term response to adapt to acute CNS insults, and on the other hand a chronic response, in which astrocytes remain in their reactive state and drive excessive neuroinflammation during autoimmune or neurodegenerative disorders [135, 43, 32, 8]. Undesirable consequences of chronic reactive astrogliosis include loss of physiological astrocyte function or gain of deleterious, disease-promoting functions, resulting in increased neurodegeneration, excessive inflammation, increased oxidative stress and exacerbation of the preexisting pathology [57, 54, 43, 135]. ***Figure 31*** summarizes this biphasic model.

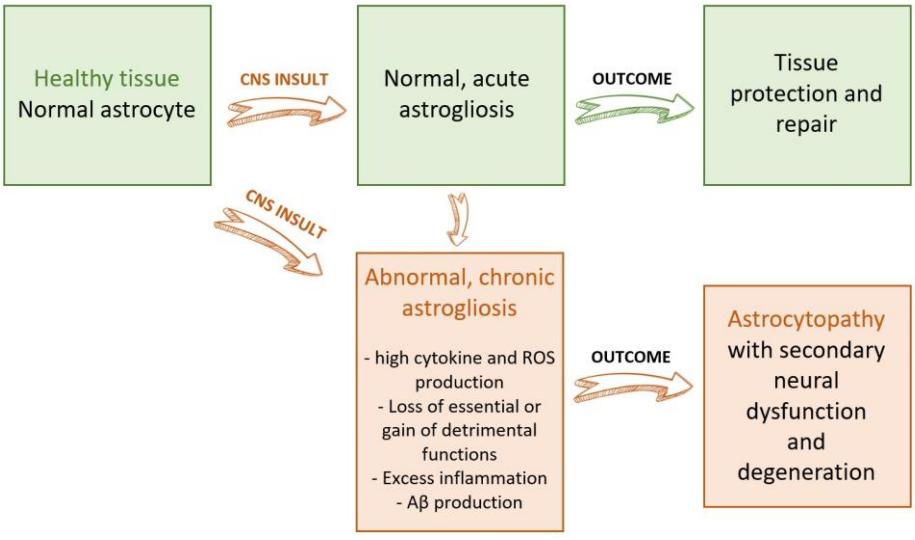

Figure 31: Scheme depicting acute and chronic reactive astrogliosis and their respective implications, describing how dysfunctional astrogliosis might be a potential contributor to neurodegenerative disease. *Adapted from Sofroniew, 2014. ROS = reactive oxygen species; Aβ = amyloid-β*

Conclusively, it seems to be important to distinguish acute astrocyte reactivity from chronic disease states. While acute astrocyte reactivity in response to short-term stressors seems to be an essential adaptive response to CNS injury and disease, chronic astrocyte exposure to harmful triggers during neurodegenerative disorders is likely to have detrimental consequences and might therefore benefit from attenuation. This is the reason we believe that the identified plant substances are of great relevance in reducing the harmful chronic low-level inflammation originating from chronic astrogliosis. However, more research is required to identify the specific circumstances under which astrocytes elicit neuroprotective or neurotoxic properties, to completely understand the role of reactive astrocytes in AD and thus investigate further therapeutic interventions.

Summary

Alzheimer's disease (AD) is a progressive, neurodegenerative disorder and the most common cause of dementia. Histopathologically, AD involves the deposition of extracellular amyloid-beta (Aβ) plaques, the intracellular aggregation of hyperphosphorylated tau as well as the onset of chronic neuroinflammation along with synaptic dysfunction. Yet, despite decades of immense efforts in the field, satisfactory therapeutic interventions for humans are still lacking. As key regulators of neuroinflammation, astrocytes play a crucial role in AD. As they are involved in Aβ production, removal and degradation, they have emerged as promising therapeutic targets. The accumulation of pyroglutamylated Aβ (Aβ3(pE)-42) in astrocytes is directly linked to glial activation and the release of pro-inflammatory cytokines, leading to early synaptic dysfunction in AD. At present, the mechanisms of Aβ3(pE)-42 uptake to astrocytes are unknown and pharmacological interventions that interfere with this process are not available.

Plant-based natural products, such as polyphenols, show potential in AD treatment due to their anti-inflammatory and antioxidant properties. However, the mechanisms underlying these positive effects are still not fully understood. Therefore, the goal of this project was to identify plant substances capable of modulating astrocytic responses to Aβ3(pE)-42.

In the first part of this study, ideal conditions for culturing primary astrocytes were established, with serum-free medium containing glucose and 0.5% dimethyl-sulfoxide set as the standard. Next, astrocytic Aβ3(pE)-42 uptake was confirmed via fluorescence and confocal microscopy, while no alterations in astrocyte reactivity markers, such as GFAP, STAT1, NLRP3, and NFκB-p65 intensities could be observed following exogenous Aβ3(pE)-42 application.

In the second part, a screening assay was developed to identify plant substances that effectively reduce astroglial Aβ3(pE)-42 uptake. Of the seventeen plant substances tested, five (quercetin, epicatechin, rutin, apigenin, and resveratrol) were found to significantly reduce Aβ3(pE)-42 uptake by astrocytes. Follow-up experiments showed that quercetin efficiently protects against early synaptic loss in neuron-glia co-cultures and rescues long-term potentiation impairment in acute mouse hippocampal slices, both of which are indicators of synaptic dysfunction in AD.

Taken together, this work highlights the potential of plant-derived polyphenols in mitigating astrocyte-related cellular dysfunctions in AD.

Zusammenfassung

Die Alzheimer-Krankheit (AD) ist eine fortschreitende neurodegenerative Erkrankung und die weltweit häufigste Ursache für Demenz. Die Krankheit ist gekennzeichnet durch die Ablagerung extrazellulärer Amyloid-beta (Aβ) Plaques, die intrazelluläre Ansammlung von hyperphosphoryliertem Tau sowie das Auftreten chronischer Neuroinflammation und synaptischer Dysfunktion. Trotz intensiver Forschungsbemühungen in den letzten Jahrzehnten gibt es bis heute kaum zufriedenstellende therapeutischen Interventionen.
Als zentrale Regulatoren der Neuroinflammation spielen Astrozyten eine entscheidende Rolle in der Pathogenese von AD. Da sie an der Produktion, der Aufnahme und dem Abbau von Aβ beteiligt sind, haben sich Astrozyten als vielversprechende therapeutische Zielstrukturen herauskristallisiert. Die Ansammlung von pyroglutamyliertem Aβ (Aβ3(pE)-42) in Astrozyten ist direkt mit der Aktivierung von Gliazellen und deren Freisetzung proinflammatorischer Zytokine verbunden, was in der Folge zur frühen synaptischen Dysfunktion bei AD beiträgt. Derzeit sind die Mechanismen der Aβ3(pE)-42-Aufnahme in Astrozyten unbekannt und es existieren keine pharmakologischen Interventionen, um diesen Prozess zu beeinflussen.
Sekundäre Pflanzenstoffe wie Polyphenole zeigen aufgrund ihrer entzündungshemmenden und antioxidativen Eigenschaften Potenzial in der Behandlung von AD, wobei die zugrundeliegenden molekularen Mechanismen weitgehend ungeklärt sind. Ziel dieses Projekts war es daher, Pflanzenstoffe zu identifizieren, die die Effekte von Aβ3(pE)-42 auf Astrozyten modulieren können.
Im ersten Teil dieser Arbeit wurden optimale Bedingungen für die Kultivierung primärer Astrozyten etabliert, wobei ein serumfreies Medium mit Glukose und 0,5 % Dimethylsulfoxid als Standard festgelegt wurde. Anschließend wurde die Aufnahme von Aβ3(pE)-42 durch Astrozyten mittels Fluoreszenz- und Konfokalmikroskopie validiert, während keine Veränderungen der Astrozytenreaktivitätsmarker GFAP, STAT1, NLRP3 und NFκB-p65 nach exogener Aβ3(pE)-42 Applikation beobachtet werden konnten.
Im zweiten Teil wurde ein Screening-Assay entwickelt, um Pflanzenstoffe zu identifizieren, die die Aufnahme von Aβ3(pE)-42 in Astrozyten effektiv reduzieren. Von den siebzehn getesteten Pflanzenstoffen zeigten fünf (Quercetin, Epicatechin, Rutin, Apigenin und Resveratrol) eine signifikante Reduktion der Aβ3(pE)-42-Aufnahme durch Astrozyten. Weitere Experimente zeigten, dass Quercetin vor frühem Verlust von Synapsen in Neuron-Glia Ko-Kulturen sowie der Beeinträchtigung der Langzeitpotenzierung in akuten Hippocampusschnitten von Mäusen schützt. Beides ist Abbild synaptischer Dysfunktion in der Pathogenese von AD.
Zusammenfassend unterstreicht diese Arbeit das Potenzial pflanzlicher Polyphenole zur Minderung der Astrozyten-vermittelten Beeinträchtigungen im Rahmen von AD.

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Declaration of Honor

I hereby declare that I have conducted the research for the dissertation submitted to the Faculty of Medicine at Otto von Guericke University for the purpose of obtaining a doctoral degree, entitled:

“Screening of plant-based natural products for reactive astrogliosis in Alzheimer's disease”

at the Institute of Pharmacology and Toxicology,

with the support of Prof. Dr. Daniela Dieterich and Dr. Michael Kreutz,

without any additional assistance and that I have used no resources other than those specified in the dissertation.

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Helene Arndt

Curriculum Vitae

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