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Organotypic slice culture models for induced alpha-synucleinopathy and exploration of the potential role of microglia in pathogenesis

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Organotypic slice culture models for induced alpha-synucleinopathy and exploration of the potential role of microglia in pathogenesis

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Tübingen, ...

To *mus musculus*.

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I. ABSTRACT

Synucleinopathies are neurodegenerative diseases characterised by the abnormal accumulation of α-synuclein (αS) in perikarya and processes of neurons, so-called Lewy bodies (LB) and Lewy neurites (LN), respectively. Increasing evidence suggests a prion-like mechanism in the formation and propagation of αS lesions throughout the brain. Introduction of αS seeds induces progressive pathology in a variety of experimental models and *in vitro* cell-to-cell spreading of αS has been demonstrated between neurons.

The aim of the present work was to study the formation, propagation, and cell-to-cell spread of αS lesions in an environment that mimics the brain environment. For this purpose, a hippocampal slice culture (HSC) model was established in which the hallmarks of α-synucleinopathies can be induced by a single treatment with αS seeds. LB- and LN-like hyperphosphorylated αS inclusions appear in a time- and concentration-dependent manner upon seed application and is followed by spreading of misfolded αS between different hippocampal regions. αS lesion-associated neurodegeneration could be monitored by neurofilament light (NfL) release into the culture medium. Potential therapeutic interference was demonstrated with an antibody directed against oligomeric and fibrillar αS, which attenuated seeding, seed-induced neurodegeneration, and spreading throughout the culture. The potential of this *ex vivo* brain culture system was then extended to adult human resection-derived brain cultures in which the induction of αS inclusions was also achieved.

Rather surprisingly, in addition to the neuronal αS pathology, abundant inclusions were found in microglia of the αS lesion-bearing brain cultures. Similar microglial inclusions were also detected in adult αS transgenic mouse models. These microglial inclusions were immunoreactive with N-terminal αS antibodies but reveal a different conformation compared to the neuronal αS inclusions, as shown by conformation sensitive amyloid dyes. Microglial inclusions occur with some delay after the neuronal inclusions but were also found at pre-symptomatic stages in transgenic mice, suggesting a potential involvement of microglial αS in the progression of α-synucleinopathies. To investigate this further, microglia were depleted in brain cultures at different timepoints in respect to seed application. Results suggest that microglial inclusions develop secondarily to neuronal inclusions, and moreover imply a role of microglia in the spreading of neuronal inclusions.

In summary, an *ex vivo* brain slice culture model system was established to study the induction and propagation of synucleinopathies. The importance of using a brain-like environment was exemplified by the discovery of an unexpected role of microglia in this process. The successful translation of the model system to human adult brain tissue will now allow to study mechanisms and therapeutic options of α-synucleinopathies in a true human adult brain environment.

II. SYNOPSIS

This synopsis serves as a mini-review to the current research in which my work was embedded.

First, I will give a brief overview on the topic of synucleinopathies, with a focus on the clinical background, which is arguably the ultimate motivation for biomedical research. I will set synucleinopathies into the broader context of neurodegenerative diseases, since they share characteristics that will be mentioned throughout the thesis. Afterwards, I will name and describe the hallmarks of synucleinopathies, most importantly the occurrence of intracellular accumulations of the protein α-synuclein (αS), after which the diseases are collectively named.

In the second part, I will go deeper into details of αS-based research, outlining the discovery of the protein and its native structure, along with an overview on the current knowledge about its physiological function. Further, I will describe the ability of αS to amplify its aggregation in a self-templated manner, which is based on the propensity of the protein to misfold and accumulate, and is influenced by genetic and post-translational modifications. Subsequently, I will focus on the consequences of intracellularly aggregated αS, which can have a toxic impact on the affected neuron, and briefly depict the proposed cell death mechanisms. Since cells have inherent defence mechanisms, I will describe cellular strategies to physiologically rid themselves of pathologic αS accumulations, and how they may fail, which can aggravate the pathology by spreading of αS to other cells. Finally, I will describe the immunotherapeutic approaches to break the vicious cycle of templated aggregation and intercellular spreading, in order to prevent neurodegeneration.

Considering synucleinopathies affect the entire brain, the third part focuses on aspects of synucleinopathies from a non-neuro-centric point of view. After mentioning the involvement of oligodendroglia and astroglia, I will focus on the role of microglia and pathology-driving neuroinflammation. In this context, I will introduce the first publication, to which I contributed, which concerns the discovery of abundant microglial inclusions in synucleinopathy mouse models, and the identification of those as containing αS in a truncated form. Since they are also detectable in pre-symptomatic mouse models, in addition to end-stage, we raise the question if these inclusions – or their formation – is involved in the development of pathology. We addressed this issue in a study outline presented at the end of this section.

The fourth part constitutes the *finale* of this thesis, where I describe my main work, namely the development and characterisation of organotypic slice culture models for induced synucleinopathy. I will start by introducing different *in vitro* and *in vivo* synucleinopathy models, along with their advantages and disadvantages, and continue by arguing for the versatile use of organotypic slice cultures. In detail, I will describe my favourite model, organotypic hippocampal slice cultures (HSCs), in which I conducted the majority of the experiments of my second (submitted) manuscript. Analogous to the *finale* of a symphony, this work entails many aspects I described in preceding synopsis chapters: In our model, we induce templated aggregation of αS in neurons which is leading to neurodegeneration. Moreover, we show inter-neuronal spreading, and demonstrate the efficacy of aggregated αS-targeted antibodies to stop the pathology. We also managed to induce the aggregation of αS in adult human brain tissue, which is a milestone for the modelling of synucleinopathy in a human-specific environment. Finally, we identified abundant microglial inclusions in our induced HSCs, which inspired the first manuscript and the study outline mentioned in the third part of the thesis.

1 SYNUCLEINOPATHIES

1.1 NEURODEGENERATIVE DISORDERS

With the world's ageing population doubling in the next 35 years (World Population Ageing 2015 Report), age-related disorders, such as neurodegenerative diseases, will become an even greater burden to our society. Neurodegenerative disorders are often characterised by abnormal accumulation of physiologically abundant proteins or peptides in the brain, such as amyloid-β (Aβ) or tau protein in Alzheimer's disease (AD), α-synuclein (αS) in Parkinson's disease (PD), transactive response DNA binding protein 43 (TDP-43) in frontotemporal dementia (FTD), or the prion protein (PrP) in Creutzfeld-Jacob's disease (CJD). Accordingly, these diseases can be described as proteopathies (Jucker and Walker 2013).

Among proteopathies, diseases can be further classified based on the accumulating protein, e.g. AD as a tauopathy, or PD as a synucleinopathy. However, there are several diseases that are summarized under these terms making such classifications broad in their definition. In this regard, 'synucleinopathies' is an umbrella term for PD, dementia with Lewy bodies (DLB), multiple systems atrophy (MSA), and, recently, pure autonomic failure (PAF) (Goedert et al. 2017).

1.2 HALLMARKS OF SYNUCLEINOPATHIES

The hallmarks of synucleinopathies are comprised of characteristic clinical symptoms, the abundant occurrence and spreading of αS inclusions, as well as pathology stage-specific biomarkers, which are measured in patient blood or cerebrospinal fluid (CSF). All of which will help to diagnose the specific disorder within the spectrum of synucleinopathies.

1.2.1 A-SYNUCLEIN INCLUSIONS

PD is the second most common type of neurodegenerative disorder in the ageing population (after AD) and the most common movement disorder (Mhyre et al. 2012). Its clinical diagnosis is based on rest tremor, bradykinesia, rigidity, and loss of postural reflexes (Jankovic 2008) (Figure 1). Other non-motoric symptoms that can occur are neuropsychiatric disturbances (Schapira and Tolosa 2010), hyposmia or anosmia (loss of smell) (Ansari and Johnson 1975), constipation (Schapira and Tolosa 2010), and REM sleep behaviour disorder (Schenck et al. 1986). Histologically, the most striking hallmarks are the degeneration of dopaminergic neurons in the *substantia nigra pars compacta* (SNpc) (Mhyre et al. 2012), and abundant so-called Lewy pathology, describing abnormal accumulation of inclusions in neuronal somas (Lewy bodies, LB) and processes (Lewy neurites, LN), which were first described by Fritz

Figure 1: Illustration of PD symptoms by William Richard Gowers (Gowers 1886).

Jakob Heinrich Lewy in 1912 (Lewy 1912). Today, PD is defined as parkinsonism (tremor, bradykinesia, rigidity) with Lewy pathology (Goedert and Compston 2018).

DLB patients' brains also exhibit Lewy pathology. In addition to the motoric symptoms of PD, DLB patients show cognitive impairment and behavioural / psychiatric symptoms (hallucinations), which typically precede the occurrence of parkinsonism. This makes DLB one of the most common types of degenerative dementia following AD (Donaghy and McKeith 2014; McKeith et al. 2017; Beach et al. 2009).

MSA patients, on the other hand, show abundant inclusions in oligodendroglia, known as Papp-Lantos bodies or glial cytoplasmic inclusions (GCI) (Papp et al. 1989; Spillantini et al. 1998), and also in neurons, named neuronal cytoplasmic inclusions (NCI) (Murayama et al. 1992). This disease is the most severe synucleinopathy and is clinically characterised by a combination of autonomic, cerebellar, parkinsonian, and cognitive symptoms (Savica et al. 2017; Spillantini and Goedert 2018).

Furthermore, PAF is a rather mild disease that occurs sporadically and progresses slowly with a relatively good prognosis. Patients suffer from hypoperfusion in the neck area, and urinary and sexual dysfunction (Garland et al. 2013). Here, Lewy pathology can be found in the sympathetic nervous system (Arai et al. 2000). However, some cases of PAF might represent an intermediate state that later develops to a central synucleinopathy, like PD or DLB (Kaufmann et al. 2004).

For many years, the significance of Lewy pathology was unknown, until 1997/98 when two discoveries revealed the nature of the inclusions. The first was a missense mutation in SNCA, the gene encoding for αS, which was found to cause familial PD (Polymeropoulos et al. 1997). The second was that LB and LN, as well as GCI, were immunoreactive against αS (Spillantini et al. 1998). In the following years, more PD causing SNCA missense mutations were found (Zarranz et al. 2004; Appel-Cresswell et al. 2013; Proukakis et al. 2013; Kiely et al. 2013; Lesage et al. 2013; Martikainen et al. 2015; Pasanen et al. 2014; Yoshino et al. 2017; Krüger et al. 1998) further supporting the importance of αS for the underlying pathogenesis.

1.2.2 PATHOLOGICAL SPREADING OF MISFOLDED A-SYNUCLEIN

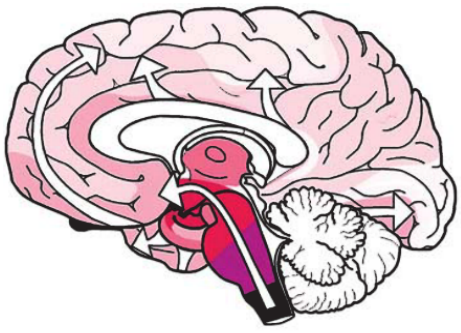

Figure 2: Schematic of topographic expansion pattern of Lewy pathology in sporadic PD patients, as suggested by Heiko Braak et al., adapted from Braak, Del Tredici, et al., 2003 *with permission from Springer Nature.*

Along with the discovery of further missense mutations in SNCA, Braak and others postulated a staging pattern of Lewy pathology in sporadic PD patients: LB and LN spread between axonally connected areas in the central and peripheral nervous system (CNS; PNS) in a stereotypic pattern of six stages, beginning in the PNS of the gut and the olfactory bulb (OB), and gradually progressing into the CNS, spreading from brainstem to multiple cortical regions in a caudal-to-rostral fashion (Braak et al. 2003b) (Figure 2). The topographic expansion pattern of the lesions reminded them of "a falling row of dominos", which they suspected to be caused by an unknown pathogen invading the nervous system (Braak et al. 2003a). Finally, two independent groups discovered that graft embryonic tissue, that had been transplanted into PD patient brains as an attempt to mitigate the loss of striatal dopamine, contained Lewy pathology more than

10 years post transplantation (Kordower et al. 2008; Li et al. 2008).

The vague idea that a pathogen or pathological process can spread from one cellular system to another was eventually consolidated when the first cell-to-cell transmission of αS aggregates was demonstrated *in vitro* (Desplats et al. 2009). Soon after, pathology-causing αS aggregates of different properties were found, that had been suspected to underlie the vast heterogeneity of synucleinopathies (Guo et al. 2013). Together with the demonstration of self-templated aggregation of αS in cell cultures (Sacino et al. 2013; Aulić et al. 2014), the new evidence underlined the resemblance of αS to prion proteins, which have been originally described by Stanley Prusiner: Prions, a slightly modified acronym for **pro**teinaceous **in**fectious particles, are misfolded proteins with high β-sheet content which can act as a template to perpetuate further misfolding of normal variants of the protein (Prusiner 1998). The similarity ultimately led to the theory of αS as a prion-like protein that is capable of acquiring alternative conformations and becoming self-propagating, similar to Aβ (Jucker and Walker 2013; Eraña 2019; Ayers et al. 2020; Prusiner et al. 2015).

1.2.3 BIOMARKERS

Diagnosing diseases and particular disease stages is key for adequate treatment. The use of brain biopsies to identify Lewy pathology or GCI, however, is impractical. A more suitable approach is the utilisation of fluid biomarkers, which are often protein levels in cerebrospinal fluid (CSF) or blood. Since CSF is part of the CNS, it makes it an ideal medium to measure the release of neurodegeneration-related molecules. Blood, on the other hand, is a more feasible source to monitor drug effects since its harvest is fairly non-invasive compared to lumbar punctures for CSF (Parnetti et al. 2019).

Due to the central role of αS in the pathology of synucleinopathies, it is not surprising that CSF and blood αS levels in PD patients deviate from healthy controls. PD patients often show reduction in total αS levels and increased oligomeric αS levels in CSF (El-Agnaf et al. 2006; Majbour et al. 2020). Surprisingly, a recent study provided evidence that skin biopsies of PD patients contained seeding-active αS species, suggesting that skin αS could be a novel biomarker for PD diagnosis (Wang et al. 2020).

As a general marker for neurodegenerative processes, the axonal cytoskeletal protein neurofilament light (NfL) was identified, which is released into the CSF when neurons die (Bacioglu et al. 2016; Preische et al. 2019). In early MSA patients (without LB), NfL levels have shown to be higher than in LB synucleinopathy patients (Singer et al. 2020; Parnetti et al. 2019), which could aid differentiating between these two diseases, whose clinical phenotypes often overlap. Moreover, disease progression and cognitive impairment of PD patients appear to be predictable by monitoring the blood levels of not only αS, but also Aβ40 and total tau (Chen et al. 2020).

2 ALPHA-SYNUCLEIN

What exactly is the protein αS? What is its physiological function? Why does it aggregate in some neurodegenerative diseases? Do its aggregation and spreading cause the clinical phenotype and the suffering of patients, or are they just an epiphenomenon, a mere side effect of another yet unknown process?

2.1 DISCOVERY

The first synuclein protein was identified in 1988 in the Pacific electric ray *Torpedo californica*. It was named after its cellular localisation in **syn**apses and **nucl**eus (Maroteaux et al. 1988). Later, two other synuclein proteins were discovered that shared a highly conserved amino-terminal domain including a variable number of 11-residue repeats and a less-conserved carboxy-terminal domain with numerous acidic residues (George 2002). With respect to their chronological discovery, the three synucleins were called α-, β- and γ-synuclein. While SNCA, the αS gene, is located at position 21 in the long arm of chromosome 4 (Shibasaki et al. 1995) the other two synuclein genes, SNCB and SNCG, are mapped to chromosomes 5 and 10, respectively (Ninkina et al. 1998; Spillantini et al. 1995). Although β- and γ-synuclein have been associated with hippocampal axon pathology in both PD and DLB, they are not found in LB (Galvin et al. 1999), and I will not focus on them further.

2.2 NATIVE STRUCTURE

αS is a small protein of only 140 amino acids (aa), abundantly expressed in neurons. Its primary sequence can be divided into three major domains: N-terminal domain (NTD), non-amyloid-beta component (NAC), and C-terminal domain (CTD). In general, αS has been called by some a "protein-chameleon" since it is an intrinsically disordered protein, able to adopt a series of different conformations dependent on the environment (Uversky 2003) (**Figure 3**). The three different domains all play their part in the structure of αS and accordingly to its function:

- The NTD (aa 1-60) is characterised by 5-6 relatively conserved repeats that acquire α-helical secondary structure upon lipid binding (Eliezer et al. 2001). It preferentially binds to phospholipid vesicles with acidic head groups (Davidson et al. 1998), and is responsible for the membrane affinity and binding of αS (Bartels et al. 2010).
- The NAC (aa 61-95) is highly hydrophobic. It contains a stretch of 12 aa which promotes fibrillisation of the full-length protein, and is essential for filament assembly (Giasson et al. 2001).
- The CTD (aa 96-140) is very flexible and natively unfolded due to its high proline content and negative charge (Chandra et al. 2003; Eliezer et al. 2001).

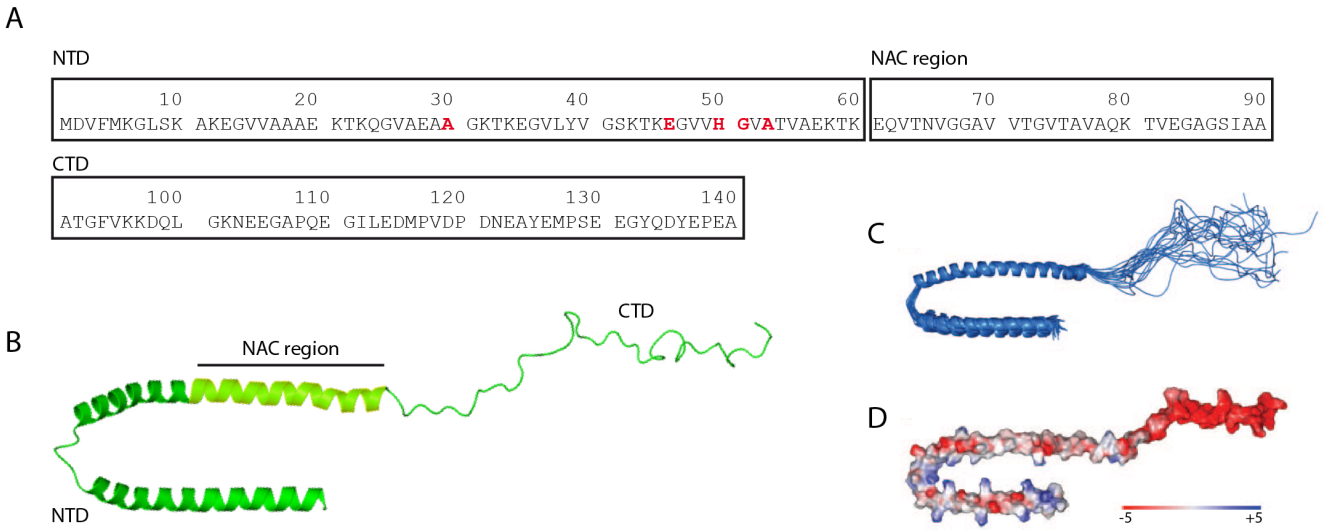

Figure 3: Primary and secondary structure of αS. (A) Sequence of αS, NTD, NAC region and CTD are labelled. Letters in red mark the position of dominantly inherited missense mutations (see 2.4.1 Nucleation and Oligomerisation - Mutation Effects). **(B)** Secondary structure showing the two α-helices of NTD and NAC region, connected by a short linker, and the unstructured CTD. **(C)** Superimposition of 20 structures reflecting the dynamic conformation changes of the CTD. **(D)** Charge distribution of αS, colour-coded by electrostatic potential, highlighting the acidic, negatively charged CTD. ***(B-D)*** *This research was originally published in the Journal of Biological Chemistry (Ulmer et al., 2005), © the American Society for Biochemistry and Molecular Biology.*

Two groups suggested that αS might exist physiologically as helix-rich tetramers (Bartels et al. 2011; Wang et al. 2011), but subsequent studies of many other groups rebutted this claim by showing that αS is an intrinsically disordered protein (Burré et al. 2013; Fauvet et al. 2012). Thus, in this thesis I will not further address the controversial topic of αS tetramers.

2.3 PHYSIOLOGICAL FUNCTION

Since the discovery of αS in the neurons of torpedo ray, where it was localised to the synapse and nucleus (Maroteaux et al. 1988), αS has also been implied a function in birds, where its expression seems to correlate with song acquisition (George et al. 1995). In humans, the protein is highly expressed in the brain, but also in red blood cells (Nakai et al. 2007), heart, pancreas, and skeletal muscles, although in lower amounts (Ueda et al. 1993). However, the loss of function of αS neither seems to be vital nor leads to a neurodegenerative phenotype in mice (Abeliovich et al. 2000). Nonetheless, it is required for survival or maturation of dopaminergic neurons in the developing murine SNpc (Garcia-Reitboeck et al. 2013; Goloborshcheva et al. 2020; Al-Wandi et al. 2010), and there is some evidence that αS suppression causes nigrostriatal degeneration in rats (Gorbatyuk et al. 2010) and non-human primates (Collier et al. 2016). Therefore, aggregation of the protein might induce some form of toxicity by inhibiting the physiological function of αS.

2.3.1 FUNCTION AT THE PRESYNAPTIC TERMINAL

Generally speaking, a chemical synapse is a junction between two neurons, and consists of three parts: 1) The presynaptic terminal of an axon which releases synaptic vesicles (SV) into 2) the synaptic cleft, which can then affect 3) the postsynaptic terminal by e.g. activation of membrane-bound receptors. The part of the

presynaptic axon forming the synapse is called synaptic bouton (Strominger et al. 2012). SV at the presynaptic terminal can be located at discrete distances from the so-called active zone at the plasma membrane, and are functionally organised in three main pools: reserve, recycling, and readily releasable pool (Vargas et al. 2017).

The localisation of αS at axon terminals and synapses (Maroteaux et al. 1988; Iwai et al. 1995) pointed to its field of action at the presynaptic terminal. In fact, around 3500 αS molecules seem to exist in individual synaptic boutons (Wilhelm et al. 2014) in an equilibrium between a soluble and a membrane-bound state, with approximately 15 % of the total αS in the membrane-bound form (Lee et al. 2002). Upon membrane binding, αS adopts α-helical conformations (Chandra et al. 2003; Jao et al. 2008; Ulmer et al. 2005) which enable it to sense and generate membrane curvature (Varkey et al. 2010; Pranke et al. 2011).

Since αS translocates to the synaptic terminal only late during development and its absence does not seem to be detrimental for synaptogenesis, its function is likely to be involved in sustained activity over time in the life of a neuron (Chandra et al. 2004; Bisaglia et al. 2009). Indeed, several studies have shown that αS interferes with intracellular vesicle trafficking (Cooper et al. 2006; Scott and Roy 2012; Medeiros et al. 2017). In more detail, gold-labelling revealed that αS clusters at the boutons of presynaptic terminals, where it is mainly located in the distal reserve and recycling SV pools instead of in proximity to the presynaptic membrane (Lee et al. 2008c; Zhang et al. 2008). Recently, it was shown that loss of αS increases the tethering of SV to the active zone at the membrane, which points to a role of αS as an orchestrator of presynaptic structure (Vargas et al. 2017). In addition to its role in SV vesicle reserve and recycling pools, αS has also been reported to promote dilation of the exocytotic fusion pore, resulting in acceleration of vesicle cargo discharge (Logan et al. 2017) and it seems to promote clathrin-dependent endocytosis (Gedalya et al. 2009).

2.3.2 FUNCTION IN THE NUCLEUS

The role of αS in the nucleus is less well explored (Bernal-Conde et al. 2020). Although it does not contain a nuclear localisation sequence (NLS), it has been reported to shuttle between the nucleus and the cytoplasm, in dependence on the availability of its NTD (Gonçalves and Outeiro 2013). Several functions of nuclear αS have been proposed that involve interactions with DNA, e.g. modulation of histone modification state (Goers et al. 2003; Kontopoulos et al. 2006; Sugeno et al. 2016), or direct DNA binding (Pinho et al. 2019; Vasudevaraju et al. 2012).

2.3.3 EFFECT ON MITOCHONDRIA

Many of the Mendelian recessive genes associated with parkinsonism are implicated in the mitochondrial damage repair pathway (Hardy 2010; Smolders and Van Broeckhoven 2020). Naturally, a whole other, non-αS-centric, field of research is concentrating on the mitochondrial point of view on diseases with parkinsonism. Interestingly, there might be an overlap with αS since the protein is reported to have a mitochondrial targeting sequence (MTS) at its NTD, and it seems to promote mitochondrial fragmentation into shorter cell organelles (Kamp et al. 2010; Nakamura et al. 2011; Devi et al. 2008; Faustini et al. 2017).

2.4 AGGREGATION OF A-SYNUCLEIN

All proteopathies, like AD, CJD, and the synucleinopathies, share a similar process, during which physiologically common proteins convert into a pathological conformer in a templated manner. After the first description of the pathological scrapie variant of prion protein (PrP^{Sc}) acting as a template for the cellular prion protein (PrP^{C}) to convert into PrP^{Sc}, the templated conformational change is often called prion-like conversion (Prusiner 1998).

As mentioned above (see 2.2 Native Structure), αS monomers exhibits dynamic conformations, which are stabilised by long-range interactions between NTD and CTD, and between NAC and CTD. However, changes in the environment, as well as post-translational modifications (PTMs) or mutations, can disturb the native structure and promote prion-like conversion and aggregation by encouraging partially folded intermediates, which will increase the propensity for self-assembly (Sahay et al. 2015; Bertoncini et al. 2005). The more exposed the NTD and the N-terminal part of the NAC are, the more aggregation prone monomeric αS conformations become (Stephens et al. 2019). Eventually, some conformational species assemble to oligomers, which in turn can aggregate in order to form several morphological different structures, like higher order oligomers, amorphous aggregates, or amyloid-like fibrils (Uversky 2003).

2.4.1 NUCLEATION AND OLIGOMERISATION

The kinetic mechanism of αS aggregate formation *in vitro* is thought to follow a three-phase nucleation-dependent polymerisation mechanism: First, in the lag phase, monomers assemble *de novo* into so-called aggregation nuclei (Wood et al. 1999). The lag phase is the rate-limiting step, where major structural changes have to take place: αS monomers undergo conformational changes from random coil to β-sheet structures and form soluble oligomers, which in time fuse to form templates capable of self-assembly (de Oliveira and Silva 2019; Serpell et al. 2000). This process can be accelerated by adding seeds, which act like pre-formed nuclei of the aggregation (Wood et al. 1999). Second, in the elongation phase, the aggregates grow exponentially by adding monomers at the fibrils' ends (Wood et al. 1999). This process is thermodynamically favourable, because it is compacting the protein, decreasing the total exposed surface (Buell et al. 2014). The

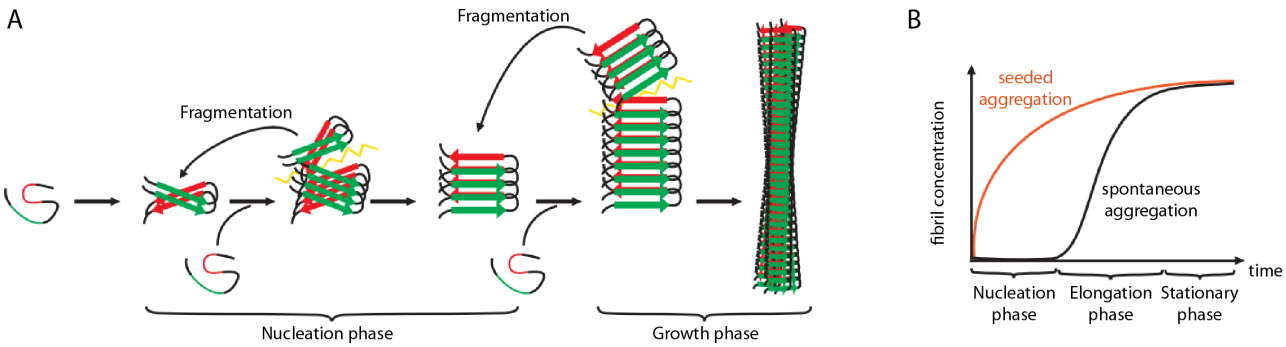

Figure 4: Schematic of aggregate formation. (A) In the nucleation phase, monomers adopt β-sheet secondary structures and assemble into transient oligomers, that can interconvert to larger aggregates and multiply by fragmentation. Through a series of intermediate states, the aggregates grow to mature fibrils. **(B)** The kinetic mechanism includes a nucleation phase, an exponential increase of fibrils in the elongation phase, and a plateau or stationary phase, resulting from the depletion of the monomer pool. Spontaneous aggregation (black) follows the scheme depicted in A. By adding seeds (orange), the nucleation phase can be significantly shortened or skipped. Modified from Jucker & Walker, 2013, *with permission from Springer Nature.*

polymerisation reaction can also involve secondary processes like multiplication of seeds by fragmentation of fibrils, or secondary nucleation events (Gaspar et al. 2017). Finally, in the stationary phase, the growing rate is decreased because the monomer pool is depleted (Wood et al. 1999) (Figure 4).

The aggregation of αS is very sensitive to its environment, such as temperature (Uversky et al. 2001), salt concentration (Munishkina et al. 2004), and pH, with mildly acidic pH increasing the aggregation propensity (Buell et al. 2014). Moreover, the presence of lipid membranes or air-water interfaces can accelerate primary and secondary nucleation processes, and mechanical agitation induces fibril fragmentation (Galvagnion et al. 2015; Marie et al. 2015; Campioni et al. 2014). Thus, dependent on the protocol, many different αS oligomers were identified *in vitro* which exhibit greatly varying structural properties, like polypeptide chain lengths varying between 5-100 units, and immensely different morphologies (spherical, elliptical, circular, tubular, flat-shaped) (Conway et al. 2000; Lashuel et al. 2002; Bousset et al. 2013; Cremades et al. 2012). Some oligomers are very transient and interconvert easily to monomers or fibrils, suggesting the existence of multiple aggregation pathways (Ghosh et al. 2015; Kaylor et al. 2005). What unites them, however, is that from early to late aggregation stages, the population of oligomeric intermediates seems to shift from α-helical to β-sheet structure (Ghosh et al. 2015; Apetri et al. 2006). Indeed, it seems that fragmented β-sheet rich fibrous aggregates of short αS fibrils are the most potent αS species that trigger prion-like conversion (Tarutani et al. 2016). Long αS fibrils, on the other hand, are rather inert species, and even show resistance to proteolysis, a trait shared with PrP^{Sc} and Aβ amyloid fibrils (Conway et al. 2000).

MUTATION EFFECTS

To this date, 7 dominantly inherited missense mutations in the SNCA gene have been discovered, which can cause familial PD: A53T (Polymeropoulos et al. 1997), A30P (Krüger et al. 1998), E46K (Zarranz et al. 2004), H50Q (Appel-Cresswell et al. 2013; Proukakis et al. 2013), G51D (Kiely et al. 2013; Lesage et al. 2013), A53E (Martikainen et al. 2015; Pasanen et al. 2014), and most recently A53V (Yoshino et al. 2017). Additionally, duplication and triplication of the wildtype αS gene are sufficient to cause a particularly severe form of PD (Singleton et al. 2003).

Interestingly, all SNCA missense mutations are located in the NTD, but none seems to promote major structural changes to the monomeric form, the only exception being A30P αS, which is less prone to adopt α-helices (Bussell and Eliezer 2001). However, A30P, G51D, and A53E mutations reduce the ability of αS to interact with acidic phospholipids (Chandra et al. 2003; Fares et al. 2014; Ghosh et al. 2014; Ysselstein et al. 2015), and there seems to be an antagonistic relationship between lipid binding of αS and aggregation into cytotoxic species (Burré et al. 2015; Cremades et al. 2012; Iljina et al. 2016). On the contrary, A53T and H50G are more likely to interact with membranes (Tsigelny et al. 2015), but also show an increase in αS aggregation (Choi et al. 2004; Ghosh et al. 2013; Serpell et al. 2000). Thus, the relationship between lipid binding, propensity to aggregate, and toxicity seems to be complex and not easily correlated.

PTM EFFECTS

αS is subject to a variety of posttranslational modifications (PTMs), which can also have an effect on the native structure and its aggregation propensity. Among the most common PTMs of αS that can be found in LB are phosphorylation, ubiquitination, truncation, and nitration.

Phosphorylation: In synucleinopathy lesions, αS is commonly phosphorylated at serine 129 (pS129) (Fujiwara et al. 2002). Consequently, antibodies directed against pS129 αS are commonly used as a surrogate marker for αS pathology in histological analyses. *In vitro* studies showed that oxidative stress and cellular toxicity can increase pS129 αS levels via an increase in casein kinase 2 activity (Smith et al. 2005; Waxman and Giasson 2008). In return, pS129 αS promotes fibril formation *in vitro* (Fujiwara et al. 2002). In line with this, promoting protein phosphatase 2A (PP2A), which de-phosphorylates pS129 αS, seemed to be sufficient to reduce αS aggregation in αS-transgenic mice and improve the disease phenotype (Lee et al. 2011). The role of another phosphorylation site, serine 87 (pS87), is controversially discussed: While some groups doubt the relevance of pS87 due to its scarcity in αS inclusions (Waxman and Giasson 2008), others stressed the occurrence of increased pS87 levels in transgenic synucleinopathy models and in human AD, LBD, and MSA brains (Paleologou et al. 2010). Phosphorylation at tyrosine 125 (pT125), on the other hand, could be neuroprotective, since it appears to inhibit toxic oligomer formation *in vivo*, and, interestingly, its levels are decreased in aged human brains (Chen et al. 2009).

Ubiquitination: Another common feature of synucleinopathy lesions is ubiquitination of phosphorylated αS, which suggests that phosphorylated αS is specifically targeted to mono- and di-ubiquitination (Hasegawa et al. 2002). In fact, together with αS, the most constantly immuno-positive protein in LB and GCI is ubiquitin (Spillantini et al. 1998; Kuzuhara et al. 1988). Strikingly, mono-ubiquitination of αS by ubiquitin ligase seven in absentia homolog (SIAH) promotes αS aggregation *in vitro* and *in vivo*, suggesting that it is contributing to LB formation (Rott et al. 2008).

Truncation: As a result of proteolytic cleavage, around 15 % of αS in LB is C-terminally truncated (Tatebe et al. 2010; Dufty et al. 2007; Mishizen-Eberz et al. 2003). Because truncation of the CTD removes a lot of repulsive negative charge it strongly enhances aggregation *in vitro* (Murray et al. 2003; Crowther et al. 1998). A prominent role is attributed to the calcium-activated protease calpain I, which cleaves monomeric wildtype αS within the NTD (aa 57) and NAC region *in vitro*, and may protect against the formation of pathologic aggregates. However, once αS is fibrillated, calpain I primarily cleaves in the CTD (aa 120), increasing the aggregation propensity and potentially contributing to toxicity (Mishizen-Eberz et al. 2003).

Nitration: With the increase in oxidative stress comes the formation of reactive oxygen species (ROS) like superoxide (O^{-}). Tyrosine residues in proteins are very vulnerable to elevated oxidative stress since O^{-} can react with nitric oxide to generate peroxy-nitrite ($ONOO^{-}$), a molecule capable of nitrating tyrosine residues converting them into 3-nitro-tyrosine (3-NT) (Giasson et al. 2000). αS in its fully nitrated form (all four tyrosine residues are nitrated) was found to accumulate in LB in PD (Giasson et al. 2000; Yu et al. 2010). However, nitrated αS does not appear to occur only at the end stage of PD; instead, it might contribute to dopaminergic neurodegeneration due to its toxicity shown *in vitro* (Yu et al. 2010). Structurally, nitration seems to stabilise αS oligomers by the formation of covalent cross-linking via oxidation of tyrosine, leading to the formation of di-tyrosine (Yamin et al. 2003; Souza et al. 2000). In fact, some argue that oxidative dimer formation is the rate-limiting step of the nucleation of αS aggregation, and that the tendency of A30P and A53T mutant proteins to dimerise accounts for their rapid fibril formation (Krishnan et al. 2003).

2.4.2 AMYLOID FIBRILS

Per definition, amyloid fibrils are fibrillary protein assemblies with a unique quaternary structure comprised of β-sheets formed by intermolecular hydrogen bonding (Fowler et al. 2007). The β-sheets are constituted by

β-strands which run perpendicular to the fibril axis, resulting in a conformation known as 'cross-β-sheet', which generates a typical X-ray fibre diffraction pattern (Sunde and Blake 1997). These characteristics have also been confirmed for the long αS filaments in LB and LN of PD patient brains (Spillantini et al. 1998; Duffy and Tennyson 1965; Araki et al. 2019; Serpell et al. 2000).

Furthermore, the aggregate must exhibit affinity for Congo red (CR), and show yellow or orange birefringence when the stained deposits are viewed with polarised light (Westermark et al. 1999). LB in PD patients' brains fit this definition since they consist of fine amyloid-like fibrils and, at least sometimes, exhibit birefringence when stained with CR (Sipe et al. 2014).

FIBRIL MORPHOLOGY

The morphology of αS fibrils is predominantly unbranched, as shown by atomic force and transmission electron microscopy (AFM; TEM). Moreover, they exhibit anti-parallel β-sheet structure, as visualised by Fourier transform infrared spectroscopy and circular dichroism spectroscopy (Conway et al. 2000). Fibrillar aggregates show a highly ordered and specifically folded central core region of 70 aa, whereas the NTD is structurally more heterogenous and the negatively charged CTD is completely unfolded (Der-Sarkissian et al. 2003). While the absence of the CTD promotes assembly (Crowther et al. 1998), deletion of NAC residues 71-82 or 66-75 completely abolishes the ability to assemble into filaments (Giasson et al. 2001; Du et al. 2003). Later, it was found that residues 71-82 are exactly located in the innermost β-sheet of the fibril core (Tuttle et al. 2016).

As discussed above, mutations and PTMs change the propensity of αS to self-assemble to oligomeric structures (see 2.4.1 Nucleation and Oligomerisation). Naturally, the effect can also be seen on the fibril level. For example, A53T and A30P mutations accelerate αS fibrillisation (Conway et al. 2000; Yonetani et al. 2009) and result in differences in fibril morphology (Giasson et al. 1999). Generally, synthetic αS pre-formed fibrils (αS pff) can vary a lot depending on distinct experimental aggregation conditions, the fibril width can range from 6-18 nm, and the length from 0.1 µm to more than 5 µm (Bousset et al. 2013; Van Raaij et al. 2008; Sweers et al. 2011; 2012a; 2012b) (**Figure 5**).

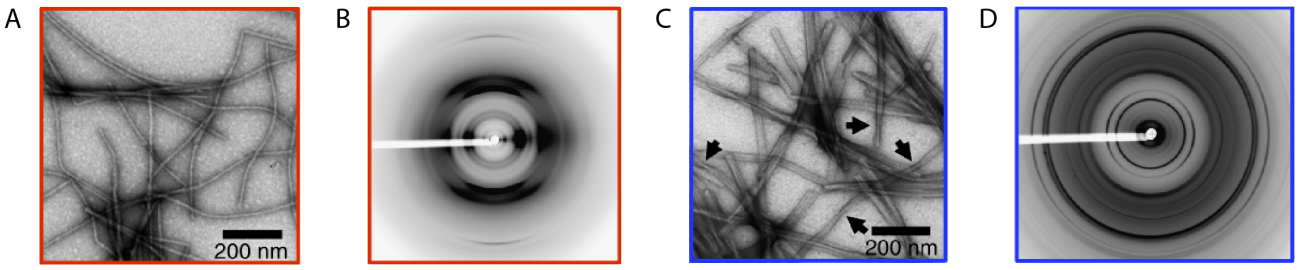

Figure 5: Examples of different polymorphs of wildtype αS pff, synthesised by Bousset et al. (A, B) Polymorph 1 ('fibrils'), width 13 nm, in TEM image (A) and its X-ray diffraction pattern (B). **(C, D)** Polymorph 2 ('ribbons'), width 18 nm, in TEM image (C) and X-ray diffraction pattern (D). The arrow heads in C) point to twists of the 'ribbon' structure, width 8 nm. Modified from Bousset et al., 2013, *with permission from Ronald Melki.*

Only very recently, the high-end method of cryogenic electron microscopy (cryo-EM) has helped to elucidate the detailed structure of diverse αS pff (Li et al. 2018a; 2018b; de Oliveira and Silva 2019; Zhao et al. 2019; Guerrero-Ferreira et al. 2018). One type of wildtype αS fibril structures showed two protofilaments intertwining into a left-handed helix, with each protofilament featuring a Greek key topology (Li et al. 2018b).

Other polymorphs of wildtype αS pff seem to be similar in exhibiting β-sheet protofilaments sharing a conserved kernel consisting of a bent β-arch motif, however, the protofilaments contact each other at different residue ranges (one at the NAC region, and the other at the pre-NAC region), thus forming distinct fibril cores (Li et al. 2018a). Interestingly, the dimer interface of the protofilaments contains most of the well-known PD missense mutations, which changes the electrostatic interactions, resulting in a different fibril cores and filament twists (Li et al. 2018b; Zhao et al. 2019; de Oliveira and Silva 2019).

Given the variety among αS pff, patient-derived αS fibrils from different synucleinopathies are expected to show at least the same heterogeneity. Indeed, analysis of amplified aggregates from PD and MSA brain extracts demonstrated that brain-derived fibrils are structurally different to all the polymorphs generated *in vitro*. Moreover, among PD cases, the structural heterogeneity was larger than between PD and MSA (Strohäker et al. 2019). Cryo-EM analysis showed that there are at least two types of filaments in MSA fibrils, and each consisted of two different protofilaments. Interestingly, MSA differed from DLB filaments, suggesting distinct conformers which characterise synucleinopathies (Schweighauser et al. 2020). To this date, there is no published cryo-EM data from human PD fibrils.

STRAINS AND SEEDING

Since nucleation-dependent polymerisation mechanisms can be accelerated by adding seeds (see 2.4.1 Nucleation and Oligomerisation), the introduction of αS seeds into *in vitro* and *in vivo* model systems efficiently drives αS aggregation and pathology (Danzer et al. 2009; Volpicelli-Daley et al. 2011). By seeding different fibril polymorphs, it became obvious that pathology formation is dependent on the introduced seed: In addition to structural variability, αS fibril polymorphs have been demonstrating differences in their seeding properties, e.g. seeding efficiency (Tarutani et al. 2018; Yamasaki et al. 2019), or cross-seeding of tau aggregates (Guo et al. 2013), and their neurotoxicity to specific neuronal populations (Peelaerts et al. 2015). Moreover, the fibrils show variations in their vulnerability to proteinase K treatment (Guo et al. 2013). In conclusion, fibril polymorphs are considered the structural basis of multiple strains of prion-like diseases (Fändrich et al. 2009; Makowski 2020).

Regarding seeding efficiency, it is possible that brain-derived αS seeds are more potent than αS pff, similar to other proteopathic seeds (Jucker and Walker 2013), while among synucleinopathy patient-extracted αS seeds, MSA strains seem to be the most potent pathogen (Tarutani et al. 2018). In fact, MSA strains provoked more severe neurotoxicity and neuroinflammation than PD strains, which is reflecting the aggressive nature of this disease in patients (Van der Perren et al. 2020).

AMYLOID DYES

For histological analysis, conformation-selective αS antibodies can be used to highlight the differences of αS aggregates (Covell et al. 2017), or conformation-sensitive dyes like CR-, or thiophene derivatives. The benzothiazoles thioflavin S (ThioS) and thioflavin T (ThioT) stain amyloid structures by adhering to fibrils. ThioT, when bound, displays a shift in excitation and emission spectra, which is a very useful feature for *in vitro* measurements of amyloid fibril formation (LeVine 1999). ThioS, on the other hand, is commonly used in histological staining of amyloid fibrils, since it does not change its excitation or emission spectra but instead results in a several-fold increase of the emission intensity, causing a high background fluorescence in solution (LeVine 1999). Of note, ThioS is not a single type of molecule but a mixture of at least six components, and

does not share the same binding sites with ThioT in amyloid deposits, but rather competes with CR derivatives (Wei et al. 2005).

CR has been used for fluorescent staining of plaques and tau neurofibrillary tangles (NFT) in post-mortem AD brain sections, but due to its hydrophilicity, it has limited brain permeability. Thus, uncharged analogues of CR and other brain-permeable fluorescent dyes had to be engineered (Wei et al. 2005). Initially designed for real-time visualisation of cerebral protein aggregates in transgenic mouse models for neurodegenerative diseases by multiphoton-microscopy (Åslund et al. 2009; Wegenast-Braun et al. 2012), the development of luminescent conjugated oligothiophenes (LCOs) emerged to be a breakthrough in histological discrimination of amyloid structures (Heilbronner et al. 2013; Brelstaff et al. 2015; Klingstedt et al. 2013; Rasmussen et al. 2017; Klingstedt et al. 2019; Strohäker et al. 2019). For oligomeric and fibrillar αS species, pentamer formyl thiophene acetic acid (p-FTAA) is often used for being at least as sensitive to accumulated αS as ThioT (Taylor et al. 2018; Kuan et al. 2019; Morgan et al. 2020). Upon binding to protein aggregates, the freedom of the p-FTAA backbone is restricted, causing the molecule to undergo a conformational shift, which specifically changes the conformation-dependent emission spectra (Åslund et al. 2009). This feature allows to distinguish between conformational strains of αS, as well as αS from PD or MSA brains (Klingstedt et al. 2019; Shahnawaz et al. 2020; Strohäker et al. 2019).

2.5 TOXICITY

The occurrence of neuronal αS inclusions positively correlates with neurodegeneration (Abdelmotilib et al. 2017), and it was demonstrated that αS inclusion-bearing neurons selectively die, using *in vivo* multiphoton-imaging in seeded mice (Osterberg et al. 2015). However, the exact nature of the neurotoxic αS species is unknown. Nevertheless, it is widely believed that it is rather an oligomeric than a fibrillar one, and that larger aggregates, even in the form of LB, have a protective role in capturing the toxic oligomers (Burré et al. 2018; Walker and Jucker 2016; Bodner et al. 2006; Tanaka et al. 2004; Olanow et al. 2004). In line with this, the very aggressive A30P and A53T mutations are prone to αS oligomerisation at the expense of fibrillisation (Li et al. 2002), and DLB patients show elevated levels of αS oligomers (Paleologou et al. 2009). In addition, many groups have shown that it is rather the pre-fibrillar structures, that increase neurotoxicity (Winner et al. 2011; Karpinar et al. 2009). Thus, the induction of mitochondrial damage and deficits, and the disruption of cellular functions seem to be the major drivers of neurodegeneration, rather than fibril formation (Mahul-Mellier et al. 2020).

2.5.1 MEMBRANE-ASSOCIATED DAMAGE

Due to the affinity to membranes, toxic αS species are bound to have a damaging effect on membrane structures, and many αS-lipid-mediated neurotoxic mechanisms have been proposed (see below). Noteworthy, all seven αS missense mutations are in the membrane-binding NTD, and several studies have reported that particularly A30P, A53E and G51D mutations decrease lipid affinity (Mori et al. 2020) and supposedly trigger αS aggregation (Mori et al. 2019).

The necessary characteristics of toxic αS oligomers to perturb cell membranes were identified to be a highly lipophilic element promoting membrane interactions, and a structured part that can insert into lipid bilayers (Fusco et al. 2017). First evidence for membrane permeabilization by oligomeric αS came from AFM and TEM

analyses, showing that it is capable of destroying synthetic vesicles (Volles et al. 2001) and the cell membrane, by forming annular, pore-like protofibrils (Lashuel et al. 2002). However, increased membrane-curvature instead of pore-like structures is a more likely mechanism of membrane rupture (Shi et al. 2015; Varkey et al. 2010). Single-vesicle fluorescence revealed the stepwise vesicle disruption and asymmetric membrane deformation upon αS binding to phosphatidylglycerol vesicles (Hannestad et al. 2020). The increased exposure of lipid acyl chains at the edges of defective membrane then facilitates membrane-oligomer interaction, resulting in stabilisation of the damage by binding of oligomers to the edges, or even enlargement of the defects by removing lipids from the edges (Chaudhary et al. 2016).

2.5.2 ORGANELLE STRESS

Membranes of cell organelles can also be affected by oligomeric αS: In yeast, the earliest defect following αS expression appears to be a block in endoplasmic reticulum (ER)-to-Golgi vesicle trafficking, and the occurrence of αS aggregates coincides with Golgi fragmentation (Cooper et al. 2006; Gosavi et al. 2002). Moreover, accumulation of αS oligomers has been shown to result in chronic ER stress and impaired ER protein quality control in a transgenic mouse model and in human PD brain tissue (Colla et al. 2012).

The presence of αS oligomeric species is associated with mitochondrial fragmentation, as well as mitochondrial impairment, mitophagy, and excessive production of ROS (Plotegher et al. 2014; Di Maio et al. 2016; Chinta et al. 2010; Ganjam et al. 2019; Vicario et al. 2018). Furthermore, oligomers are able to promote calcium-induced mitochondrial swelling and depolarisation, and accelerated cytochrome c release (Luth et al. 2014), which is thought to be one of the earliest steps in apoptotic cell death (Liu et al. 1996), and fibrillar αS binding to mitochondria correlates with defects in cellular respiration (Wang et al. 2019).

2.5.3 DNA DAMAGE AND PARTHANATOS

Several mechanisms that ultimately lead to cell death have been proposed for synucleinopathies. One of them is based on the observation that overexpression of αS seems to have a detrimental effect on αS-modulated transcription, which is implied in downregulation of DNA repair gene transcription (Paiva et al. 2017). The aggregation of αS in soma and neurites reduces its availability in the nucleus, which in turn impairs DNA repair, particularly the repair of double-strand breaks, and thus contributes to programmed cell-death mechanisms (Schaser et al. 2019).

DNA damage is the primary activator of the enzyme poly(ADP-ribose)polymerase-1 (PARP-1) that catalyses the addition of poly(ADP-ribosyl) (PAR), a PTM of many proteins involved in gene expression, maintenance of genomic stability, and cell death (Krietsch et al. 2013; Aredia and Scovassi 2014; Beneke 2012; Bürkle 2006; Bürkle and Virág 2013). Indeed, PAR levels were found to be increased in the CSF of PD patients in two independent patient cohorts (Kam et al. 2018). Excessive PARP-1 activity can be detrimental by two mechanisms: First, in order to PARylate proteins, the enzyme cleaves the ADP-ribose moiety from NAD+, which leads to depletion of NAD+ levels. This has detrimental consequences for ATP production, resulting in impaired cell functions (Alano et al. 2010). Second, PAR itself can serve as a protein-binding platform and act as a signalling molecule that induces cell death (Andrabi et al. 2006). The cell death program, also named ‘parthanatos’, after Thanatos, the Greek personification of death (Andrabi et al. 2008), involves the release of the flavoprotein apoptosis inducing factor (AIF) from the intermembrane space of mitochondria (Susin et al. 1999) and its nuclear translocation where it mediates chromatin condensation and large-scale fragmentation

of DNA (Ye et al. 2002). In line with this, αS was found to activate nitric oxide synthase (NOS), resulting in DNA damage and PARP-1 activation. This was shown to have two downstream effects: First, AIF was released from mitochondria and initiated parthanatos (Kam et al. 2018; Adamczyk et al. 2005). Second, activation of PARP-1 lead to PARylation of the molecular transcription factor EB (TFEB), a positive regulator of autophagosome formation and fusion to the lysosome. The PARylation led to the nuclear export of TFEB and thus reduction of autophagosomal degradation of aggregated αS (Mao et al. 2020). In agreement with these findings, pharmacological inhibition of PARP-1 was shown to reduce cytotoxicity in PD *in vitro* and *in vivo* models (Mao et al. 2020; Outeiro et al. 2007; Martire et al. 2015).

2.5.4 SYNAPTOPATHY

Another, increasingly popular, term to describe the cell death mechanisms in diseases like PD, is synaptopathy. It implies that disruptions in synaptic structure and function are the major determinant of brain disorders (Brose et al. 2010; Bridi and Hirth 2018). Synaptic dysfunction is thought to occur already at the early stages of PD, and oligomeric αS species have been implicated by binding the vesicular protein synaptobrevin-2, and thus preventing the formation of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex, the core fusion machinery for vesicle fusion. This would lead to decreased neurotransmitter release and possible loss of connectivity, ultimately resulting in neuronal dysfunction and death (Schulz-Schaeffer 2010; Choi et al. 2013). In addition, interrupted synaptic transmission could be caused by a high concentration of extracellular αS which seems to correlated with a reduction of the firing rate (Hassink et al. 2018). Synaptic vesicle trafficking may also be negatively impacted by αS oligomers, which have been shown to modulate microtubule stability (Cartelli et al. 2016; Alim et al. 2004) and impair the interaction between kinesin and microtubules (Chen et al. 2007; Prots et al. 2013).

2.6 DEGRADATION

Since synucleinopathies are age-related disorders, the progressive, age-dependent decline of protein degradation efficiency could play a synergistic role in the accumulation of αS aggregates (Kaushik and Cuervo 2015; Xilouri et al. 2013). The two main pathways for intracellular proteolysis, lysosomal and proteasomal clearance, are likely to work in complementary ways in the removal of αS species, which could depend on PTMs, localisation of the αS species, and pathological state of the cells (Stefanis et al. 2019).

2.6.1 PROTEASOMAL DEGRADATION

The proteasome degrades intracellular proteins mainly through the ubiquitin system, following the conjugation of a chain of at least four ubiquitin molecules (Pickart 2000). Thus, the accumulation of ubiquitinated αS (see 2.4.1 Nucleation and Oligomerisation – PTM Effects) might signal that the proteolytic system is overwhelmed, resulting in aberrant ubiquitination (Tofaris et al. 2003). Some of the αS oligomers can be degraded by the proteasome (Emmanouilidou et al. 2010b), but oligomers can also interact with the proteasome complex, leading to a decreased activity of degradation (Ghee et al. 2000; Lindersson et al. 2004). Moreover, it is possible that the proteasome fragments αS assemblies into smaller aggregates (Cliffe et al. 2019), which might be more toxic than the large fibril species (see 2.5 Toxicity) (**Figure 6**). In any case,

proteasomes are thought to be the primary site for the degradation of phosphorylated αS (Arawaka et al. 2017).

2.6.2 LYSOSOMAL DEGRADATION

Lysosomes degrade intracellular proteins though the autophagic pathway, which involves the creation of a double membrane structure engulfing intracellular organelles or proteins, creating autophagosomes, which then fuse with lysosomes to degrade their contents. The degradation of extracellular proteins after internalisation happens via the endo-lysosomal pathway (Stefanis et al. 2019).

Lysosomal inhibition or deficiency promotes intracellular accumulation of αS, however only in transgenic αS overexpression models, suggesting that the autophagy-lysosomal pathway is activated to handle specifically accumulated αS (Desplats et al. 2009; Yu et al. 2009; Ebrahimi-Fakhari et al. 2011). Stimulation of the autophagy-lysosomal pathway, on the other hand, leads to increased clearance of αS oligomers (Decressac et al. 2013; Crews et al. 2010). Of further note, accumulation of αS can also inhibit autophagy (Tanik et al. 2013; Winslow et al. 2010). Even more interestingly, aggregation of αS in autophagosomes can provoke a partial block of the autophagosome-to-lysosome fusion, leading instead to its secretion. This type of secretion mediated through autophagosomes is termed exophagy (Ejlerskov et al. 2013) (Figure 6).

The endo-lysosomal pathway, conversely, processes mainly extracellular αS species, which are internalised through endocytosis, sorted through recycling endosomes, and finally delivery to lysosomes for degradation by cathepsins (Mellman 1996; Karpowicz et al. 2017; Konno et al. 2012; Lee et al. 2008a; McGlinchey et al. 2020). Several candidate proteins for the receptor-mediated endocytosis of oligomeric extracellular αS have been suggested (Ihse et al. 2017; Mao et al. 2016; Shrivastava et al. 2015). Furthermore, intracellular αS can be targeted to endo-lysosomal degradation by mono-ubiquitination by the ubiquitin-ligase neuronally expressed developmentally down-regulated gene 4 (NEDD4) (Tofaris et al. 2011). NEDD4 also ubiquitinates misfolded αS assemblies, suggesting that it may promote the clearance of aggregated αS (Davies et al. 2014) (Figure 6).

2.6.3 EXTRACELLULAR CLEAVAGE

Extracellular αS can also be cleaved in the extracellular space (ECS) by several proteases, like neurosin (also known as kallikrein 6), which is expressed by oligodendro- and astroglia, and whose levels correlate inversely with αS accumulation in brains with DLB (Yamanaka et al. 1999; Tatebe et al. 2010; Spencer et al. 2013). Another group of proteases that have been shown to cleave extracellular αS are matrix metalloproteases (MMPs) (Sung et al. 2005), but, furthermore, they exhibited adverse effects by cleaving the CTD and thus enhance αS aggregation (Levin et al. 2009; Vekrellis et al. 2011). On the other hand, Plasmin, a distinct extracellular serine protease, is able to degrade extracellular αS by mainly cleaving the NTD (Kim et al. 2012) (Figure 6).

2.7 SPREADING

Ever since Braak's postulation of staging pathology, based on the discovery of LB and LN spread between anatomically connected areas (see 1.2.2 Pathological Spreading of Misfolded α-Synuclein), cell-to-cell-transmission of αS was suspected to contribute to the pathology. The transmission would require active or passive transport across the axon to connected areas, the release of αS into the ECS and uptake by recipient cells, where it would seed the prion-like conversion and aggregation of αS.

2.7.1 TRANSPORT AND RELEASE

The study of αS in cell cultures showed that αS can be transported both anterogradely and retrogradely, but with more efficiency in the anterograde direction (Brahic et al. 2016). Interestingly, in a mouse model with induced synucleinopathy by pff injection into the OB, αS inclusions appears to spread in a retrograde manner for the first 9 months, and then switched to anterograde propagation (Mezias et al. 2020). Notably, it appears that αS fibrils are not enclosed in vesicles when transported in anterograde direction (Roy et al. 2007).

Although αS lacks a secretory sequence, the protein can be detected in the plasma and CSF of individuals with PD (El-Agnaf et al. 2006), indicating that αS is nevertheless secreted into the ECS. There is evidence of neurons secreting monomeric and oligomeric αS through unconventional (Golgi-independent) exocytosis (Lee et al. 2005; Jang et al. 2010; Lee et al. 2014). Exocytosis can be enhanced by overexpression, mutations, or reduced clearance of αS through cellular degradation mechanisms (Alvarez-Erviti et al. 2011; Fernandes et al. 2016a; Lee et al. 2013). In addition, as discussed above (see 2.6.2 Lysosomal Degradation), aggregated αS might be released by exophagy (Ejlerskov et al. 2013) (Figure 6).

2.7.2 UPTAKE

The uptake of extracellular αS can take place both in the soma, the dendrites, or the axon compartment (Brahic et al. 2016), and it does not seem to require synaptic contacts (Freundt et al. 2012). Interestingly, the uptake of fibrils by neurons resembles the entry of viruses into host cells, since αS fibrils – as well as PrP^{Sc} (Horonchik et al. 2005) – seem to interact with the cell surface protein group of heparan sulfate proteoglycans (HSPGs), like viruses (Bieri et al. 2018; Marsh and Helenius 2006; Holmes et al. 2013). Other receptors for αS fibrils have been proposed, such as the Na+/K+ transporting subunit α3 (α3-NKA) (Shrivastava et al. 2015) and lymphocyte activating gene-3 (LAG3) (Mao et al. 2016; Angelopoulou et al. 2020) (Figure 6). However, the relevance of LAG3 is controversial since it is predominantly expressed by microglia, not neurons (Zhang et al. 2014), thus potentially mediating αS fibril uptake by glia cells, rather.

Since neurons are not phagocytic cells, their uptake of protein aggregates by endocytosis is unlikely. However, it has been demonstrated in cell cultures and animal models, that extracellular αS of various forms – monomers, oligomers, fibrils – can be internalised by neurons (Lee et al. 2008a; Desplats et al. 2009), and internalised αS fibrils were found to co-localise with endosomal markers (Brahic et al. 2016). Since protein aggregates are huge – with single fibrils ranging from 0.1 µm to more than 5 µm, depending on the protocol (Bousset et al. 2013; Van Raaij et al. 2008; Sweers et al. 2011; 2012a; 2012b), the uptake by clathrin-coated pits (for particle sizes <200 nm (Traub 2009)) becomes unlikely. Thus, an alternative mechanism for the uptake of such large aggregates by neurons is macropinocytosis, a clathrin-independent endocytosis mechanism. It

involves the polymerisation of actin to form ruffles at the membrane surface that can fuse back to the plasma membrane, by which it is encapsulating extracellular fluid and proteins into large vacuoles of up to 5 µm (Holmes et al. 2013; Lim and Gleeson 2011; Zeineddine and Yerbury 2015). Indeed, interaction of HSPGs with αS fibrils was shown to induce uptake via macropinocytosis *in vitro* (Holmes et al. 2013) (Figure 6).

After entering the cell, αS fibrils are trapped in an endosome, surrounded by lipid membranes, without direct access to soluble cytosolic αS. Escaping the endosome could be a relatively rare, rate-limiting event, contributing to the slowness of the development of synucleinopathies (Bieri et al. 2018). Yet, there is evidence that αS aggregates can induce the rupture of endosomal/lysosomal vesicles, similar to some viruses and bacteria (Freeman et al. 2013) (Figure 6). Of note, several conformational fibrillar αS strains were able to induce vesicle rupture, but not monomeric or oligomeric forms (Flavin et al. 2017).

2.7.3 DIRECT TRANSFER

Some studies suggest, that in addition to classical exo- and endocytosis, αS could be transmitted from one neuron to the other via mechanisms that will not expose it to the ECS (Figure 6). However, direct transfer would not allow for antibodies to bind free αS (see 2.8 Immunotherapy). Thus, alternative transfers might exist, but not exclusively (Bieri et al. 2018).

One of those alternative transfer mechanisms could be by tunnelling nanotubes (TNTs), as it has been suggested for PrP^{Sc} spreading (Gousset et al. 2009). TNTs are F-actin containing membranous bridges, that have also been demonstrated between embryonic stem cell (ESC)-derived astroglia (Rostami et al. 2017). In cell culture, they have been observed to transfer αS fibrils inside lysosomal vesicles from donor to acceptor cell, where they were able to seed soluble αS aggregation (Abounit et al. 2016; Dieriks et al. 2017) (Figure 6). An additional opportunity for αS to travel across TNTs is via the association to mitochondria, which can be tunnelled to adjacent cells in need in order to increase their ATP production (Valdinocci et al. 2020; Lu et al. 2017).

Another mechanism is the release of exosomes. Exosomes are membrane-bound extracellular vesicles, formed intracellularly inside a multi-vesicular body, which fuses with the cell membrane to release its exosomes into the ECS (Klumperman and Raposo 2014) (Figure 6). In the brain, exosomes are physiologically implicated in intercellular communication of neurons, triggering localised signalling events and regulating synapse formation (Chivet et al. 2013). Curiously, high levels of αS were found in exosomes that have been isolated from PD patients' plasma (Shi et al. 2014; Fu et al. 2020), and the secretion of αS-containing exosomes seems to be enhanced by neuronal activity in a calcium-dependent manner (Emmanouilidou et al. 2010a). Synaptic connectivity appears to be crucial, which we learned from exosomal transmission of tau (Wang et al. 2017), that is thought to spread in a similar way to αS (Vasili et al. 2019). Moreover, tau seems to be predominantly secreted in *ectosomes*, which are large exosomes, originating directly from the plasma membrane (Dujardin et al. 2014). Nevertheless, it remains to be seen if tau transmission mechanisms hold true for αS. In any case, the relevance of exosomal αS transfer for spreading of pathology is controversial and needs to be further elucidated since some studies suggest that intracerebral (i.c.) injection of exosomes isolated from DLB patients can induce αS aggregation in neuronal cultures and wildtype mice (Ngolab et al. 2017; Stuendl et al. 2016), but others have shown that exosomes neutralise the seeding capacity of its misfolded αS content (Karampetsou et al. 2020). Moreover, the internalisation of exosomes by microglia and

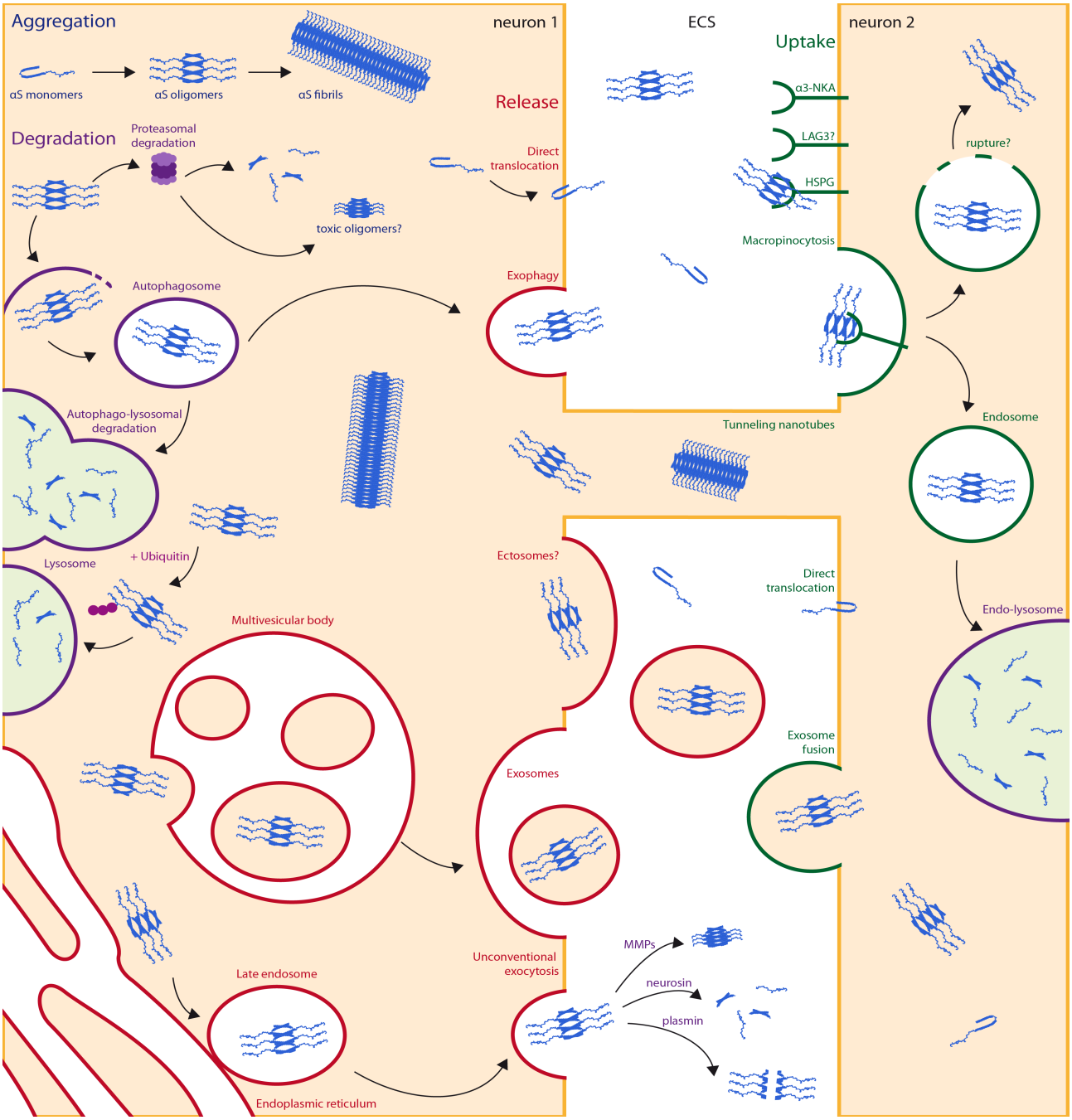

Figure 6: Schematic representation of possible interneuronal spreading and degradation pathways of αS. Aggregation of αS from monomers via oligomeric species to fibrils is shown in blue (for details, see 2.4.1 Nucleation and Oligomerisation; **Figure 4**). Degradation pathways are shown in purple. Oligomeric species can be degraded by the proteasome, which might result in the production of toxic oligomers. Another degradation pathway involves the encapsulation into autophagosomes which fuse with lysosomes for digestion of their content. Oligomers also can also be directly targeted by ubiquitin ligases to lysosomal degradation. Proposed release mechanisms are shown in red. The small αS monomers are able to directly translocate through the cell membrane. Oligomeric species can be released by the fusion of autophagosomes with the cell membrane (exophagy), budding of the cell membrane (ectosomes), the fusion of multivesicular bodies resulting in release of its vesicles (exosomes), or via unconventional exocytosis, which involves the uptake of αS species into late endosomes and their fusion with the cell membrane. In the ECS, αS oligomers can be cleaved by matrix proteases like MMPs, neurosin, or plasmin. The uptake mechanisms are shown in green. Uptake of αS species can take place by receptor-mediated macropinocytosis, or the fusion of exosomes with the cell membrane. Another possibility is the direct transfer of αS oligomers and possibly αS fibrils through the formation of tunnelling nanotubes, or direct translocation of monomeric αS. Endosomal αS can be degraded by fusion of its encapsulating vesicle with lysosomes, or it might rupture the endosome to be released into the cytoplasm.

astroglia, the brain cell types responsible for maintaining the brain microenvironment homeostasis, is thought to contribute to the removal of αS species (Filippini et al. 2019).

Importantly, the major caveat of many studies is the assumption that fibrillar αS is extracellularly present. In fact, αS in human CSF exosomes was only found in oligomeric and monomeric forms, and it is not sure if fibrils exist outside cells, at all (Koh et al. 2018). Monomeric αS, on the other hand, seems to be able to cross the membrane by direct translocation (Lee et al. 2008b) (Figure 6), an unconventional secretion and uptake mechanism that has also been described for monomeric tau (Katsinelos et al. 2018).

2.7.4 PRION-LIKE PROPAGATION

Numerous studies have been conducted demonstrating the spreading pathology by introducing an αS seed into one specific site, and expecting the amplification of αS aggregates in a connected region by recruitment of endogenous αS in a templated-manner (Ma et al. 2019). First, it was demonstrated that injecting brain homogenate of symptomatic αS-transgenic mice into the brain of younger transgenic mice will lead to early symptoms of PD and LB-like lesions, which could be prohibited by the injection into SNCA knockout mice (Luk et al. 2012b; Mougenot et al. 2012). These results could be replicated with injections of αS-containing LB extracts from the brain tissues of post-mortem PD patients into wildtype mice (Recasens et al. 2014). Second, injection of synthetic αS pff, but not soluble αS, into the striatum or *substantia nigra* (SN) would lead to spreading of αS inclusions in anatomically interconnected brain regions in both transgenic and wildtype mice (Masuda-Suzukake et al. 2013; Paumier et al. 2015; Luk et al. 2012a; 2012b; Masuda-Suzukake et al. 2014). But not just intracerebral injections of αS seeds can lead to brain pathology. Instead, injection of recombinant monomeric and oligomeric αS into the olfactory bulb of wildtype mice was sufficient to induce the formation of αS aggregates in interconnected brain areas (Rey et al. 2013). Furthermore, intramuscular, intravenous, intraperitoneal, enteric, urogenital injections and oral intake of αS fibrils could also lead to transmission of pathology from peripheral neurons to the brain (Sacino et al. 2014; Kuan et al. 2019; Lohmann et al. 2019; Rey et al. 2016b; Kim et al. 2019; Challis et al. 2020; Ding et al. 2020).

The strongest evidence for αS propagation, however, originates from *in vitro* studies, where neuronal somas can be spatially separated from their axonal projections in microfluidic devices (Taylor et al. 2005). This approach allows for αS uptake in the isolated compartment of neuronal somas, and demonstrated the subsequent axonal (anterograde and retrograde) transport, release and uptake by the secondary neuron in a third compartment (Freundt et al. 2012; Brahic et al. 2016; Bieri et al. 2018). All these experimental findings provide evidence that some αS species are capable of transcellular spreading, acting as an aggregation seed in the cell they enter, and thus propagating pathology in a prion-like manner.

2.7.5 SELECTIVE VULNERABILITY

Historically, the distribution of αS lesions in the CNS of patients was thought to result from vulnerability differences of different brain regions, e.g. neurons with highly branched axons, slow tonic activity, and elevated mitochondrial stress are more prone to αS aggregates (Surmeier et al. 2017). Since it was not longitudinal data but post-mortem studies that identified the stereotypical distribution of pathology, there was no direct evidence for the sequential pathology development of different brain regions (Peng et al. 2020). Thus, the concept of prion-like cell-to-cell transmission is controversial for some, since the apparent spread of αS could be due to selective vulnerability of specific neuronal populations, induced by neuroinflammation of

oxidative stress (Walsh and Selkoe 2016). In all likelihood, the truth lies somewhere in between, with network diffusion models showing that both anatomical connectivity as well as regional vulnerability, which correlates with SNCA expression, are major contributors to the pathogenic αS spread (Henderson et al. 2019).

2.8 IMMUNOTHERAPY

The ultimate aim of neurodegenerative disorders research is the development of disease modifying therapies for patients. In the case of synucleinopathies, there are high hopes in finding a way to neutralise the pathogenic αS species, or enhance its clearance, in order to slow down or halt the disease progression. Indeed, therapeutic suppression of αS could be a viable approach, since αS deficiency, e.g. in mice with a naturally occurring deletion of SNCA or αS knockout lines, is rather mild, and it would furthermore reduce the amount of toxic αS assemblies (Visanji et al. 2016).

Since αS oligomers can be secreted (Lee et al. 2005; Jang et al. 2010; Lee et al. 2014) and propagate extracellularly (Freundt et al. 2012; Brahic et al. 2016), they are fortunately recognisable by αS-directed antibodies, and thus may provide a rationale for immunotherapy.

2.8.1 A-SYNUCLEIN EPITOPES

First studies of active immunisation (vaccination) of transgenic mice with human αS showed that the animals produced high affinity antibodies, which were capable of decreasing the accumulation of aggregated αS in neurons and reducing neurodegeneration (Masliah et al. 2005). Interestingly, the antibodies recognised epitopes within the CTD (Masliah et al. 2005), presumably because this domain is completely unfolded (Der-Sarkissian et al. 2003) and antibodies will not require conformational specificity to recognise all forms of αS (Bae et al. 2012). Thus, the first passive immunisation studies were also aimed at the CTD in transgenic mice, and demonstrated impaired αS accumulation, reduced neuroinflammation and -degeneration, and improved motor function (Masliah et al. 2011; Bae et al. 2012; Games et al. 2014). Later, experiments with NTD-targeting antibodies also succeeded in reducing Lewy pathology, inflammation, and neurodegeneration (Tran et al. 2014; Shahaduzzaman et al. 2015). However, only few studies exist in which different antibodies have been compared side by side in the same animal model, and since animal models differ in their pathology, it is difficult to draw general conclusions about the most effective epitope targeting (Bergström et al. 2016).

Other partially successful approaches were targeted to recognise oligomeric αS or protofibrils to prevent potential interference with physiological protein function (Fagerqvist et al. 2013; Vaikath et al. 2015; El-Agnaf et al. 2017). However, a recent study demonstrated that the majority of αS antibodies do not show specificity for one type of αS species, and, importantly, none of the tested antibodies could differentiate between oligomers and fibrils (Kumar et al. 2020). Hence, cross-reactivity is as a major caveat for immunotherapies, since the aged brain is characterised by many aggregates of different proteins, many of which adopt similar conformations (Vaikath et al. 2019).

2.8.2 MECHANISM OF ACTION

The action mechanism of antibodies can be divided into two general categories: blocking or clearance.

Antibodies binding to αS can neutralise or shield the protein, and thus block the toxicity and uptake of extracellular αS into non-phagocytic cells, like neurons. Several immunotherapy studies have shown *in vivo* that their applied antibodies blocked deleterious immune cell activation, e.g. by increased expression of anti-inflammatory cytokines and reduced inflammation (Shahaduzzaman et al. 2015; Mandler et al. 2015). Others demonstrated inhibition of uptake and seeding (Tran et al. 2014; Näsström et al. 2011), or prevented the formation of more toxic truncated species by shielding the cleavage site for proteolysis (Masliah et al. 2011; Games et al. 2014).

On the other hand, antibodies bound to extracellular αS can be recognised by Fc receptors on phagocytic cells, primarily microglia in the brain, resulting in clearance by endocytosis and lysosomal degradation of the αS-antigen complex (Masliah et al. 2011). In transgenic animal studies, the administration of αS recognising antibodies reduced accumulation of αS in neurons and astroglia and reduced neurodegeneration and behavioural deficiencies (Bae et al. 2012; Masliah et al. 2011). Interestingly, in the comparative study performed by Bae and colleagues, only antibodies targeting the CTD lead to effective microglial clearance (Bae et al. 2012).

3 GLIA IN SYNUCLEINOPATHIES

Neuroinflammation and dysfunctional glial cells are typical pathological traits of synucleinopathies. In the course of synucleinopathy progression, astroglia and non-myelinating oligodendroglia will be filled with αS, impairing their physiological functions, and an increasing number of microglia will collect neuronal debris and become dysfunctional (Halliday and Stevens 2011; Heneka et al. 2014). This makes it unlikely that neuronal death can be alleviated without improving other aspects concerning the glial population.

3.1 A-SYNUCLEIN IN OLIGODENDROGLIA AND ASTROGLIA

As discussed above (see 1.2.1 α-Synuclein Inclusions), the brains of MSA patients contain abundant αS inclusions in oligodendroglia, called GCI. It is still not clear where they originate, but there is evidence that oligodendroglia express αS, which could argue for an oligodendroglial origin of GCI (Culvenor et al. 2002; Asi et al. 2014). However, other groups did not find any αS mRNA in primary oligodendroglia cell culture and *in situ* hybridisation of human brain tissue, and suggested that αS is rather taken up from the external environment or from neurons (Miller et al. 2005; Kisos et al. 2012; Reyes et al. 2014).

At advanced PD stages, on the other hand, αS deposits are found in astroglia (Braak et al. 2007), particularly in protoplasmic astroglia, the type which envelopes neuronal cell bodies and synapses and makes up the grey matter, but not fibrous astroglia, which wrap nodes of Ranvier and oligodendroglia and makes up the white

matter (Song et al. 2009). Curiously, in PD patient tissue, there are more αS-immunoreactive astroglia in brain regions that lack LB (Braak et al. 2007).

While astroglia do indeed express αS (Kim et al. 2013b), there is also evidence for direct transfer of αS from neurons to astroglia, leading to inflammatory responses of astrocytes by pro-inflammatory cytokine and chemokine release (Lee et al. 2010). Astroglia also express a variety of receptors that modulate inflammation, for example toll-like receptor (TLR)-4, which can recognise pathogen-associated molecular patterns and has been shown to mediate αS-induced inflammatory responses in astroglia (Fellner et al. 2013; Rannikko et al. 2015). However, upon uptake of αS oligomers, astroglia will try to degrade the endocytosed material via the lysosomal pathway. Failure to degrade the aggregates results in sustained intracellular deposits and mitochondrial damage (Lindström et al. 2017). Moreover, it has been shown that diseased astroglia attempt to unload their αS burden through TNTs to surrounding astroglia (Rostami et al. 2017).

3.2 MICROGLIA IN SYNUCLEINOPATHIES

Microglia are in the unique position of being the only brain cell population derived from progenitors originating in the yolk sac during embryogenesis, and invade the brain before the blood-brain barrier (BBB) forms (Alliot et al. 1999). Since microglia are the resident immune cells in the brain, their causal relationship to brain diseases is among the most popular topics in current neurodegeneration research.

Microglia were identified only in 1919, when the work of Nicolás Achúcarro and Pío del Río-Hortega, two alumni of the Santiago Ramón y Cajal school, determined them as a unique brain cell type, different from oligodendroglia (contrary to what Ramón y Cajal believed) (Tremblay et al. 2015). Río-Hortega described microglia as "characterised by their small, dark nucleus enveloped by scant protoplasm and its long, tortuous, ramified expansions adorned with lateral spines" (del Río-Hortega 1919b) (Figure 7). He further discovered that microglia are regularly distributed throughout the brain (del Río-Hortega 1919a), and observed that these cells are highly dynamic, transforming from "resting" to "activated", phagocytic cells in response to destructive processes of the nervous tissue (del Río-Hortega 1919b).

3.2.1 ACTIVATION

To this day, Río-Hortega's descriptions of microglia states remain valid: In a resting, quiescent state, microglia have long, ramified processes with small cell bodies. In response to an insult or various neurodegenerative diseases, however, microglia become 'activated', meaning they display morphological changes, like taking on an amoeboid shape with shorter processes and larger cell bodies. Moreover, they proliferate and alter their gene expression and surface markers, a state called microgliosis. Yet, it is important to note that the term 'activated microglia' may be oversimplified, since microglia can have a very heterogenous nature and various, complex effector functions (Joers et al. 2017; Tansey and Goldberg 2010). Furthermore, recent evidence suggests that morphological changes do not necessarily indicate activation but instead microglial impairment, meaning they can no longer efficiently perform their functions (Heppner et al. 2015).

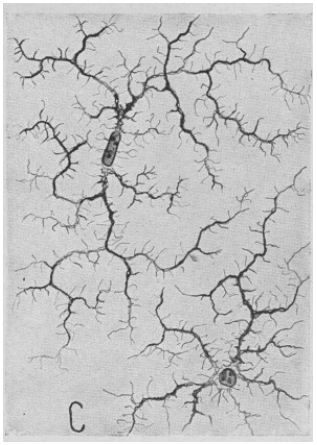

Figure 7: Original drawing by Pío del Río-Hortega of human microglia. Adapted from Sierra et al., 2016, *with permission from John Wiley and Sons.*

Microglia can be activated through various pattern recognition receptors, such as TLRs, which promote the synthesis of cytokines, inflammatory mediators, growth factors and cell surface molecules. Depending on the range of expressed and secreted molecules, the response of microglia can be classified as either pro- or anti-inflammatory (Joers et al. 2017). Regarding αS-dependent activation and phagocytosis, the receptors TLR-2 and -4 were found to play crucial roles: While TLR-4 signalling mediates αS phagocytosis, it is also required for αS-dependent activation of microglia (Stefanova et al. 2011; Fellner et al. 2013). TLR-2, on the other hand, is specifically activated by extracellular oligomeric αS, but not fibrillar or monomeric αS, and leads to inflammatory responses (Kim et al. 2013a; 2020). Importantly, microglia are known to be directly activated by aggregated and nitrated αS (Zhang et al. 2005; Reynolds et al. 2008). Monomeric αS, on the other hand, seems to modulate microglia towards an anti-inflammatory phenotype (Li et al. 2020).

3.2.2 INFLAMMATION

In PD, numerous studies have highlighted the role of neuroinflammation and microgliosis, and their contribution to degeneration of dopaminergic neuron terminals in early stages of the disease (Ouchi et al. 2005; Tansey and Goldberg 2010; Wu et al. 2002). In post-mortem tissue from advanced PD patients with healthy embryonic dopaminergic neuron implants, activated microglia in the healthy grafts preceded diffuse monomeric αS staining and αS aggregates, indicating that microglial activation may contribute, or even be causative, to the development of αS pathology and spread (Warren Olanow et al. 2019). On a side note, not only microglia but also infiltrating T cells from the peripheral immune system appear to play a crucial role in neuroinflammation in PD and DLB (Iba et al. 2020).

Importantly, microgliosis is not an acute but a sustained response in PD, and not limited to areas with significant neuronal death (Ferreira and Romero-Ramos 2018; Imamura et al. 2003). Instead, activated microglia can be regarded as sentinels of neuropathological changes. In the serum and CSF of PD patients, both pro- and anti-inflammatory cytokines have been reported, suggesting that pro- and anti-inflammatory microglia coexist (Joers et al. 2017). The differences within the microglial population could be a result of variation in their environment, which is influenced by the neuronal activity in the area, and also the type of αS aggregation at each stage (Sanchez-Guajardo et al. 2010). Moreover, different anatomic regions seem to contain varying amounts of microglia, since, within the same region, more microglia are found in myelinated areas compared to non-myelinated (Lawson et al. 1990; Mittelbronn et al. 2001). In addition to their abundance, microglia also differ in their expression profile from one brain region to another, and from grey matter to white matter (de Haas et al. 2008; Melief et al. 2012). This makes microglial response during synucleinopathies a very dynamic one, which has consequences on the neuronal fate. For example, in transgenic αS mice, microglia were activated in a regionally-specifically pattern, with an early inflammatory response in regions containing the axon terminals and cell bodies of the nigrostriatal pathway (Watson et al. 2012)

3.2.3 PHAGOCYTOSIS

All major brain cell types are capable of clearing extracellular αS aggregates by internalisation, however, microglia – the main phagocytic cells of the brain – show by far the highest rate of degradation (Lee et al. 2008b). Phagocytosis is receptor-ligand-induced, in particular by Fcγ receptors (FcγRs), which cluster by binding to IgG-opsonised complexes (García-García and Rosales 2002). Additionally, the FcγR clusters trigger

a signal transduction cascade that enhances phagocytosis, which makes antibody-αS complex internalisation extremely efficient (García-García and Rosales 2002; Bae et al. 2012). In the absence of antibodies, phagocytosis is often mediated by direct binding of TLR-4 to different types of αS species, or TLR-2 to oligomeric αS species, specifically (Kim et al. 2013a; Stefanova et al. 2011). Interestingly, even in an antibody-unbound state, FcγRs can also be involved in αS internalisation and clearance by phagosomes by altering intracellular trafficking (Cao et al. 2012).

The internalisation of αS activates microglia, and reportedly leads to subsequent stimulation of NADPH oxidase (Zhang et al. 2005). This, in turn, enhances αS-mediated neurotoxicity by microglial proliferation and secretion of further pro-inflammatory molecules (Su et al. 2009). At the same time, microglia can have a neuroprotective role in αS clearance: after being activated by neuron-released αS, microglia engulf αS into autophagosomes for degradation via selective autophagy, mediated by TLR-4 (Choi et al. 2020). Which pathway will dominate could depend on the αS species, since activation with monomeric αS seems to increase phagocytosis, while exposure to aggregated αS decreases it (Park et al. 2008; Choi et al. 2015).

Defects in microglial phagocytosis are associated with ageing in mice, which suggests that the age-related pathogenesis of synucleinopathies could be the result of insufficient removal of extracellular αS (Bliederhaeuser et al. 2016; Scheffold et al. 2016). Moreover, microglial degradation efficiency upon phagocytosis is dependent on the extracellular environment. Bacterial lipopolysaccharides (LPS), for example, can activate microglia in a way that leads to a decrease of degradation, resulting in the accumulation of internalized αS aggregates in the microglia cytoplasm (Lee et al. 2008b). More evidence for the relevance of microglial lysosomal function on neuronal degeneration is provided by genetic studies showing that many loci associated with PD are directly or indirectly connected to the autophagy-lysosomal pathway (Tremblay et al. 2019).

3.2.4 MICROGLIAL A-SYNUCLEIN INCLUSIONS

In reference to:

Gaye Tanriöver*, Mehtap Bacioglu*, Manuel Schweighauser*, Jasmin Mahler, Bettina M. Wegenast-Braun, Angelos Skodras, Ulrike Obermüller, **Melanie Barth**, Deborah Kronenberg-Versteeg, K. Peter R. Nilsson, Derya R. Shimshek, Philipp J. Kahle, Yvonne S. Eisele, Mathias Jucker. **Prominent microglial inclusions in transgenic mouse models of α-synucleinopathy that are distinct from neuronal lesions.** Acta Neuropathologica Communication 8, 133 (2020).

https://doi.org/10.1186/s40478-020-00993-8

*equal contribution

In our recent study, we found indeed abundant microglial inclusions, in addition to neuronal αS pathology, in several transgenic mouse models of synucleinopathy (Tanriöver et al. 2020). Initially focused on the differences of neuronal αS inclusions within the transgenic lines, we observed an overall similar appearance of the pathogenic brain tissue between the transgenic lines. However, within the same tissue, strikingly

different inclusions were identified, which could be categorised into being of neuronal or of microglial origin. The abundance of microglial inclusions was unexpected, since they had not been reported previously to a comparable degree. To this point, ubiquitin- and p62-positive microglial inclusions had been identified (Henrich et al. 2018), and hypertrophic microglia were found to engulf cells and Lewy-like αS aggregates-containing processes (Thakur et al. 2017). In patients, microglial inclusions had been described as neuromelanin granules that were internalised and remained non-degraded (McGeer et al. 1988; Orr et al. 2005), and only very recently, microglial αS inclusions were identified in the OB of PD patients (Stevenson et al. 2020). Our study shows that these microglial inclusions are structurally distinct from neuronal inclusions by using the amyloid dye p-FTAA (see 2.4.2 Amyloid Fibrils – Amyloid Dyes) which emits a different fluorescent spectrum when bound to microglial inclusions, indicating that the amyloid morphologies differ. Moreover, they are ThioS positive, and showed immunoreactivity with antibodies recognising the NTD of αS, but not the CTD. In fact, they remained largely pS129 αS negative, which we ascribed to possible C-terminal truncation. Lastly, we found microglial inclusions to be present already at pre-symptomatic stages of pathology in transgenic mouse models and in a seeded synucleinopathy mouse model. The presence of αS inclusions in microglia, especially at pre-symptomatic stages, poses the question if αS can also be freed from microglia and contribute to spreading in one way or another. In line with this, it has been suggested that microglia are able to contribute directly to αS transmission via exosomal αS release (Xia et al. 2019; Guo et al. 2020). Additionally, there is evidence that microglia play a role in affecting neuron-to-neuron transfer of αS (George et al. 2019).

3.2.5 MICROGLIA DEPLETION

In reference to:

Melanie Barth, Gaye Tanriöver, Marc Welzer, Luc Bousset, Ronald Melki, Derya R. Shimshek, Mathias Jucker. ***Microglia depletion and the differential effect on α-synuclein seeding in long-term murine hippocampal slice cultures.*** In preparation.

To learn more about the impact microglia have on αS pathology and spreading, methods to eliminate microglia have been developed. One of these approaches is the use of colony-stimulating factor 1 (CSF1)-receptor (CSF1R) inhibitors: CSF1R is expressed by macrophages and osteoclasts, and by microglia in the brain (Patel and Player 2009; Erblich et al. 2011; Nandi et al. 2012), and it has two ligands, CSF1 and interleukin-34 (Lin et al. 2008). Since CSF1 regulates proliferation, differentiation, and survival (Patel and Player 2009), depletion of the receptor via CSF1R knockout results in mice devoid of microglia (Erblich et al. 2011; Ginhoux et al. 2010). However, these mice rarely survive until 5-6 weeks of age (Erblich et al. 2011), which makes a transient approach by reversible receptor inhibition more valuable.

One of the lead oral inhibitors of CSF1R is PLX3397, currently in phase I-III clinical trials as a treatment for a variety of cancers (Cannarile et al. 2017). Elimination of microglia with PLX3397 showed no detrimental effect on locomotion, cognition, or behaviour in mice, despite microglial depletion of > 90 % up to 2 months (Elmore et al. 2014). Since PLX3397 inhibits not exclusively CSF1R, a more refined compound was developed, named PLX5622, which proved to be sufficient to eliminate microglia and sustain the elimination in a concentration-dependent manner (Dagher et al. 2015).

Microglial depletion with PLX5622 has been reported to reduce inflammation-related neuronal loss or cognitive deficits both in transgenic AD mice and AD patients (Dagher et al. 2015; Spangenberg et al. 2016). On the other hand, microglia seem to be critical in the host defence against prion disease, as PLX5622 treatment is associated with faster development of pathology and earlier death in prion mouse models (Carroll et al. 2018). In a graft mouse model for PD, deletion of microglia with PLX5622 prior to graft transfer was found to increase host-to-graft transfer of αS, emphasising the importance of removal of aggregated proteins by microglia (George et al. 2019).

The consistent abundancy of microglial inclusions in transgenic mice (Tanriöver et al. 2020) directed us to look at the role of microglia during αS seeding and spreading in our *ex vivo* synucleinopathy model (Barth et al, *in preparation*). For the study outline, we made use of PLX5622 for microglia depletion in an *ex vivo* seeding model. Pilot experiments were conducted to examine an effect of microglia depletion at different stages of the development of αS pathology, as measured by the amount of pS129 positive neuronal and ThioS positive microglial αS inclusions throughout the cultures. Preliminary results suggest that microglial inclusions indeed come secondarily to neuronal inclusions, and further indicates that microglia influence the progression of neuronal pathology. Further analyses and experiments need to be conducted to confirm these early findings, regarding the role of microglia and their effect on seeding and spreading of neuronal inclusions.

4 MODEL SYSTEMS FOR SYNUCLEINOPATHIES

Studying human diseases is often easier by focusing on smaller aspects of the disease and investigate the underlying mechanisms. Depending on the scientific question, various model systems can be employed, which can be categorised broadly into *in vitro*, *in vivo*, *ex vivo*, or *in silico* models. *In vitro* models are often based on cell cultures and very targeted to a specific biochemical or cell biological question. *In vivo* systems, on the other hand, exploit the complex organisms of experimental animals to advance in finding a treatment possibility for the human disease. An alternative to these approaches is the use of *ex vivo* models, which involve the culturing of organotypic tissue outside the living organism, and combine advantages of *in vitro* and *in vivo* studies. This makes them useful for scientific questions that require more complexity than *in vitro* models but also more accessibility than *in vivo* models. The fourth type, *in silico* models, is inarguably the most modern one, and employs mere simulations of biological processes and interaction networks in a virtual environment in order to infer or predict the outcome of a manipulation from available biological data by the use of computational power.

Here, I will focus on the advantages of the classical biological models, which are conducted *in vitro*, *in vivo*, or *ex vivo*.

4.1 *IN VITRO* MODELS

In vitro models are simplified systems which do not fully reproduce the complexity of diseases, but can be highly useful in understanding pathophysiological mechanisms that underlie neurodegeneration, or for high-throughput screening of new therapeutic compounds. Over the years, several cellular models that mimic

important aspects of αS biology have been developed and contributed to our comprehension of synucleinopathy pathogenesis (Delenclos et al. 2019).

4.1.1 CELL CULTURES

Cell cultures allow for the study of large quantities of homogenous cells in an isolated and tightly controlled environment. Depending on the scientific question, different cell models can be utilised. For example, yeast cells (*Saccharomyces cerevisae*), although in some ways very different from multi-cellular organisms, let alone mammals, have been proven to be extremely useful in deciphering highly conserved cellular mechanisms like protein folding or degradation, or the effect of PTMs on protein structure and interaction (Botstein et al. 1997; Franssens et al. 2010; Delenclos et al. 2019). Immortalised human cell lines, such as H4, HEK-293, or SH-SY5Y, are widely used because they are easy to manipulate to express fluorescent tags or biosensors, which enables live imaging of αS oligomerization (Klucken et al. 2006; Holmes and Diamond 2017). For neuron-specific questions, primary neuronal cultures are often used, which are prepared from embryonic or early post-natal mouse or rat pups. Since these cultures simulate a neuronal environment, they can provide physiologically relevant results (Delenclos et al. 2019). Primary neurons are particularly useful when grown in microfluidic chambers that can separate neuronal soma from axonal endings, which enables the direct study of axonal transport of αS for cell-to-cell spreading (Freundt et al. 2012; Taylor et al. 2005; Brahic et al. 2016).

To achieve a more complex environment, co-cultures of neurons, astrocytes, and/or microglia can be used. This is also interesting in a multifluidic platform system, which provides a device to study interaction of the different cell types in response to a stimulus (Park et al. 2018). Moreover, microfluidic systems allow for completely controlling the microenvironment, which makes them an excellent tool to study cell-cell communication by soluble factors (Fernandes et al. 2016b).

4.1.2 IPSCS AND ORGANOIDS

New possibilities to develop physiologically relevant disease models in a culture dish were created by the use of patient-derived induced pluripotent stem cells (iPSCs), which allow for studying patient-specific risk factors or disease-specific mutations, and thus provide the opportunity to test for individualised therapeutic approaches (Kouroupi et al. 2017). Many studies were done on the basis of iPSC-derived neurons from patients with Mendelian forms of PD, e.g. SNCA triplication (Piper et al. 2018). The derivation of midbrain dopaminergic neurons from patients with SNCA triplication was a breakthrough in the field (Byers et al. 2011; Devine et al. 2011). These neurons exhibit PD-related phenotypes like the upregulation of genes related to oxidative stress and protein aggregation, lysosomal dysfunction, and increased ROS production, as well as decreased neurite outgrowth and lower neuronal activity (Mazzulli et al. 2016; Deas et al. 2016; Oliveira et al. 2015). Moreover, microglia derived from iPSCs with SNCA triplication, but not with mutant A53T αS, showed impaired phagocytosis (Haenseler et al. 2017). Genome engineering of iPSCs, e.g. with Clustered Regularly Interspaced Short Palindromic Repeats interference (CRISPR), can generate isogenic cell lines with corrected mutations or with introduced genetic variants to understanding their functional consequences (Hockemeyer and Jaenisch 2016).

However, iPSC-derived neurons usually grow in monolayers, which do not represent their architecture *in vivo*. Moreover, their epigenetic profile naturally does not resemble that of mature neurons, which hinders αS accumulation and aggregation, unless the cells are maintained in culture for years (Delenclos et al. 2019).

Thus, the development of cerebral organoids, or 'mini-brains', grown from human iPSCs, constituted a milestone for 3D brain-like human model systems (Lancaster and Knoblich 2014). Contrary to 2D neuron cultures, organoids contain more mature neurons, and can even resemble particular brain areas with specific cell populations, for example cerebellum (Muguruma et al. 2015), forebrain (Qian et al. 2016), or midbrain containing dopaminergic neurons (Paik et al. 2018; Jo et al. 2016). This makes brain organoids a highly suitable method to model proteopathies (Gonzalez et al. 2018; Smits et al. 2019).

4.2 *IN VIVO* MODELS

In order to study the progression of synucleinopathies in a more complex organism, numerous animal models have been developed. While rodents, particularly mice, remain the most frequently used animals, some non-mammalian animals are also widely utilized to study simpler pathologic mechanisms. Since none of these animals naturally develop synucleinopathies, they are being manipulated by transgenic (over-) expression of wildtype or mutant αS, transduction of SNCA-carrying viral vectors, or injection of αS seeds for pathology induction.

4.2.1 TRANSGENIC NON-MAMMALIAN MODELS

Caenorhabditis elegans (nematode), *Drosophila melanogaster* (fruit fly), and *Danio rerio* (zebrafish) are relatively simple non-mammalian organisms that can serve as synucleinopathy models. Each of these models provides certain advantages and disadvantages (Visanji et al. 2016). *C. elegans*, for example, has exactly 302 neurons, with 6 neurons in the nerve ring, its "brain". Since its body is transparent, it is the ideal transgenic model for fluorescent visualisation of cellular processes, e.g. inclusion formation of fluorescently labelled αS aggregates (Van Ham et al. 2008). In drosophila, on the other hand, the GAL4/UAS system is widely used for targeted transgene expression, which makes this model particularly useful for genetic screens (Butler et al. 2012). Zebrafish are relatively new synucleinopathy models, but due to their transparent bodies, they are very convenient for continuous *in vivo* imaging, such as monitoring the degenerative and neurotoxic effect of transgenic neuronal αS expression (O'Donnell et al. 2014). However, complex human diseases can hardly be fully modelled in these simplistic models. Therefore, non-mammalian systems often bridge the gap between *in vitro* and mammalian model studies (Visanji et al. 2016).

4.2.2 TRANSGENIC MOUSE MODELS

Rodents, such as mice, are considerably more complex organisms than non-mammalian animals, and the cellular architecture of human and mouse brain tissues are rather well-conserved (Hodge et al. 2019). Thus, transgenic mouse models, which mimic the toxic gain of function of αS, have advanced the understanding of the cellular pathology in humans.

To reproduce the degeneration of dopaminergic neurons in PD patients, transgenic mice have been generated that overexpress wildtype or mutant human αS under control of the dopaminergic neuron-specific tyrosine hydroxylase (TH) promoter (Matsuoka et al. 2001; Rathke-Hartlieb et al. 2001; Richfield et al. 2002; Wakamatsu et al. 2007; Tofaris et al. 2006). However, in all of these mouse lines, αS pathology remains

restricted to dopaminergic neurons and does not spread to neurons that do not express the transgene (Recasens et al. 2017).

Conversely, transgenic mouse lines that express αS under the control of pan-neuronal promoters, like Prnp or Thy1, develop strong synucleinopathy, which eventually leads to an age-dependent lethal locomotor phenotype. Among these animal models are the models we used in our study (Tanriöver et al. 2020): The Prnp-h[A53T]αS line, in the literature also referred to as 'M83', expresses h[A53T]αS under the control of the PrP promoter, which drives expression in most CNS neurons. These mice remain healthy until the age of 7 months, and slowly, but progressively, develop a dramatic motor phenotype, including trembling and paralysis of the legs, within 16 months of age (Giasson et al. 2002). The Thy1-h[A53T]αS, on the other hand, line expresses human αS with an A53T mutation (h[A53T]αS) under the control of the Thy1 promoter, which drives reliable neuron-specific expression in many CNS neurons. These mice show an early onset and progressive decline of motor performance, leading to severe impairments at 8-9 months of age (van der Putten et al. 2000). Another line, Thy1-h[A30P]αS, expresses human αS with an A30P (h[A30P]αS) mutation under the control of the Thy1 promoter, which develop motor disabilities from the age of 15-16 months on (Fournier et al. 2009; Kahle et al. 2000; Neumann et al. 2002). Finally, the Thy1-mαS line was developed in an attempt to resolve some of the questions regarding the differences in the αS protein sequence between humans and mice and its contribution to the disease process. These mice which overexpress murine αS (mαS) under the control of the Thy1 promoter and start to develop severe motor deficits at around 6-7 months of age (Rieker et al. 2011).

Generally, overexpression of αS appears to be is associated with αS aggregation, which can be accelerated by PD-linked mutations. As a result, affected cells suffer from mitochondrial dysfunction, oxidative stress, and activation of cell death pathways. Therefore, these transgenic models are very useful for testing therapeutic strategies designed to limit αS aggregation (Visanji et al. 2016). However, the widespread transgene expression in all these mouse lines does not permit the distinction between cell-autonomous development of synucleinopathy and local propagation within the brainstem (Recasens et al. 2017).

4.2.3 VIRAL INJECTION MODELS

Another approach to recapitulate key features of PD pathology in rodents can be accomplished by injecting viral vectors, adeno-associated viral (AAVs) or lentiviral, to induce tissue-targeted αS overexpression (Kirik et al. 2002; Klein et al. 2002; Lo Bianco et al. 2002). Interestingly, the administration of human αS AAVs into the vagal system did not exclusively lead to the presence of the protein the afferent and efferent fibres of the vagal system, the medulla oblongata. Instead, human αS was found also in pons, midbrain, and forebrain, sequentially (Ulusoy et al. 2013), which provided evidence of inter-neuronal αS transfer *in vivo*. Moreover, the sequential distribution of αS in these vagal AAV injection models reflects the distribution pattern of human disease development, as opposed to most transgenic models whose pathology remains restricted to brainstem areas (Recasens et al. 2017).

4.2.4 SEEDING MODELS

Progressive synucleinopathy can be also induced by injection of pre-existing αS aggregate seeds, which was first shown by intracerebral inoculation of αS transgenic young mice with brain extracts containing αS aggregates or αS pff (Mougenot et al. 2012; Luk et al. 2012b) (see 2.7.4 Prion-Like Propagation). Importantly, αS pathology has also been demonstrated in wildtype mice following pff injection (Luk et al. 2012a). These

seeding models permit the mapping of αS aggregates spread along neuroanatomical pathways upon seed injection into a defined brain region. Moreover, they are useful for assessing if αS aggregates can traffic from the periphery to the CNS. Notably, some groups prefer injections targeting the OB or the vagal nerve, since the Braak staging of αS pathology emphasizes these two areas as possible starting sites of PD pathogenesis (Rey et al. 2013; 2016a). However, the translational relevance of these models has to be taken with care, since there is no evidence that PD or any other synucleinopathy develops via the introduction of exogenous αS aggregates (Visanji et al. 2016).

4.2.5 GRAFT MODELS

The first strong evidence supporting the hypothesis that αS can act in a prion-like manner in humans came from PD patients who received embryonic neural tissue grafts to replace the lost dopaminergic neurons of the SN: More than a decade after surgery, the young grafts had developed αS pathology (Kordower et al. 2008; Li et al. 2008). Based on these clinical findings, rodent models have been developed that involve grafting of embryonic midbrain neurons into the striatum of a mouse or rat overexpressing human αS, where the presence of human αS in the grafted naïve cells is used as a read-out for αS transfer (Desplats et al. 2009; Kordower et al. 2011; Angot et al. 2012; Hansen et al. 2011). However, the presence of accumulated αS in the grafted cells could be also explained by selective neuronal vulnerability induced by neuroinflammation or oxidative stress (Visanji et al. 2016).

Ultimately, despite the efforts to develop mouse models with synucleinopathy features, none of the transgenic mouse lines fully recapitulates the human behavioural phenotype, neuropathology, and pathophysiology. From a epigenetic point of view, some modules of co-expressed genes in mice were also identified in humans, showing strong between-species conservation of cellular functions (Miller et al. 2010). On the other hand, many sets of genes were differently expressed, in addition to epigenetic histone marks, non-coding transcript levels, and missing sequence homologues (Lin et al. 2014). Even between homologous human and mouse cell types, major differences were apparent (Friedman et al. 2018; Hodge et al. 2019). Efforts to develop human-like models were made by grafting human iPSCs or differentiated ESCs into mouse brains (Hasselmann et al. 2019; Mancuso et al. 2019). Using this method, grafts of human ESC-derived dopaminergic neurons, transplanted into the striatum of a αS-seeding rat model, were shown to develop phosphorylated αS, thus demonstrating interspecies host-to-graft transfer (Hoban et al. 2020), similar to the rodent graft model.

In conclusion, every *in vivo* model offers an opportunity to study a different aspect of the disease. Non-mammalian models are ideal for large-scale screenings, after the validation in *in vitro* models. Transgenic mice provide a model to study the effect of therapeutic strategies directed to limit αS aggregation. Seeding models and viral inoculation models can be applied to wildtype or transgenic lines, and serve as platforms to understand the propagation of pathology and how to halt it. And finally, chimeric human-rodent graft models will provide the next step to translatability.

4.3 EX VIVO MODELS

One great disadvantage of studies solely conducted *in vivo* is the limited accessibility, given by the difficulty to directly look into a living animal – with the exception of *C. elegans* and zebrafish – and keep it alive for

longitudinal studies. Moreover, *in vivo* experiments require high amounts of animal lives and suffering, which is against the 3R principle (reduce, refine, replace) of animal protection policies (Kirk 2018).

Ex vivo model systems, on the other hand, are positioned on the intersection between *in vivo* and *in vitro* models, combining advantages of both systems. *Ex vivo* means that tissue has been taken out of an organism and placed into an external environment under culturing settings, with minimal alteration of natural conditions. Due to the maintenance of the complex histoarchitecture of the tissue, *ex vivo* models are as close to *in situ* as possible ('organotypic') while granting access for extensive manipulation and observation that often cannot be achieved in live animals.

4.3.1 BRAIN SLICE CULTURES

The breakthrough for *ex vivo* brain cultures, also known as organotypic brain slice cultures, was made by Beat Gähwiler's group, by placing brain slices on coverslips in rotating test tube with constant medium flow (Gähwiler 1981). Later, the protocol was modified and optimized by Luc Stoppini and colleagues, who established organotypic brain slices cultures on semipermeable membranes (Stoppini et al. 1991).

Typically, slice cultures are derived from neonatal animals (postnatal day 0 – 7; P0 – P7). This period is ideal since the essentials of the cytoarchitecture are already established in most brain areas, and the brain is larger and easier to dissect (Gähwiler 1997). Since one dissected brain can be chopped to several slice cultures (depending on the size of the brain area), organotypic brain slice cultures are contributing to the principle of 3R, by markedly reducing the number of experimental animals.

Many CNS areas have been successfully cultured. Among others, there are cultures of the cortex (Annis et al. 1994; Caeser et al. 1989; Giesing and Zilliken 1980; Leiman and Seil 1986; Molnár and Blakemore 1991; Plenz and Kitai 1996; Romijn et al. 1988; Toran-Allerand 1991; Yamamoto et al. 1989), cerebellum (Audinat et al. 1990; Chédotal et al. 1996; Gähwiler 1984; Gähwiler and Llano 1989; Mouginot and Gähwiler 1995; Notterpek et al. 1993), brainstem (Eustache and Gueritaud 1995), spinal cord (Braschler et al. 1989; Delfs et al. 1989; Phelps et al. 1996; Streit and Lüscher 1992), retina (Feigenspan et al. 1993; Reh and Radke 1988), and more specific areas like hippocampus (Gähwiler 1984), raphe nuclei (Hochstrasser et al. 2011; Miller Jonakait et al. 1988), thalamus (Bolz et al. 1990), hypothalamus (Belenky et al. 1996; Gähwiler 1984; Gähwiler and Dreifuss 1979; Shinohara et al. 1995; Tominaga et al. 1994; Wray et al. 1993), suprachiasmatic nucleus (Wray et al. 1993), striatum (Hölsi and Hölsi 1986; Liu et al. 1995; Østergaard et al. 1995; 1991; Studer et al. 1994), SN (Holmes et al. 1995; Jaeger et al. 1989; Kida 1986; Østergaard et al. 1990), and *locus coeruleus* (Knopfel et al. 1989). However, despite the maintenance of the synaptic connection within the slice culture, cells lose their target innervation because they have been axotomized. Since axotomy causes neuronal cell death, it is the major disadvantage of the slice culture system. Moreover, loss of afferent connections to neurons within the culture in combination with loss of efferent connections to neurons outside the area will result in re-organisation of intrinsic axons (Humpel 2015a).

4.3.2 HIPPOCAMPAL SLICE CULTURES

The human hippocampus resembles the shape of a seahorse, which inspired its name (genus *Hippocampus*). While in humans, it is buried deep within the medial temporal lobe, rodent hippocampi are relatively large and cashew shaped structures, and lie just beneath the neocortex. A cross-section of the hippocampus (both

human and rodent) exposes the so-called 'tri-synaptic loop' of hippocampal anatomical connectivity: the major cortical input is provided by the entorhinal cortex (neuronal layer II + III) with its strongest projections via the performant path to the dentate gyrus (DG) (synapse 1). The DG projects to the cornu ammonis 3 (CA3) region with the mossy fibre pathway (synapse 2). CA3 projects to the CA1 region via the Schaffer collaterals (synapse 3), and CA1 projects back to the entorhinal cortex (neuronal layer V) completing the loop. In addition to this circuit, CA3 axons sent collaterals onto other CA3 neurons (Knierim 2015) (Figure 8 A).

Interestingly, in hippocampal slice cultures (HSCs), only little synaptic rearrangement has been observed in consequence of afferent entorhinal fibres being cut (Gähwiler 1997). However, many aspects of the development of the hippocampal formation occur during the first weeks after birth, in particular the granule cells of the DG, but also partially pyramidal neurons of the CA regions, which receive most of their synaptic connections during this time (Buchs et al. 1993). This plasticity leads to a couple of changes in cultures compared to *in situ*. First, the density of synapses in the distal parts of dendrites, which would usually receive afferents from extra-hippocampal regions *in situ*, remains markedly lower throughout the cultivation period (Buchs et al. 1993), whereas otherwise, the dendritic spine density of pyramidal cells very closely matches the values found *in situ* (Gähwiler 1997). Second, mossy fibre projections establish supragranular collaterals in reaction to the deafferentation (Zimmer and Gähwiler 1984), but otherwise stay mainly preserved when transverse HSCs are prepared, since they run perpendicular to the longitudinal axis of the hippocampus (Frotscher et al. 1995). Lastly – and curiously – pyramidal neurons of CA1 seem to spread horizontally within the slice, on the contrary to CA3 neurons which remain "piled up" in a compact manner (Buchs et al. 1993). Nevertheless, in general, the neuronal architecture and connectivity remains as *in situ* (Figure 8 B). Moreover, pyramidal cells exhibit several forms of short- and long-term synaptic plasticity in HSCs (Debanne et al. 1995).

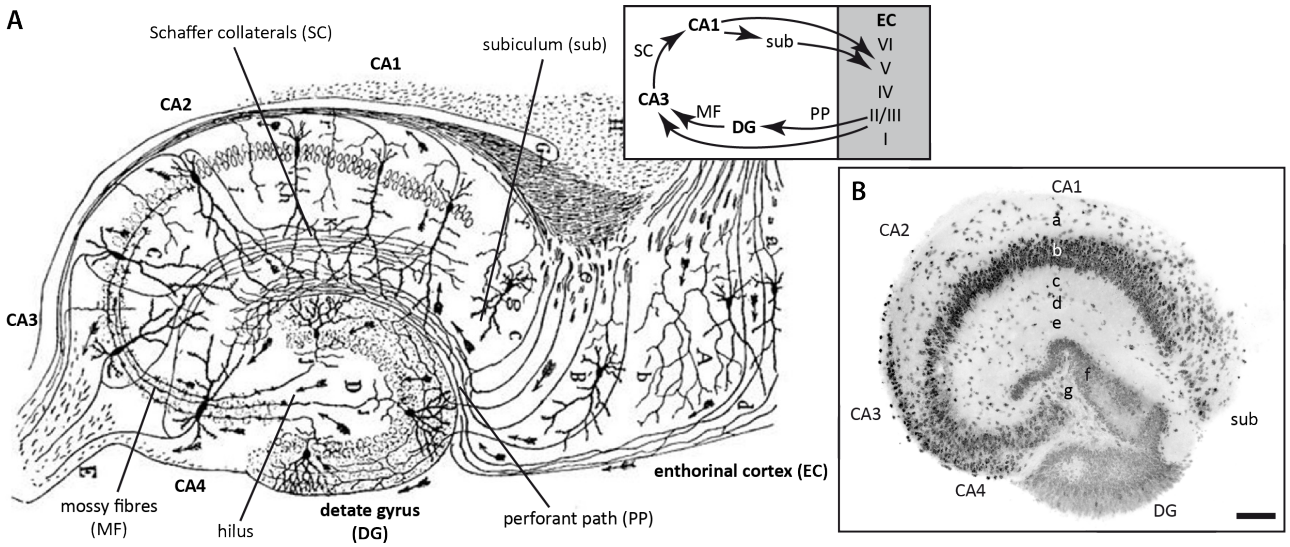

Figure 8: Hippocampal anatomy and connectivity. **(A)** Detailed and schematic sketch of the tri-synaptic loop of hippocampal connectivity. Cortical input is provided by the entorhinal cortex (EC) layer III via the performant path (PP) to the dentate gyrus (DG). DG projects via mossy fibres (MF) to CA3; alternatively, EC layer II can project directly to CA3. CA3 pyramidal neurons extend all along the Schaffer collaterals to CA1. CA1 neurons project either directly or via the subiculum (sub) back to EC layer V. Modified from Ramón y Cajal, 1909, *with permission from Springer Nature*. **(B)** Preservation of hippocampal cyto-architecture and neuronal layers in HSCs. Immunofluorescence staining against neuronal nuclei (NeuN) showing all major hippocampal areas and their strata, namely subiculum (sub), cornu ammonis 1-4 (CA1-4) consisting of a) stratum (str.) oriens, b) pyramidal layer, c) str. radiatum, d) str. lacunosum, and e) str. moleculare; and dentate gyrus (DG), composed of f) granular layer and g) hilus. Scale bar 200 µm.

While all neurons and glial cell types survive, they do so with a couple of changes, to arrange with the *ex vivo* situation. The adjustments of neurons have been described above. Accordingly, oligodendroglia, which are a very neuron-dependent cell type, also display an organotypic distribution and myelinate axons during the first two weeks *ex vivo* as they do *in situ*, with exception of the fibre projections that are missing (Berger and Frotscher 1994). Astroglia, on the other hand, seem to lose their layer-specific distribution, and do not seem to reach full maturation in HSCs (Derouiche et al. 1993). Microglia, conversely, are initially highly activated following explantation and migrate towards the axotomized, dying neurons, but gradually regain a resting state after around nine days *ex vivo* (Hailer et al. 1996; Heppner et al. 1998). On the whole, microglia preserve their morphological features as well as their interactions with other cell types (Skibo et al. 2000).

A few days after being transferred to the membrane inserts, the organotypic sections attach to the membrane, and cells grow out from the edge of the culture. Macroscopically, the slices flatten and become transparent, which is an important criterion for evaluating if the slices are well-cultured (Humpel 2015a). Brain slices can be cultured for many months, but the question remains if cultures derived from neonatal donors can ever represent a mature adult situation. Some groups succeeded in culturing adult hippocampal tissue for around a week (Wilhelmi et al. 2002). However, it is highly demanding and requires rather thin sections (100 – 120 µm instead of 300 – 400 µm), and still, sensitive neurons, such as dopaminergic or cholinergic neurons, did not show prolonged survival even when incubated with growth factors (Humpel 2015b).

4.3.3 HUMAN SLICE CULTURES

The application of organotypic slice culture techniques to human brain tissue, obtained after surgery of epilepsy-patients, could be the bridge across the knowledge gap between learning from experimental setups in immature rodent and adult human brain cultures. So far, only few groups have succeeded in maintaining the cytoarchitecture and electrophysical properties of cortical slices for weeks (Eugène et al. 2014; Schwarz et al. 2019). The key seems to be replacing artificial culture medium with human CSF (Schwarz et al. 2017; Wickham et al. 2020).

4.3.4 EX VIVO MODELS FOR NEURODEGENERATIVE DISEASES

In reference to:

Melanie Barth, Mehtap Bacioglu, Gaye Tanriöver, Niklas Schwarz, Renata Novotny, Janine Brandes, Marc Welzer, Sonia Mazzitelli, Lisa M. Häsler, Manuel Schweighauser, Thomas V. Wuttke, Deborah Kronenberg-Versteeg, Karina Fog, Malene Ambjørn, Luc Bousset, Ronald Melki, Philipp J. Kahle, Derya R. Shimshek, Henner Koch, Mathias Jucker. ***Induced α-synucleinopathy in long-term murine slice cultures and its translation to adult human resection-derived brain slice cultures.*** Submitted.

Transgenic murine HSCs have been used to study mechanisms of prion diseases (Falsig and Aguzzi 2008) tauopathies (Duff et al. 2002; Mewes et al. 2012), and Aβ aggregation (Novotny et al. 2016). Very recently,

there have also been efforts to establish a viral-transduction synucleinopathies model in wildtype HSCs (Croft et al. 2019).

Our study aimed at the profound establishment of a seed-induced synucleinopathy model in HSCs. We could induce pS129 and p-FTAA positive αS inclusions in HSCs derived from Thy1-h[A53T]αS (A53T) and wildtype mice by one-time application of an αS seed, which was either brain homogenate from symptomatic A53T mice or αS pre-formed fibrils (αS pff). Depending on the seed, the induced neuronal inclusions either had a long, smooth fibrillar appearance (αS pff) or consisted of short filamentous structures (A53T brain homogenate). In addition, we also saw microglial inclusions that were both p-FTAA and ThioS positive, consistent with the observations we had made in transgenic mouse lines (Tanriöver et al. 2020). Compared to wildtype, seeded A53T cultures developed faster neuronal and microglial inclusions, and the abundancy of the inclusions was dependent on seed concentration. To further develop this model, we injected αS pff into CA3 and were able to see the pathology spread to CA1, which could be prohibited by surgically disconnecting the two regions. Thus, we established a synucleinopathy seeding model that was able to mimic neuronal and microglial inclusion formation in wildtype and A53T brain tissue. Furthermore, it demonstrated a time-dependent inclusion formation, matching the time course of *in vivo* seeding. The extent of pathology induction was reliably dependent on seed concentration. Moreover, the accessibility of HSCs allowed us to demonstrate the propagation between interconnected hippocampal regions, which was completely inhibited when neuronal connections were severed (Barth et al., *submitted*).

In the meantime, other groups have made efforts to develop similar synucleinopathy models: Elfarrash and colleagues described a model for interneuronal spread by injecting αS pff into DG of HSCs (Elfarrash et al. 2019), while Roux and others applied a larger volume of αS pff on top of cerebellar slices as a proof-of-principle study (Roux et al. 2020). All three models, including ours, demonstrate the seeded induction of pS129 positive neuronal inclusions in a time-dependent manner. While Roux and colleagues showed neurotoxic effects upon αS pathology by analysing synaptophysin levels, a synaptic vesicle protein (Roux et al. 2020), we used a novel clinical biomarker, NfL, to analyse the culture medium in a longitudinal manner. Increased levels of NfL in CSF and blood have been observed in αS transgenic mice and PD patients (Parnetti et al. 2019; Bacioglu et al. 2016), and also in our induced synucleinopathy HSC model, showing that αS pathology and the associated neurodegeneration can be monitored directly by NfL levels in the culture medium.

Moreover, we were able to block seeding and spreading of the pathology by using an antibody, which recognises the αS CTD of oligomeric and fibrillar species. As a secondary outcome, the antibody treatment also prevented the release of NfL into the culture medium, which provides further evidence for the effectiveness of immunotherapy against αS aggregates. Our experiments thus demonstrate the utility of this model system as a tool to study the effects of disease-modifying drugs.

Finally, we addressed the possibility of inducing synucleinopathies in human slice cultures, the same way we achieved in murine HSCs. In a collaboration with the authors of previously mentioned resected human slice culture techniques (Schwarz et al. 2017; 2019), we injected αS pff into human slice cultures, and co-expressed hA53T-αS in the otherwise “wildtype” tissue, using an adeno-associated virus (AAV). The combinatorial treatment enabled the seeded induction of pS129- and p-FTAA positive neuronal αS inclusions in adult human brain tissue after only 7 days. This observation, confirming findings from neonatal murine cultures in an aged human brain environment, highlights the translational relevance of murine *ex vivo* models.

5 DISCUSSION

It was only two decades ago, when Spillantini and others discovered that Lewy bodies (LBs) and Lewy neurites (LNs), the key histopathological hallmark of PD and DLB, consisted of accumulated αS (Spillantini et al. 1998). Starting around the same time, several missense mutations in SNCA, the gene encoding αS, were identified (Zarranz et al. 2004; Appel-Cresswell et al. 2013; Proukakis et al. 2013; Kiely et al. 2013; Lesage et al. 2013; Martikainen et al. 2015; Pasanen et al. 2014; Yoshino et al. 2017; Krüger et al. 1998; Polymeropoulos et al. 1997). Ever since, evidence has been accumulating to support the importance of αS for the pathogenesis underlying PD, MSA and DLB, collectively, which were hence called synucleinopathies (Goedert et al. 2017).

Braak and colleagues were the first to describe the staging pattern of Lewy pathology in PD patients between anatomically connected brain areas (Braak et al. 2003b). While the authors suspected an unknown invading pathogen to be the cause for the spreading (Braak et al. 2003a), two groups independently noticed in grafted embryonic tissue, more than 10 years after being transplanted into PD patient brains, the spreading and aggregation of αS (Kordower et al. 2008; Li et al. 2008). Soon after, αS was also found to spread *in vitro* between connected neurons (Danzer et al. 2009; Desplats et al. 2009). This finding was reproduced *in vivo* by intracranially injecting αS seeds in αS transgenic and wildtype mice but not αS knockdown mice (Luk et al. 2012b; Mougenot et al. 2012; Masuda-Suzukake et al. 2013; Paumier et al. 2015; Luk et al. 2012a; Masuda-Suzukake et al. 2014; Recasens et al. 2014). Interneuronal transport of αS could be further demonstrated directly in cell culture *in vitro* systems, e.g. using microfluidic chambers (Aulić et al. 2014; Freundt et al. 2012; Brahic et al. 2016; Bieri et al. 2018). However, while *in vivo* studies do not provide easy access for manipulation and investigation of cellular events, *in vitro* models do not reflect the complexity of brain tissue composition, since they often consist of only one cell type.

First study:
Characterisation of an *ex vivo* model for synucleinopathies (Barth et al., *submitted*)

The aim of this thesis was to develop and characterise an *ex vivo* synucleinopathy model in slice cultures. This method bridges advantages of *in vivo* and *in vitro* models by maintaining the complex histoarchitecture of the cultured tissue and allowing for direct access of the organotypic brain slice. Murine hippocampal slice cultures (HSCs) are the most stable brain slice cultures since they contain a nearly-closed loop of neuronal connectivity. This has two advantages for the establishment of our model: First, since only afferents of the entorhinal cortex will be axotomized, there is very limited dissection-induced neurodegeneration and only little synaptic rearrangement (Gähwiler 1997; Frotscher et al. 1995). Second, the well-studied hippocampal tri-synaptic loop (Knierim 2015) facilitates the observation of αS lesions spreading from an injected region to a connected area.

In this work, we succeeded in reproducing the hallmarks of synucleinopathies, such as the formation of LB-like neuronal αS inclusions, and templated amplification and spreading of misfolded αS between interconnected regions. The direct access to cultures and conditioned medium allowed us to measure the release of NfL longitudinally, which was increased upon abundant αS inclusion formation, indicating neurodegeneration (Bacioglu et al. 2016; Preische et al. 2019). The application of an antibody directed against oligomeric and

fibrillar αS prohibited seeding, neurodegeneration, and spreading throughout the culture., providing further evidence for the efficacy of immunotherapies for synucleinopathies.

One limitation of using murine slice cultures is the fact that they are derived from neonatal animals, and thus lack the aspect of ageing, an important contributor to neurodegenerative diseases. In an effort of translation to the human disease, we made use of the human brain slices, which had recently been established (Schwarz et al. 2017; 2019). Excitingly, we were able to reproduce the induction of αS inclusions by the application of an αS seed and viral overexpression of mutant A53T αS in aged human brain tissue. We believe, the here achieved translation from neonatal murine to adult human slice cultures is, thus, a major step towards a true human synucleinopathy model. Today, it is the closest *ex vivo* model of human α-synucleinopathies.

Second study:
Discovery of abundant αS inclusions in microglia (Tanriöver et al., 2020)

One of the most salient observations in this study was the plenitude of ThioS-positive microglial inclusions in high seed-concentration treated murine HSCs. Interestingly, in the subsequent *in vivo* study (Tanriöver et al. 2020), we discovered the same phenotype across different transgenic mouse models, which ruled out a culture artefact. These inclusions were not only positive for the amyloid dye ThioS but also for p-FTAA, which allows for differentiation of distinct amyloid-conformations (Taylor et al. 2018; Kuan et al. 2019; Morgan et al. 2020; Åslund et al. 2009). In this way, we were able to clearly distinguish neuronal from microglial inclusions, which showed characteristic fluorescence spectra. This implies the existence of at least structurally different aggregates in these two cell types. By using domain-specific αS antibodies, we identified the inclusions to contain C-terminally truncated αS species. Finally, microglia filled with inclusions were also observed in pre-symptomatic transgenic mice and seeded transgenic mice, which may suggest a role of microglial αS in the pathology process, instead of a mere bystander and end-stage symptom.

From these unexpected findings, several questions arise: How do microglia obtain these aggregates? Are they acquired from neurons or generated inside the microglia themselves? Furthermore, (how) are these inclusions relevant to the pathogenesis of synucleinopathies? Under homeostatic conditions, microglia express αS only at low levels (Austin et al. 2006). However, it is possible that microglia upregulate αS expression upon activation, thereby contributing to the formation of αS aggregates. On the other hand, microglial inclusions might result from uptake of neuron-released αS or phagocytosis of neuronal components (Choi et al. 2020; Thakur et al. 2017). Possibly, the originally neuronal aggregates are C-terminally truncated in their new cellular environment, leading to structural rearrangement and the here observed changes in p-FTAA emission spectra. Alternatively, microglia could take up neuronally-secreted oligomeric αS species from exosomes (Xia et al. 2019), which only in their new host cell assemble to filamentous αS aggregates. As it is well known that different cellular environments influence the composition and conformation of proteopathic seeds (Jucker and Walker 2018), these aggregates might differ in seeding potency and compactness from their neuronal counterparts, as seen in oligodendroglial αS aggregates (Peng et al. 2018).

Determining the relevance of microglial αS inclusions to pathogenesis, may be challenging. Neuroinflammation is a typical pathological trait of synucleinopathies, and microglia are key players (Halliday and Stevens 2011). Oligomeric αS species have been shown to activate microglia, which can either lead to inflammation (via TLR-2) (Kim et al. 2013; 2020), or to phagocytosis (via TLR-4) (Stefanova et al. 2011; Fellner et al. 2013) with subsequent removal of the pathogen by autophagy (Choi et al. 2020). Interestingly, exposure

to monomeric αS seems to increase phagocytosis, while aggregated species decreases it (Park et al. 2008; Choi et al. 2015).

Outlook / third study:
What is the contribution of microglial to synucleinopathies? (Barth et al., *in preparation*)

In an attempt to shine light on the influence of microglial αS on pathogenesis, we started a pilot study to examine the importance of microglia during αS seeding and spreading in our *ex vivo* synucleinopathy model. The causality between microglia, neuronal inclusions, and microglial inclusions was addressed by temporarily depleting microglia in A53T transgenic HSCs at different time points before or after adding an αS seed. Preliminary results support the notion that microglial inclusions develop secondarily to neuronal inclusions. This suggests that microglial inclusions might be dependent on the uptake of neuronal αS. Further results imply that microglia might influence the progression or spreading of neuronal αS pathology, once set in. Possibly, microglia contribute directly to αS transmission via exosomal αS release, which are taken up by neurons (Xia et al. 2019; Guo et al. 2020). On the other hand, however, with the current experimental setup we cannot rule out microglial influence on neurons via the extracellular environment, by changing it to a more neuroinflammatory one, which affects neuron-to-neuron αS transfer (George et al. 2019). Further experiments need to be conducted to assess the microglial influence with regard to their neuroinflammatory effects.

The role of microglia in synucleinopathies is not well-examined, and the studies described here contribute to a still young field of research. However, the work included in this dissertation leads to the straightforward conclusion that *ex vivo* findings have a high chance to be reproduced in their *in vivo* counterparts – shown by the induction of neuronal αS inclusions first in murine and later in human slice cultures, as well as the discovery of abundant ThioS positive microglial αS inclusions *ex vivo* and subsequently *in vivo*. Optimistically speaking, this method will pave the way for translatable discoveries in the field of synucleinopathy research, providing new tools for developing novel treatments for these diseases.

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III. PUBLICATIONS

1 DESCRIPTION OF PERSONAL CONTRIBUTION

I. Induced α-synucleinopathy in long-term murine slice cultures and its translation to adult human resection-derived brain slice cultures

Melanie Barth, Mehtap Bacioglu, Gaye Tanriöver, Niklas Schwarz, Renata Novotny, Janine Brandes, Marc Welzer, Sonia Mazzitelli, Lisa M. Häsler, Manuel Schweighauser, Thomas V. Wuttke, Deborah Kronenberg-Versteeg, Karina Fog, Malene Ambjørn, Luc Bousset, Ronald Melki, Philipp J. Kahle, Derya R. Shimshek, Henner Koch, Mathias Jucker.

submitted

Personal contribution: Experimental design and planning of the study (together with M. Bacioglu, GT and RN); maintenance and preparation of hippocampal slice cultures (HSCs) (together with GT, with assistance from RN, JB, MW, and SM); performance of seeding experiments (together with GT) and collection of HSCs for analysis; histological staining of HSCs; (confocal) microscopic data acquisition (pS129, ThioS, iba1, p-FTAA); histological characterization of α-synuclein inclusions in HSCs (Fig 1, S1, S4); design and performance of the biochemical work (together with M. Bacioglu; Fig 1D); histological quantification of α-synuclein inclusions (Fig. 1E, Fig. 2, S4); collection of conditioned HSC medium and analysis of NfL Simoa raw data (Fig. 3, 5C); injection (together with GT) and lesioning of HSCs, and analysis of α-synuclein spreading (Fig. 4); performance of co-treatment experiments with antibody and α-synuclein seed, histological analysis and quantification of inclusions (Fig. 5); quantification of HSC sections (S4 D-F); statistical analysis (with help from GT and MJ); drawings, figure design and preparation; writing manuscript (together with M. Bacioglu, GT, RN, and MJ).

Others: NS, TVW and HK prepared human slice cultures; GT designed and performed treatment of human slice cultures; M. Bacioglu and MS designed and performed *in vivo* seeding experiments; LMH performed immunoassays for alpha-synuclein and neurofilament light; KF and MA contributed antibodies and provided experimental input and performed CISBIO assay; LB and RM contributed pre-formed fibrils; DRS and PJK provided tg and ko mouse models.

II. Prominent microglial inclusions in transgenic mouse models of α-synucleinopathy that are distinct from neuronal lesions

Gaye Tanriöver*, Mehtap Bacioglu*, Manuel Schweighauser*, Jasmin Mahler, Bettina M. Wegenast-Braun, Angelos Skodras, Ulrike Obermüller, **Melanie Barth**, Deborah Kronenberg-Versteeg, K. Peter R. Nilsson, Derya R. Shimshek, Philipp J. Kahle, Yvonne S. Eisele, Mathias Jucker.

Acta Neuropathologica Communication 8, 133 (2020).

Personal contributions: Performance of histology (together with GT, M. Bacioglu, MS, JM, and UO) and design and writing of the manuscript (together with all authors).

Others: GT, M. Bacioglu, MS, DK-V, and YSE designed and performed mouse experiments; M. Bacioglu and MS designed and performed intracerebral injetions; GT, M. Bacioglu, MS, JM, BMW-B, and AS. analysed data; KPRN provided the amyloid-binding dye pFTAA; DRS provided Thy1-h[A53T]αS and Thy1-mαS mice; PJK provided Thy1-h[A30P]αS mice.

III. Microglia depletion and the differential effect on α-synuclein seeding in long-term murine hippocampal slice cultures

Melanie Barth, Gaye Tanriöver, Marc Welzer, Ania Alik, Ronald Melki, Derya R. Shimshek, Mathias Jucker.

in preparation

Personal contribution: Experimental design and planning of the study (with help from GT); preparation of hippocampal slice cultures; design and performance of seeding and depletion treatments (with help from GT and MW); collection of cultures for analysis and performance of histological stainings; (confocal) microscopy data acquisition (pS129, ThioS, iba1); quantification of time-dependent inclusions in neurons and microglia (Fig. 1, 3); quantification of microglia in depleted HSCs (Fig. 2, 3); descriptive statistical analysis; figure design and preparation; writing manuscript (together with MJ).

Others: AA and RM contributed pre-formed fibrils; DRS provided tg mouse model.

2 INDUCED A-SYNUCLEINOPATHY IN LONG-TERM MURINE SLICE CULTURES AND ITS TRANSLATION TO ADULT HUMAN RESECTION-DERIVED BRAIN SLICE CULTURES

Induced α-synucleinopathy in long-term murine slice cultures and its translation to adult human resection-derived brain slice cultures

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ABSTRACT

Proteopathic lesions in the brain are a hallmark of age-related neurodegenerative diseases, and develop at least a decade before the onset of clinical symptoms. Thus, an understanding of the initiation and propagation of such lesions is key for pre-symptomatic therapeutic endeavours. The development of proteopathic lesions is largely cell-non-autonomous and depends on the brain's microenvironment. To this end, we have developed long-term hippocampal slice cultures from postnatal mouse brains and succeeded in seeded induction of α-synuclein (αS) inclusions through one-time application of αS seed-containing brain extract or synthetic αS fibrils. Induction of αS inclusions in neuronal cell bodies and processes is apparent as early as 7 days post-seeding, and is dependent on the type of αS seed and on the culture's genetic background (wildtype vs human A53T-αS genotype). Formation of αS inclusions and associated neurodegeneration could be monitored by neurofilament light chain protein release into the culture medium, a neurodegenerative fluid biomarker commonly used in preclinical animal studies as well as in clinical settings. Local microinjection of αS seeds resulted in spreading of the αS inclusions to neuronally connected hippocampal sub-regions, which was inhibited by addition of human αS seed-recognizing antibodies to the culture medium. We then applied the knowledge and parameters from the murine cultures to surgical resection-derived adult human long-term brain slice cultures from 22 – 61-year-old donors. Consistently, in these human slice cultures αS inclusions could be induced within 7 days post-seeding in combination with viral A53T-αS expressions. Postnatal murine and adult human *ex vivo* brain slice cultures are easily adaptable to other cerebral proteopathies, are accessible for manipulation and imaging, and allow mechanistic dissection and targeting of lesion development in mouse and even aged human brain environments.

INTRODUCTION

Synucleinopathy is a collective term for neurodegenerative diseases such as Parkinson's disease (PD), dementia with Lewy bodies (DLB) and multiple system atrophy (MSA), which are all characterised by the abnormal accumulation of filamentous α-synuclein (αS). Initial evidence for a cell-non-autonomous mechanism underlying synucleinopathies came from the discovery of Lewy pathology in graft embryonic tissue in *post mortem* PD brains (Kordower et al. 2008; Li et al. 2008). As a result, prion-like transmission of αS lesions was proposed and the concept of seeded aggregation – a nucleation-dependent process similar to the one described for prions and Aβ – was suggested (Olanow and Prusiner 2009; Jucker and Walker 2013). This concept gained further support by *in vitro* findings showing that the exogenous application of αS fibrils induced Lewy body-like pathology in cultured neurons (Danzer et al. 2009; Desplats et al. 2009; Volpicelli-Daley et al. 2011; Fernandes et al. 2016b). Similarly, several groups reported induction and spreading of αS lesions *in vivo* by performing inoculation studies in mice using recombinant αS preformed fibrils (αS pff) or brain homogenates containing aggregated αS (Sacino et al. 2014; Schweighauser et al. 2015; Masuda-Suzukake et al. 2013; Challis et al. 2020; Kim et al. 2019; Luk et al. 2012a; 2012b).

The induction and propagation of proteopathic lesions is largely dependent on the interaction between the seed and the host environment (Jucker and Walker 2018). Spreading of αS lesions occurs along neural pathways, and cell-to-cell transfer is cell-type dependent and promoted by neural activity (Masuda-Suzukake et al. 2013; Rey et al. 2016b; Peng et al. 2018; Yamada and Iwatsubo 2018). Thus, seed propagation is best studied in a living environment that closely mimics the adult or aged brain. While mice have been instrumental in the past to study such prion-like propagation of pathogenic seeds (Jucker and Walker 2018), mouse models are time-consuming and costly, and experimental manipulations to mechanistically understand propagation of seeds are challenging *in vivo*.

Studying propagation of pathogenic seeds in humans is even more intricate. Human stem-cell-derived organoids and 3D-culture systems have been developed (Koh et al. 2018; Park et al. 2018; Gonzalez et al. 2018) to mimic the human brain environment but they remain controversial regarding their purity, maturity, and cell subtype-identity. Grafting of human stem-cell derived tissue into adult mice is another exciting new development (Mancuso et al. 2019; Hasselmann et al. 2019), however the influence of the murine host on the human transplant needs to be addressed further.

To this end we developed long-term slice cultures from postnatal mouse brain that allow the seeded induction and propagation of murine and human αS inclusions in a complex cellular brain environment. We found spreading of αS lesions along neural pathways, which could be blocked by a human αS seed-recognizing antibody. To foster translation, progression of α-synucleinopathy and associated neurodegeneration in these cultures was monitored by assessing neurofilament light chain (NfL) protein levels in the culture medium, a biomarker used in preclinical animal studies as well as in clinical settings. Finally, we applied the knowledge and parameters from the murine cultures to resection-derived adult human brain tissue cultures and succeeded in inducing αS inclusions in a true adult human brain environment.

RESULTS

Induction of αS inclusion in murine hippocampal slice cultures

Murine hippocampal slice cultures (HSC) were prepared from new-born mice and grown in culture for 10 days to stabilize. αS inclusions were induced by one-time application of αS seed-rich aged Thy1-hA53TαS transgenic (tg) mouse brain homogenate or alternatively by synthetic αS pff to HSC prepared from either tg or wt mice (tg HSC; wt HSC) (Fig. 1A). Five weeks later, all cultures had developed abundant perikaryal and neuritic αS lesions positive with an antibody specific for phosphorylation at serine 129 (pS129), a surrogate marker of αS assembly *in vivo* (Fujiwara et al. 2002). Untreated control cultures and cultures treated with brain homogenate from wt mice did not develop any αS inclusions (Fig 1B). While the tg brain homogenate-induced inclusions that presented as short filamentous and distinct cytoplasmic and neuritic inclusions, αS pff treatment resulted in longer, smooth-looking often uninterrupted fibrillar structures that filled the whole neuronal soma and extended along neurites (Fig 1B, inserts).

The neuronal αS inclusions stained positive for the amyloid-binding dye pFTAA, but much less so for Thioflavin S (ThioS). Nevertheless, ThioS-positive inclusions were numerous but the vast majority was localized to microglia (Fig. 1C, D), consistent with recent *in vivo* observations in αS tg mice (Henrich et al. 2018; Tanriöver et al. 2020). The microglial inclusions appeared wool-like and mostly in vicinity to the nucleus, and their overall appearance was different from the neuronal inclusions. Analysis of the sarkosyl-insoluble HSC fractions of both αS pff and tg brain homogenate-treated cultures confirmed the presence of aggregated αS (Fig. 1D).

αS inclusions are dependent on genotype, time, and seed

The induction of αS lesions was faster and more abundant in tg HSC compared to wt HSC (Fig. 1E) and was absent in $Snca^{-/-}$ HSC (Supplementary Fig. 1). In both tg and wt HSC, there was an increase in the number of αS lesions between 1 week and 5 weeks after seed application (Fig. 1E; note the exception of αS pff in tg HSC as discussed below). No induced αS lesions were observed 1 hour after seed application (Fig. 1E). Using the same brain homogenate as seed, we observed that the emergence of αS lesions in tg HSC matched the time course of *in vivo* seeding in adult mice of the same genotype (Thy1-hA53TαS tg mice) pointing to a common fundamental mechanism of αS induction in HSC and in mice *in vivo* (Supplementary Fig. 2).

Titration of αS pff revealed a striking increase of pS129- (largely neuronal) and ThioS-positive (largely microglial) αS lesions with increasing amount of seeds (Fig. 2). Tg brain homogenate appeared 100 x less potent (Fig. 2) but also contained 1 000 x fewer monomeric αS equivalents (typically 5 µg/ml; measured by immunoassays after formic acid treatment) compared to undiluted αS pff (5 mg/ml). This observation suggests that tg brain-derived αS seeds are in fact more seeding potent than their synthetic counterparts, which is also in line with direct measurements of αS aggregates (Supplementary Fig. 3). Importantly, the difference in morphological appearance between inclusions induced by αS pff and tg brain homogenate (see above) remained independent of the seed dilutions.

Lesion-related neurodegeneration can be monitored by NfL release in medium

To further investigate on the unexpected finding that αS pff-treated tg HSC showed more inclusions after one week compared to five weeks post seeding (Fig. 1E) a detailed time-course of αS pff-induced αS lesions in tg HSC was established (Supplementary Fig. 4). Results revealed an increase of mainly neuronal pS129-positive inclusions up to 2-3 weeks post-seed application followed by a decline up to week 5. The emergence of ThioS-positive (largely microglial) inclusions occurred after an initial delay at 2-3 weeks concomitant with thinning of the cultures and an overall unhealthy appearance. However, such a degenerative phenotype of the cultures was only observed for the highest concentration (350 µM) of αS pff and was restricted to the tg HSCs (Supplementary Fig. 4 F).

In light of this degenerative HSC phenotype, a 1:10 αS pff (35 µM) dilution was used in all following experiments and the analysis was performed for the first 3 weeks post-seeding. To assess the extent of subtler neurodegeneration the release of neurofilament light (NfL) into the culture medium was measured. Increased levels of NfL have previously been found in CSF and blood of Thy1-hA53TαS tg mice and has been linked to the induction and spreading of αS lesions (Bacioglu et al. 2016). Indeed, an increase in NfL upon induction of αS pathology was found in tg HSCs (Fig. 3). NfL increase was not imminent, but progressed and peaked at week 2 to 3 (Fig. 3B).

Seed-induced synucleinopathy propagates to interconnected regions

To establish the suitability of HSC as a model system to study the propagation of αS aggregates, ATTO-550-labelled αS pff were injected into the CA3 region, in order to see if locally induced αS inclusions spread to the distant CA1 via Schaffer collaterals, and/or retrogradely via mossy fibres to the dentate gyrus (DG) (Fig. 4). Indeed, upon local seed injection, the formation of filamentous pS129-positive inclusions in neurites around the CA3 injection site could be observed already after 1 day (Fig. 4B). It is important to note that αS pff are not phosphorylated upon uptake (Gribaudo et al. 2019; Pieri et al. 2016) and the pS129-positive signal can be attributed to aggregated endogenous αS. Four days post-injection, somatic αS inclusions appeared in the DG. At 7 days post injection, both DG and CA1 neurons exhibited somatic αS inclusions with neuritic inclusions along the Schaffer collaterals and mossy fibres, suggesting both anterograde and retrograde spreading (Brahic et al. 2016). The occurrence of lesions in CA1 neurons indicated spreading to neighbouring neurons (Fig. 4C, D).

To rule out the possibility of diffusion of the applied seeds from CA3 to these regions, Schaffer collaterals and mossy fibres were surgically disconnected (Fig. 4E). As a result, hardly any spreading across the incision site was apparent (Fig. 4F). Presuming that a simple mechanic cut would not impair diffusion of seeds, these results indicate that the observed formation of αS inclusions in distal regions spreading must occur via neural connection.

Spreading of αS inclusions can be blocked by αS antibody

The efficiency of antibodies to block αS aggregates from seeding was tested by mixing antibodies with αS pff and applying the mix onto tg HSC. Analysis was performed 3 weeks later (Fig. 5A). In particular one human antibody (HLu-3) prevented the formation of the majority of αS-inclusions (Fig. 5A, B). Also, ThioS-positive

inclusions were largely prevented (αS pff / control antibody: 0.344 ± 0.120 % ThioS area of HSC area; αS pff / αS antibody: 0.003 ± 0.003 % ThioS area of HSC area; mean ± SD, n = 6) highlighting a link between neuronal and microglial αS inclusions. Consistently, NfL levels in the medium of cultures treated with αS antibody were lower than NfL levels in the control cultures (Fig. 5C).

To study whether the antibody was also able to block αS aggregation when present in the culture medium, HLu-3 was added to the culture medium 7 days before injection of αS pff into CA3. Again, there was robust blocking of αS aggregation one week after seed application (Fig. 5D, E).

Next, the ability of the αS antibody HLu-3 to prevent the spreading of pathology from the initial injection site throughout the culture was tested. To this end, αS pff seeds were injected into CA3 and the culture medium was continuously supplemented with αS antibody once the seeds were applied (Fig. 5F). Following this treatment scheme, there was no apparent difference between αS antibody and control antibody treated cultures after one week, indicating an insufficient blocking of the initial seeds (Fig. 5G; such insufficient blocking is in contrast to the more complete blocking when the antibody is already present at the time of seeding, see Fig. 5E). Most interestingly, beginning at 3 weeks and more robustly at 5 weeks post-injection, there was a decrease of αS inclusions in the entire cultures treated with HLu-3 antibody (Fig. 5H), including areas CA1 and DG, suggesting an inhibiting effect of the αS antibody on the spreading of pathology.

Induction of αS inclusion in resected human brain slice cultures

Having established the conditions needed to induce αS lesions in murine HSC, we next addressed the possibility of adapting these findings to cultures derived from human adult tissue. To this end, we have built upon a recent milestone finding in the development of human slice cultures (Schwarz et al. 2019). Cultures are derived from resected adult brain tissue and are stable *in vitro* for up to 21 days when cultured in human CSF (Schwarz et al. 2019; Wickham et al. 2020) (Fig. 6A-C). As before for the murine cultures, αS pff were applied on top of each culture in the same concentration as previously used for the mouse cultures (1 µl, 35 µM). In contrast to the murine cultures, however, αS pff were already applied after 3 days in culture (compared to 10 days for the murine culture) because of the overall shorter culturing time-span for human cultures. To mimic the high levels of endogenous mutated αS in the murine tg cultures, hA53T-αS was expressed at day 1 in culture using an adeno-associated virus (AAV-hA53T-αS) (Fig. 6).

Consistent with the murine cultures, one week after seed application pS129-positive inclusions were apparent in AAV-hA53T-αS-treated human cultures (Fig. 6D, E). In contrast, lesions were absent in cultures treated with either αS pff or AAV-hA53T-αS alone, or in untreated controls (Fig. 6F). The pS129-positive inclusions were neuronal and also positive for pFTAA. No pFTAA-positive microglial inclusions were observed. Similar to observations in murine cultures, the induced αS inclusions in these human cultures appeared as smooth, fibrillar structures in the cytoplasm with pathology also extending into the processes (Fig. 6D, E).

DISCUSSION

Cerebral proteopathies such as Alzheimer´s disease and Parkinson´s disease are human age-related disorders that do not naturally arise in animals (Walker and Jucker 2017). Proteopathic lesions develop many years, if not decades before the first symptoms occur, and lesions at death are likely to be different from the ones driving the disease. The progression of filamentous pathology and neurodegeneration is at least in part attributed to a non-cell-autonomous process (Peng et al. 2020; Jucker and Walker 2018). Taking these observations into consideration is important when modelling cerebral proteopathies.

Here we have established mouse and human brain slice cultures of α-synucleinopathies. While murine brain slice cultures now allow to study the formation, dynamics, impact and targeting of the inclusions in a brain-like environment, it needs to be acknowledged that these cultures are derived from postnatal brain and therefore lack the aspect of aging. Moreover, recent transcriptome studies have revealed essential differences in disease-relevant genes between mouse and human cell populations (Zhou et al. 2020; Lin et al. 2014; Hodge et al. 2019; Friedman et al. 2018; Miller et al. 2010). Thus, the here described translation of the slice culture model from mouse to human is an important step forward and now allows to confirm findings derived from mouse cultures in a true aged human brain environment.

Murine brain slice cultures

Organotypic murine slice cultures have previously been shown to be beneficial in modelling proteopathies using viral overexpression of disease-related proteins (Croft et al. 2019). The present results expand these endeavours and we show that one-time seeding of brain-derived or synthetic αS seeds is sufficient for the induction and subsequent spreading of αS lesions in hippocampal murine slice cultures. Overall, neuronal inclusions in the cultures (cytoplasmic and neuritic inclusions) and their biochemical properties (phosphorylation of S129 and sarkosyl insolubility) are identical to those observed in αS-transgenic or wildtype mice in which the lesions have been induced by seeded aggregation (Sacino et al. 2014; Masuda-Suzukake et al. 2013; Elfarrash et al. 2019; Luk et al. 2012b; 2012a). In turn, αS lesions in culture and mice resemble Lewy pathology in humans (Goedert et al. 2013).

The dynamic of αS lesion induction, its dependence on the host genotype and properties of the seed are similar in organotypic slice cultures and *in vivo*. The induction of αS lesions in wt cultures was much slower and the resulting lesion burden of pathology was lower than what we observed in the tg cultures. A similar observation was also made in mouse models (Luk et al. 2012b; 2012a), which can be linked to enhanced vulnerability of neuronal populations with high αS expression levels (Courte et al. 2020; Taguchi et al. 2014). Moreover, the morphology of the induced lesions was dependent on the αS seed (in the present study, mouse brain-derived or synthetic αS assemblies), again similar to what has been described for seeded induction in mice (Peelaerts et al. 2015; Luk et al. 2012b; Masuda-Suzukake et al. 2013; Bétemps et al. 2014; Tarutani et al. 2018; Lau et al. 2020). This observation is consistent with prion-like templated propagation (Jucker and Walker 2013) and structural differences between pff and brain-derived human αS seeds (Schweighauser et al. 2020). In both, human and murine cultures as well as *in vivo*, highly concentrated αS pff are more seeding active compared to tg brain material. Hence, when comparing the specific activity of αS pff and tg brain-derived aggregates

(seed activity per αS molecules), brain-derived αS seeds appear to be more potent in seeding aggregation consistent with other proteopathic seeds (Jucker and Walker 2018).

The finding that αS lesions in murine brain cultures are strongly positive for the amyloid-binding dye pFTAA is in line with recent *in vivo* data using pFTAA and other luminescent-conjugated oligothiophenes (Kuan et al. 2019; Shahnawaz et al. 2020). This not only confirms the amyloid nature of the inclusions, but it also opens up the possibility to use these fluorescent dyes for live imaging of αS lesions and for spectral discrimination of αS conformers (Shahnawaz et al. 2020; Klingstedt et al. 2019; Morgan et al. 2020). It should be noted that live-imaging of αS lesions dynamics in mice is challenging (Osterberg et al. 2015) and has never been achieved in combination with spectral analysis.

As recently reported for αS tg mice (Tanriöver et al. 2020), we also found αS-like inclusions in microglia, which are pFTAA- and ThioS-positive (but largely pS129-negative). The appearance of the αS-like inclusions in microglia was always linked to neuronal inclusions in magnitude and location, but microglial inclusions developed with a delay of 2-3 weeks compared to neuronal inclusions. Although final proof of their nature and conformation remains to be established, our observations in the cultures suggest that the microglial inclusions contain αS of neuronal origins (Tanriöver et al. 2020). Intriguingly, targeting αS seeds with HLu-3 αS antibody also blocked the formation of microglial inclusions, highlighting a link between neuronal and microglia inclusions which is in line with recent findings that microglia are involved in transfer of αS lesions in brain (George et al. 2019).

Modelling proteopathic lesions in a brain-like environment bears the advantage that the cell-to-cell transfer of aggregates between interconnected regions occurs in a similar way to that in mice and humans (Peng et al. 2020; Jucker and Walker 2018) and therefore allows for studying the propagation of pathology across neuronal networks. Immunotherapy targeting aggregated αS is a vigorously pursued therapeutic strategy, although its mechanism of action remains to be established. Because of the presumption that antibodies do not readily enter intact neurons, αS antibodies that neutralise αS seeds are thought to capture the seeds extracellularly when transferred from cell to cell (Masliah et al. 2005; Tran et al. 2014; Masliah et al. 2011; Bae et al. 2012; Bergström et al. 2016). The observation that HLu-3 αS antibody was capable of reducing the number of αS-inclusions in our culture model 5 weeks after the induction, but not after 1 week, suggests that this human antibody inhibits the spreading of αS seeds apart from preventing their initial cellular uptake. Although, at present, our results do not provide conclusive explanation on the mechanisms involved, these experiments nevertheless demonstrate the utility of this culture system as an important tool for studying the effects of disease-modifying drugs in a novel pre-clinical model.

Proteopathic lesions develop before clinical symptoms occur and thus early biomarkers that mirror lesion development and associated neurodegeneration are essential for diagnosis and for monitoring disease progression. Our results show that αS lesions and associated neurodegeneration in murine slice cultures can be monitored directly by NfL levels in the culture medium, thus providing an important finding for translational research. An increase of NfL in CSF and blood has also been observed in αS-tg mice as well as in human α-synucleinopathies (Bacioglu et al. 2016; Parnetti et al. 2019). NfL measurements in cultures provide a significant advantage over measurements in animal models (i.e. NfL in CSF). For instance, longitudinal measurements are possible and changes of NfL levels can be directly related to lesions as well as to any therapeutic efforts.

Slice cultures from resected adult human brain

The induction of αS lesions in slice cultures derived from adult human brains is remarkable and will now set the stage to study αS lesion dynamics and cellular responses in a genuine human brain environment. The current findings that αS lesion induction is dependent on the viral expression of mutated αS (here A53T-αS) is not surprising since the induction of lesions in murine culture within one week was also only observed in the tg host line. Robust preservation of the neural cytoarchitecture and electrophysiological properties of resected human brain cultures have been reported up to 21 days *ex vivo* if cultures were grown in human CSF (Schwarz et al. 2017; 2019). Thus, it will be interesting to see whether this time frame will be long enough to induce αS lesion without additional overexpression of A53T-αS. Along the same line, further research will reveal whether microglia inclusions will also develop in the human cultures upon extended cultivation.

The present cultures were derived from three adult individuals (22-61 years old) of both sexes. Although this small sample size does not allow any conclusions about effects of age and gender, these parameters could be addressed by increasing the donor sample size. In particular it will be interesting to study whether αS lesion induction is more prominent in aged donors compared to younger donors.

Although the induction of αS lesions in adult human brain cultures is a major step forward in modelling proteopathic pathology in humans, there are still limitations that need to be considered and overcome before the model can be used in a routine lab environment. The availability of suited resection tissue is limited and long-term cultivation of human cultures appears more difficult compared to the murine cultures. In addition, human CSF is needed to nurture the cultures whose availability is again limited, and NfL in CSF might interfere with the measurements of culture-derived NfL. Thus, at present, the human slice culture model is best used to confirm findings from the mouse cultures in a true aged human brain environment. However, resected human cultures are still in their infancy (Schwarz et al. 2017) and current efforts aim at improving these culture conditions to make it a tool with a wide range of applications.

Conclusions and future perspective

We have established an attractive cellular model system for studying α-synucleinopathy that contains essential elements of *in vivo* tissue complexity and replicates key aspects of disease. We further provided proof-of-principle evidence for their clinical application in screening antibodies that prevent the spread of αS lesions. As was the case in animal models and in humans, NfL measurements can be used as reliable readouts of disease in these slice cultures. Given the limitations of studying human disease in the context of an animal brain, we successfully translated this model from mouse to human and reported the first induction of human αS lesions in a true adult human brain environment. Although our study was focussed on the induction of α-synucleinopathies, these slice culture models can be expanded to other proteopathies. Slice cultures allow for easy access and manipulation of the tissue while keeping the 3Rs (reduce, refine, replace) guiding principles. In particular, live-imaging of the pathology development with fluorescent amyloid dyes combined with single cell transcriptomics offer great potential to study human neurodegenerative diseases in new light.

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METHODS

Mice

Wildtype C57BL/6J, heterozygous Thy1-hA53TαS tg (van der Putten et al. 2000), and Snca$^{-/-}$ (Abeliovich et al. 2000) pups were used for preparation of HSC. Thy1-hA53TαS tg mice overexpresses human mutant (A53T) αS under control of a Thy1-promoter element. Experimental procedures were carried out in accordance with the veterinary office regulations of Baden-Wuerttemberg (Germany) and were approved by the local Animal Care and Use Committees.

Preparation of mouse hippocampal slice cultures (HSC)

HSCs were prepared from pups at postnatal day 4-6 (P4-6) according to previously published protocols (Mayer et al. 2005; Novotny et al. 2016). After decapitation, brains of the pups were aseptically removed, the hippocampi were dissected and cut perpendicular to the longitudinal axis into 350 µm sections with a tissue chopper. Carefully selected intact hippocampal sections were transferred into petri dishes containing an ice-cold buffer solution (minimum essential medium (MEM) supplemented with 2 mM GlutaMAX^{TM} at pH 7.3). Three sections were placed onto a humidified porous polyethylene (PTFE) membrane insert (Merck Millipore, PICM0RG50) and into a 6-well plate with 1.2 ml culture medium (20 % heat-inactivated horse serum in 1x MEM complemented with GlutaMax^{TM} (1 mM), ascorbic acid (0.00125 %), insulin (1 µg/ml), $CaCl_2$ (1 mM), $MgSO_4$ (2 mM) and D-glucose (13 mM) adjusted to pH 7.3) per well. HSCs were kept at 37°C in humidified CO_2-enriched atmosphere. The culture medium was completely changed three times per week.

Preparation and treatment of human slice cultures

Approval (#338/2014BO2) of the ethics committee of the University of Tübingen as well as written informed consent was obtained from all patients and allowed spare tissue from resective surgery to be used for *ex vivo* human slice cultures. Human neocortical slice cultures were prepared according to a previously published protocol (Schwarz et al. 2019). Access tissue from temporal lobe was obtained from patients who had undergone resective brain surgery (patient 1: 61 years old, female, epilepsy due to ganglioglioma; patient 2: 49 years old, male, epilepsy due to cortical tubers; patient 3: 22 years old, male, epilepsy due to hippocampus sclerosis). Tissue was transported from surgery room to the laboratory in oxygenated (95% O2 and 5% CO2) ice-cold artificial cerebrospinal fluid (aCSF; 110 mM $C_5H_{14}ClNO$, 26 mM $NaHCO_3$, 10 mM D-glucose, 11.6 mM Na-ascorbate, 7 mM $MgCl_2$, 3.1 mM Na-pyruvate, 2.5 mM KCl, 1.25 mM NaH_2PO_4, and 0.5 $CaCl_2$). The tissue was cut into 250 µm slices perpendicular to the cortical surface using a vibratome. Afterwards the slices were kept in aCSF equilibrated with carbogen for 30 min at room temperature before they were transferred onto culture membrane inserts (Merck Millipore, PICM0RG50) in a 6-well plate for cultivation. For the first hour following the slicing procedure the slices were cultured in 1.5 ml NSC media (48% DMEM/F-12 (Life Technologies), 48% Neurobasal (Life Technologies), 1x N-2 (Capricorn Scientific), 1x B-27 (Capricorn Scientific), 1x Glutamax (Life Technologies), 1x NEAA (Life Technologies) + 20 mM HEPES. From then on, human slice cultures were grown in human cerebrospinal fluid (hCSF) obtained from normal pressure hydrocephalus patients via lumbar puncture (Schwarz et al. 2019). Approval of the ethics committee of the University of

Tuebingen as well as written informed consent from all patients was obtained (#338/2016A). The hCSF was collected, pooled and centrifuged at 2 000 x g at 4 °C for 10 min. The supernatant was kept at -80 °C and thawed at RT before changing the medium. Cultures were kept in 1.5 ml hCSF at 37°C in humidified CO_2-enriched atmosphere. The hCSF was completely changed three times per week.

For αS overexpression, AAV1/2-CMV/CBA-human-A53T-α-synuclein-WPRE-BGH-polyA virus (AAV-hA53T-αS) (Vigene Biosciences, titer of $5x10^{12}$) was injected evenly spaced, once per 5 mm^2 of the slice, using a picospritzer (PDES-O2DX/NPI electronics, Tamm, Germany).

Brain-derived αS seeds and αS pre-formed fibrils

For brain-derived seeds, brain homogenates were prepared as previously described (Schweighauser et al. 2015). In short, fresh frozen pooled brainstems from spontaneously ill male and female Thy1-hA53TαS tg mice (7-8 months old, n = 3 brainstem per homogenate) and corresponding non-tg controls (20 months old, n = 2) were used. Homogenisation at 10 % (w/v) was done in sterile PBS (Pre-cellys, 4 × 10 s at 5,500 rpm), followed by vortexing and centrifugation at 3 000 x g for 5 min at 4°C. The supernatants were collected, aliquoted and stored at -80°C until use.

Expression in *E. coli*, purification and quality control of human recombinant monomeric wt αS was done as previously described (Bousset et al. 2013). For fibril formation, soluble wt αS was incubated in Tris-HCl buffer (50 mM Tris-HCl, pH 7.5, 150 mM KCl) at 37 °C under continuous shaking for 5 days and formation of fibrils was assessed with Thioflavin T. The fibrils were quality checked by transmission electron microscopy after negative staining before and after fragmentation. Their limited proteolytic pattern was also assessed (Bousset et al. 2013). The average size of the fibrils after fragmentation 47 ± 5 nm was derived from length distribution measurements and their average molecular weight (16 200 kDa) was derived from analytical ultracentrifugation sedimentation velocity measurements. The fibrils (350 µM) were aliquoted (6 µl per tube), flash frozen in liquid nitrogen and stored at -80°C. Fibrils were labeled with NHS-ester ATTO-550 as previously described (Gribaudo et al. 2019).

Seeding of the cultures

Murine HSCs were kept for 10 days *in vitro* (DIV-10) without any experimental treatment. At DIV-10, 1 µl of αS pff (350 µM or dilutions thereof) or brainstem homogenate of A53T tg or wt mice was pipetted on top of each culture. Human slice cultures were kept until DIV-3 without any experimental treatment. At DIV-3, 1 µl of αS pff (35 µM) was pipetted on top of the culture.

Local injection of αS pff into HSCs was performed into cornu ammonis 3 (CA3) at DIV-10. CA3 was identified as the region next to dentate gyrus (DG), which can be observed by light microscopy (dense cell layer resembling a horseshoe). Microinjection pipette (Science Products GmbH, GB150TF-10) was pulled using a micropipette puller (Sutter instruments, Model P-97; settings: 1 cycle, heat=520, pull=50, velocity=50, time=250). To immediately visualise the injection, 0.3 µl FastGreen dye (Carl Roth) was added to a 6 µl aliquot of ATTO-550-labelled or unlabelled αS pff (35 µM), upon loading the pipette. The loaded pipette was then inserted into the holder of a picospritzer (PDES-O2DX/ NPI electronics, Tamm, Germany), and the very end of the tip was carefully broken under visual guidance with sterile forceps, until a pressure pulse of 10 ms was

able to release a small droplet of αS pff from the tip. Post-injection microscopic examination of the broken tip revealed an opening size of 25-40 µm. HSCs in their 6-well-plates were placed under the light microscope, and the pipette was carefully inserted into CA3. With a pressure pulse of 10 to 20 ms (10 ms for larger tip openings, 20 ms for smaller tip openings), a small volume of αS pff was injected. After having injected all slices, (approximately 30 min later) the medium was changed.

Antibody treatment

The antibody HLu-3 is a human IgG1-recognizing αS. The epitope of the antibody was determined to be amino acid 113-115 of human αS, using arrays of overlapping linear peptides at Pepscan (Pepscan Zuidersluisweg 2 8243 RC Lelystad, The Netherlands). The affinity to human, mouse and cynomolgus αS monomers is determined to be 31 nM by surface plasmon resonance (BIAcore® 3000). HLu-3 has approximately a 200-fold avidity shift in the binding to fibrillated forms of human αS (determined by competition ELISA). HLu-3 has no cross-reactivity to beta- & gamma-synuclein. Reference item used in the study was a negative isotype-matched control, anti-HIV-1 gp120 human IgG1 antibody (b12).

HLu-3 or control antibody were either mixed with human αS pff (1:1 in PBS to obtain a final concentration of 35 µM) and drop seeded on top of each HSC at DIV-10, or added to the medium. For the latter, antibodies were added to pre-warmed culture medium upon medium change (final concentration of 100 nM or 350 nM). Depending on experimental scheme, antibody-supplemented medium was used 7 days prior to the application of αS pff seeds (-7 days post-injection, dpi) or 30 min after αS pff seeds (0 dpi). Thereafter, the cultures were treated with antibody-supplemented medium with every medium change.

Histological analysis of cultures

Cultures were fixed with 4 % paraformaldehyde (PFA) in PB at pH 7.4 for 2 h (HSCs) or for 24 h (human slice cultures). After fixation, HSCs were rinsed 3 times with 0.1 M PBS for 10 min and stored in PBS at 4°C for up to 1 month until sectioning. Human slice cultures were rinsed 3 times with PBS supplemented with 0.2 % TritonX-100 (PBST) and incubated with PBST overnight for permeabilization. The membrane carrying the fixed HSCs was cut out and mounted onto a planar agar block. With a vibratome (Leica VT 1000S Vibratome, Leica Bio-systems), the cultures were sliced into 50 µm sections. Typically, 5-6 intact sections per HSC were obtained and collected in PBS to be stained within one week. Human slice cultures were stained unprocessed.

Antigen retrieval was performed by heating the sections in 10 mM citrate buffer (1.8 mM citric acid, 8.2 mM trisodium citrate, pH 6.0) at 90°C for 35 min (HSCs) or overnight at 4°C and subsequently 30 s at 90°C (human slice cultures). Sections were blocked with 5 % NGS (HSCs, 2h) or 1 % NGS (human slice cultures, overnight at 4°C) and 0.3 % PBST. For detection of αS phosphorylated at Ser-129, a rabbit monoclonal pS129 antibody (Abcam, EP1536Y, Cat# ab51253, 1:1000) was used. For microglia detection rabbit monoclonal Iba1 (Wako Chemicals GmbH, Cat# 019-19741, 1:250) and for neuronal staining and structure mouse monoclonal NeuN antibody (Millipore GmbH, Cat# MAB377, 1:500) and chicken anti-MAP2 (Abcam, Cat# ab5392, 1:500) were used. Following Alexa-fluorophore-conjugated secondary antibodies were applied in a concentration of 1:250: goat-anti-rabbit Alexa-568 (Thermo Fisher, Cat# A11011); goat-anti-mouse Alexa-488 (Thermo Fisher, Cat# A11001); goat-anti-mouse Alexa-568 (Invitrogen, Cat# A11004); goat-anti-rabbit Alexa-633 (Thermo Fisher,

Cat# A21070); goat-anti-chicken Alexa-488 (Invitrogen, Cat# A21467). DAPI counterstaining was performed at a concentration of 1:500.

For staining with amyloid binding dyes pentamer formyl thiophene acetic acid (pFTAA) (Klingstedt et al. 2011; Ries et al. 2013) and thioflavin S (ThioS), sections were incubated for 1 h with either freshly prepared pFTAA (1.5 mM in de-ionized water, used at 1:500 in PBS) or ThioS (Sigma-Aldrich, Cat# T1892; 1 % w/v ThioS in milliQ H_2O). ThioS-stained sections were washed 2 x in 70 % EtOH and for 10 min. Slices were transferred on glass slides and coverslipped with Dako Fluorescence mounting medium (Biozol Diagnostika, Cat# S3023).

Sections were analyzed using an Axioplan2 imaging microscope (Zeiss, Jena, Germany) and digitised with an AxioCam HRm black and white camera (Zeiss) using AxioVision 4.8 software (Zeiss). With a Plan Neofluar 10x/0.50 objective lens (Zeiss), 16-bit RGB mosaics of the whole culture were obtained with a resolution of 170 pixels / µm. High resolution images were acquired using a Zeiss LSM 510 META (Axiovert 200M) confocal microscope with an oil immersion 40×/1.3 or 63×/1.4 Plan Apochromat objective and LSM software 4.2 (Carl Zeiss). Sequential excitation of fluorophores was performed using lasers with the wavelength 405 nm (DAPI), 488 nm (Alexa-488 coupled secondary antibodies, ThioS, pFTAA), 543 nm (Alexa-568 coupled secondary antibodies, pFTAA), and 633 nm (Alexa-633 coupled antibodies).

Quantification of immunohistochemical stainings

For the quantification of the pS129-positive inclusions in HSCs, whole culture mosaic images were acquired on an Axioplan2 imaging microscope as described above. Cultures with sectioning artefacts or cultures that were injected in the wrong site (e.g. into CA2 instead of CA3) were excluded from the analysis with FIJI ImageJ (version 2.0.0-rc-64/1.51s). Images were blinded, colour channels were split, background was subtracted (rolling ball radius 50 pixels), and the intensity threshold was manually adjusted. On each mosaic, the percentage of pS129-positive signal over the whole culture was calculated. To quantify the pS129 signal percentage in the injection site, the region of interest (ROI) for CA3 was selected and stored before channels were split. Background was subtracted and intensity threshold was adjusted for the whole culture. Then, the total of pS129-positive signal in the ROI of every mosaic image was measured.

For the quantification of both pS129 and ThioS in hippocampal subregions images were acquired using an LSM 510 META (Axiovert 200M) confocal microscope with a 20×/0.8 Plan Apochromat objective and LSM software 4.2 (Carl Zeiss), using sequential excitation of fluorophores, as described above. Stacks of 50 µm were taken and maximum intensity projections (MIP) images produced. Colour channels were split and a fixed intensity threshold was applied to both red and green channel. To exclude unspecific staining of ThioS, the particle size of signal in the green channel was limited to 20 – 200 μm^2. On each image, the selected area over the total image area was calculated.

Biochemical analysis of cultures

Slice culture homogenates were prepared from treated or untreated cultures. Cultures were removed from the membrane, pooled (n = 16) and immediately frozen on dry ice and stored at -80°C until use. Frozen slice cultures were homogenised with a syringe in 160 µl sterile PBS, aliquoted and stored at -80°C until further use. For immunoassays (Western blotting) homogenates in PBS were shifted to high salt (HS) buffer (50 mM Tris-

HCl pH 7.5, 750 mM NaCl, 5 mM EDTA, 1 % phosphatase and protease inhibitor cocktails). 100 µL of homogenate were incubated on ice in *N*-lauroylsarcosyl (Sigma, Cat# 61747, Saint-Quentin-Fallavier, France) at a final concentration of 10%, and were left on ice for 15 min before they were loaded on a 10 % sucrose cushion and ultracentrifuged at 186 000 x g for 1 h 10 min at 4°C. The supernatant was collected, and the pellet was resuspended in sample buffer, and sample buffer was also added to the supernatant. Proteins were separated in a 4-12 % SDS NuPage Gel and electroblotted onto Amershan nitrocellulose membranes (VWR International Merck Eurolab, Cat# 10600001). Membranes were incubated with 0.4 % PFA for 30 min, and then saturated with 5 % dry milk in 0.1 % PBS-Tween20 (0.1 %). Monoclonal rabbit antibody against pS129 (AbCam, Cat# ab51253) at 1:1000 dilution to detect phosphorylated αS species, monoclonal mouse antibody against αS (BD Transduction Laboratories, Cat# 610786) at 1:1000 dilution to detect total αS, and rabbit β-actin antibody (AbCam, Cat# ab8227) as a loading control were used. Membranes were then incubated for 1 h with anti-rabbit antibody or anti-mouse antibody at 1:20 000 for 1 h at room temperature. Samples were visualized with chemiluminescence using SuperSignal West Dura Extended or Pico (both Thermo Scientific).

Immunoassay for total αS measurements in brain homogenate

For αS measurements, brain homogenates were extracted as follows: aliquots were thawed on ice, mixed 1:3.2 with cold formic acid (FA) (minimum 96% purity; Sigma, St. Louis, MO, USA), sonicated for 35 seconds at 4°C, and spun at 25,000g at 4 C for 1 hour. The supernatant was equilibrated (1:20) in neutralization buffer (1 M Tris base, 0.5 M Na_2HPO_4, 0.05% NaN_3).

Concentrations of human αS in brain homogenates were determined with an electrochemiluminescence-linked immunoassay using the MSD Human α-Synuclein Kit (Meso Scale Discovery, Gaithersburg, MD, USA), or by Single Molecule Array (Simoa) technology using the Simoa™ Human Alpha-Synuclein Discovery Kit (Quanterix, Billerica, MA, USA) according to manufacturer's instructions. FA-soluble brain homogenates were diluted up to 1:10 000 in Diluent 35 (Meso Scale Discovery) or 1:100 in Alpha-Synuclein Sample Diluent (Simoa) before the measurement, and analyzed in duplicates on a Mesoscale Sector Imager 6000 or a Simoa HD-1 Analyzer. MSD DISCOVERY WORKBENCH software 3.0 or Simoa Software Version 1.5 for HD-1 Analyzer was used for data analysis. Internal reference samples were used as controls on every plate.

Immunoassay for aggregated αS measurements in brain homogenate and pre-formed fibrils

αS aggregates were measured using a HTRF-FRET assay developed by Cisbio (#6FASYPEG, Cisbio). αS pff and tg brain homogenate were serially diluted and a HTRF-FRET signal measured on a PHERAstar (BMG LABTECH) using 337 nm laser excitation, simultaneous dual emission 665 nm / 620 nm and HTRF technology. Data is reported as 665 nm / 620 nm x 10 000. αS pff needed to be diluted > 50 000 fold to be on the proper side of the hook effect of the assay, whereas the tg brain homogenate did not show hook effect issues at any dilutions. For αS pff, a dilution of 204 800-fold resulted in a signal of approximately 20 000, whereas the tg brain homogenate only was diluted 25-fold to reach a similar aggregation level. The difference in dilutions (e.g. 204 800-fold vs 25-fold) to reach a similar aggregation level was used to estimate the relative αS aggregation level per volume of sample. Results over several dilutions were combined to reach the final result of 8 000 x more aggregates in αS pff relative to tg brain homogenate per volume.

NfL immunoassay

Culture medium was collected, aliquoted, and kept at -80°C until use. NfL concentrations were determined by Single Molecule Array (Simoa) technology using the highly sensitive Simoa™ NF-Light Advantage Kit (Quanterix, Billerica, MA, USA) according to manufacturer's instructions (Preische et al. 2019). Medium samples were pre-diluted 1:10 or 1:50 in NF-Light sample diluent and measured in duplicates on a Simoa HD-1 Analyzer (Quanterix). Internal reference samples were used as controls on every plate.

Culture thickness

While sectioning the freshly fixed cultures with a vibratome (see above), the amount of 50 µm sections was assessed. Although the first and last section sometimes were not exactly 50 µm, the amount of sections x 50 µm equals roughly the culture thickness at the time of fixation.

Intracerebral injection of αS tg mice

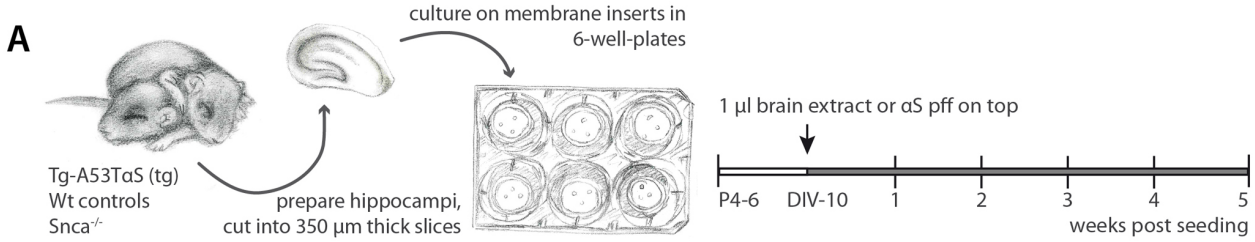
Male and female 3–4-month-old Thy1-hA53TαS tg mice were anesthetized using a mixture of ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight) in saline. 2.5 µl of brain homogenate (see above) was then infused bilaterally into the dorsal hippocampus (AP −2.5 mm, L ± 2.0 mm, DV −1.8 mm) by stereotactic injection. Injection speed was 1.25 µl/min, and the needle was slowly removed after being kept in place for an additional 2 min. The surgical area was cleaned with sterile saline and the incision was sutured. Mice were kept under infrared light for warmth and monitored until recovery from anesthesia.

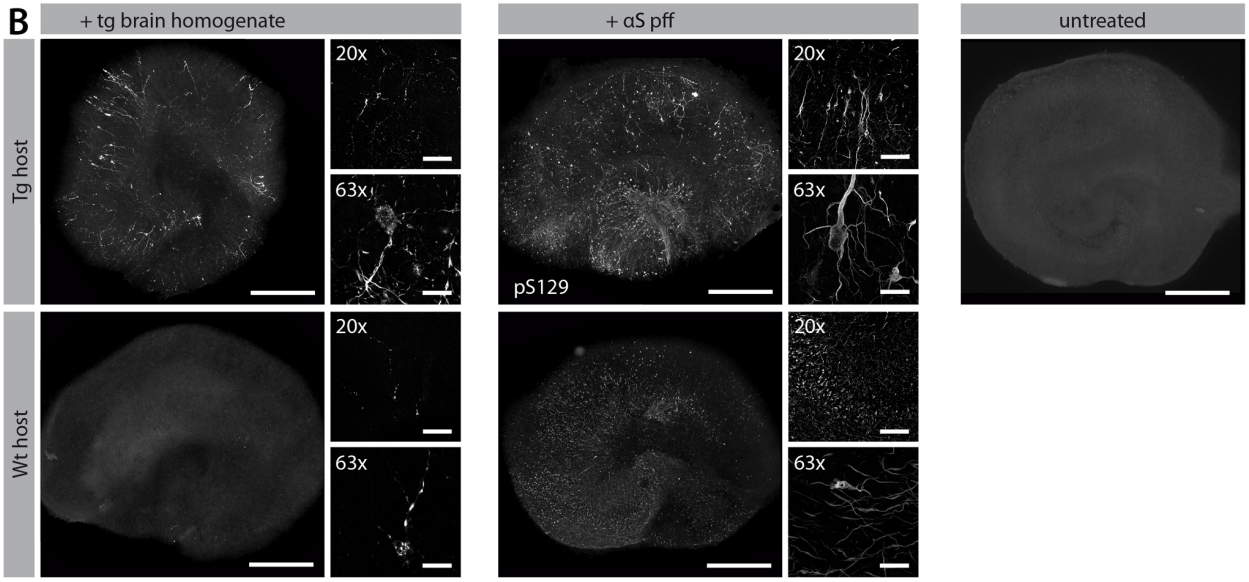
After incubation periods of up to 30 days, mice were perfused for 5 min with ice-cold PBS. Brains were removed and immersion-fixed in 4 % PFA in 0.1 M PB at pH 7.4 for 48 h, and then placed in 30 % sucrose in PBS for 48 h. Brains were frozen in 2-methylbutane, cooled with dry ice, and then serially cut into 25 µm thick sagittal sections using a freezing-sliding microtome. The sections were collected in cryoprotectant (35 % ethylene glycol, 25 % glycerol in PBS) and stored at −20 °C until use. Immunohistochemical staining of αS inclusions was done as described for HSCs (see above).

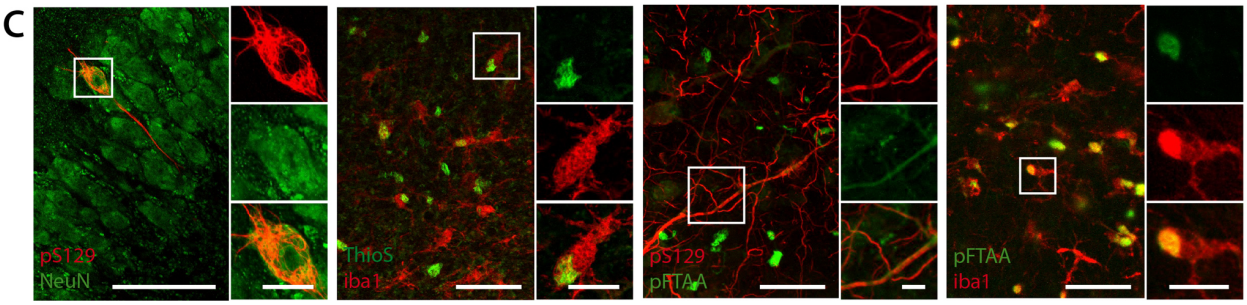
Statistical Analysis.

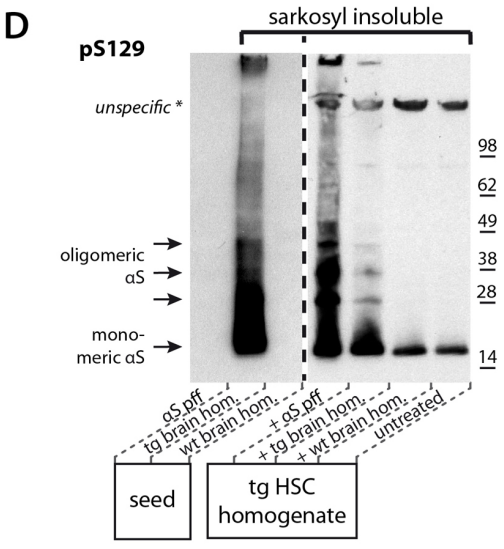
GraphPad Prism (v.5) was used for statistical analyses. The statistical tests were applied as indicated in the figure legends.

FIGURES









E

Time post seeding	Tg	Wt	Snca$^{-/-}$
	pS129 positive area [%]		
Tg brain homogenate			
5 weeks	0.64 ± 0.23	0.01 ± 0.00	-
1 week	0.22 ± 0.09	-	-
1 hour	-	-	-
αS pff			
5 weeks	2.00 ± 0.26	1.79 ± 0.20	N/A
1 week	4.77 ± 1.14	0.01 ± 0.00	N/A
1 hour	-	-	N/A
Wt brain homogenate			
5 weeks	-	-	-
untreated			
5 weeks	-	-	-

Figure 1: Induction of αS inclusions in mouse hippocampal slice cultures. (A) Treatment scheme for hippocampal slice cultures (HSCs). Hippocampi of 4-6-day old Tg-A53TαS (tg), wildtype (wt) or *Snca*$^{-/-}$ mice were sectioned into 350 µm thick slices and plated onto semi-permeable culture membranes. After 10 days *in vitro* (DIV-10), 1 µl of either wt or tg brain homogenate, or αS preformed fibrils (αS pff) at 350 µM were added on top of each culture. All three cultures within one well received the same treatment. Five weeks later, the cultures were collected for analysis. **(B)** Immunofluorescence staining for pS129 revealed a myriad of inclusions in both somas and throughout the processes in the tg host, and smaller, but still abundant inclusions in the wt host, at 5 weeks after treatment with αS pff. Note the difference in appearance of inclusions between tg brain homogenate-treated and αS pff-treated cultures, the former having a more punctate, filamentous appearance of the pS129-inclusions upon treatment with tg brain homogenate while αS pff treatment resulted in longer, smooth-looking pS129-inclusions. Scale bars represent 500 µm (overview), 100 µm (20x), and 20 µm (63x). **(C)** Immunofluorescence double staining in αS pff treated tg cultures for pS129 (red) and NeuN or pFTAA (green) as well as ThioS or pFTAA (green) and iba1 (red). Note that pS129-positive inclusions are predominantly present in neurons (NeuN), while ThioS-positive aggregates rather appeared to be associated with microglia (iba1). On the other hand, pFTAA stained both pS129-positive aggregates in neurites and microglial inclusions (ThioS). Scale bars represent 50 µm, and 10 µm for inserts **(D)** Immunoblot of sarkosyl-insoluble material of seed and HSC homogenates. Monomeric αS phosphorylated at S129 was present as a band at around 15 kDa in brain extract as well as tg HSCs seeded with αS pff or tg brain homogenate, but not αS pff. Multimeric species were also present as higher molecular bands as well as ubiquitinated αS at around 29 kDa. pff = αS pff; tg = tg brain homogenate; wt = wt brain homogenate; untr = untreated; n = 15-16 pooled HSCs per group. **(E)** Quantification of pS129-positive inclusions (in percentage of culture area) in dependence of time, seeding agent (rows) and host (columns). Mean ± SEM; n = 5 HSCs per group and timepoint; - = no inclusions; N/A = not done.

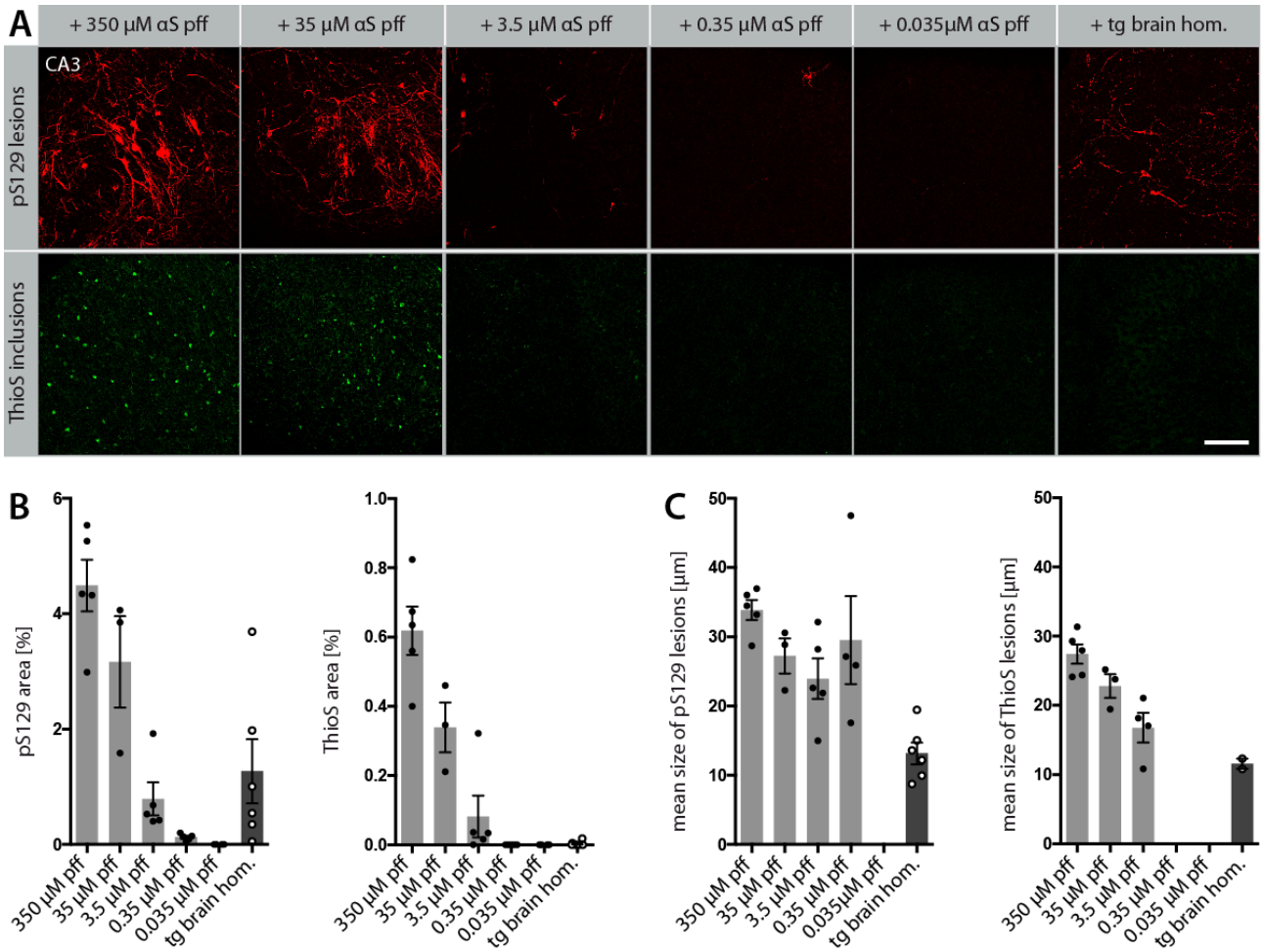


Figure 2: Induction of αS inclusions is dependent on the seed concentration. See Figure 1 for treatment scheme. **(A)** Immunofluorescence staining for pS129 and ThioS in tg HSCs of CA3 at 5 weeks post-seeding. The concentration of αS pff (undiluted, 10-fold to 10 000-fold diluted) determines the abundance αS inclusions positive for pS129 (red, largely neuronal) and ThioS (green, largely microglial) in tg HSCs. Brain homogenate shows approximately the seeding activity of 1:100 αS pff. However, the seed concentration in the brain homogenate is likely much lower compared to the one of αS pff (see Results). Scale bars represent 50 µm. **(B)** Quantification of pS129-positive and ThioS-positive inclusions in CA3 of tg HSCs treated with different concentrations of αS pff (light grey bars) or with tg BS homogenate (dark grey bar). Mean ± SEM and individual values are shown as circles; n = 3-5 HSCs per group. **(C)** Quantification of the size of individual pS129- or ThioS-positive inclusions in CA3 of tg HSCs treated with different concentrations of αS pff (light grey) or with tg brain homogenate (dark grey). Mean ± SEM and individual values are shown as circles; n = 3-5 HSCs per group.

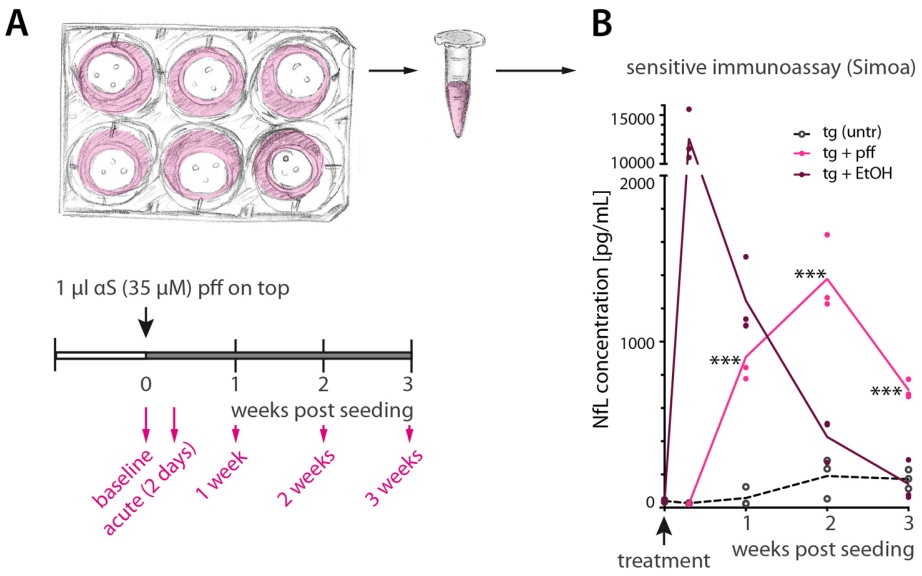


Figure 3: Increased levels of Neurofilament light chain (NfL) in response to the induction of αS lesions. (A) Schematic of a well-plate containing HSCs and medium collection scheme. Each well contains 3 cultures of the same treatment. The entire culture medium (1.2 ml) of a well was collected (and immediately replaced with new one) each before seeding (baseline), 2 days (acute), 1, 2, and 3 weeks after seeding. **(B)** Longitudinal measurement of NfL in culture medium of tg HSC measured by Single Molecule Assay (Simoa), a sensitive immunoassay. Cultures were treated with 35 µM αS pff (pink), or EtOH (dark red), or left untreated (black, dotted line). While EtOH treatment had an acute toxic effect, αS pff treated cultures released NfL gradually into the medium, with a peak release at 2 weeks post seeding. Mean ± SEM is shown; n = 3-4 wells with medium per group; two-way-ANOVA repeated measurements (treatment $F(1, 4) = 79.86$, $p = 0.0009$; time $F(3, 12) = 78.57$, $p < 0.0001$; interaction $F(3, 12) = 51.69$, $p < 0.0001$) with Sidak's corrections for multiple comparisons between treatment groups (within time points),***$p < 0.001$.

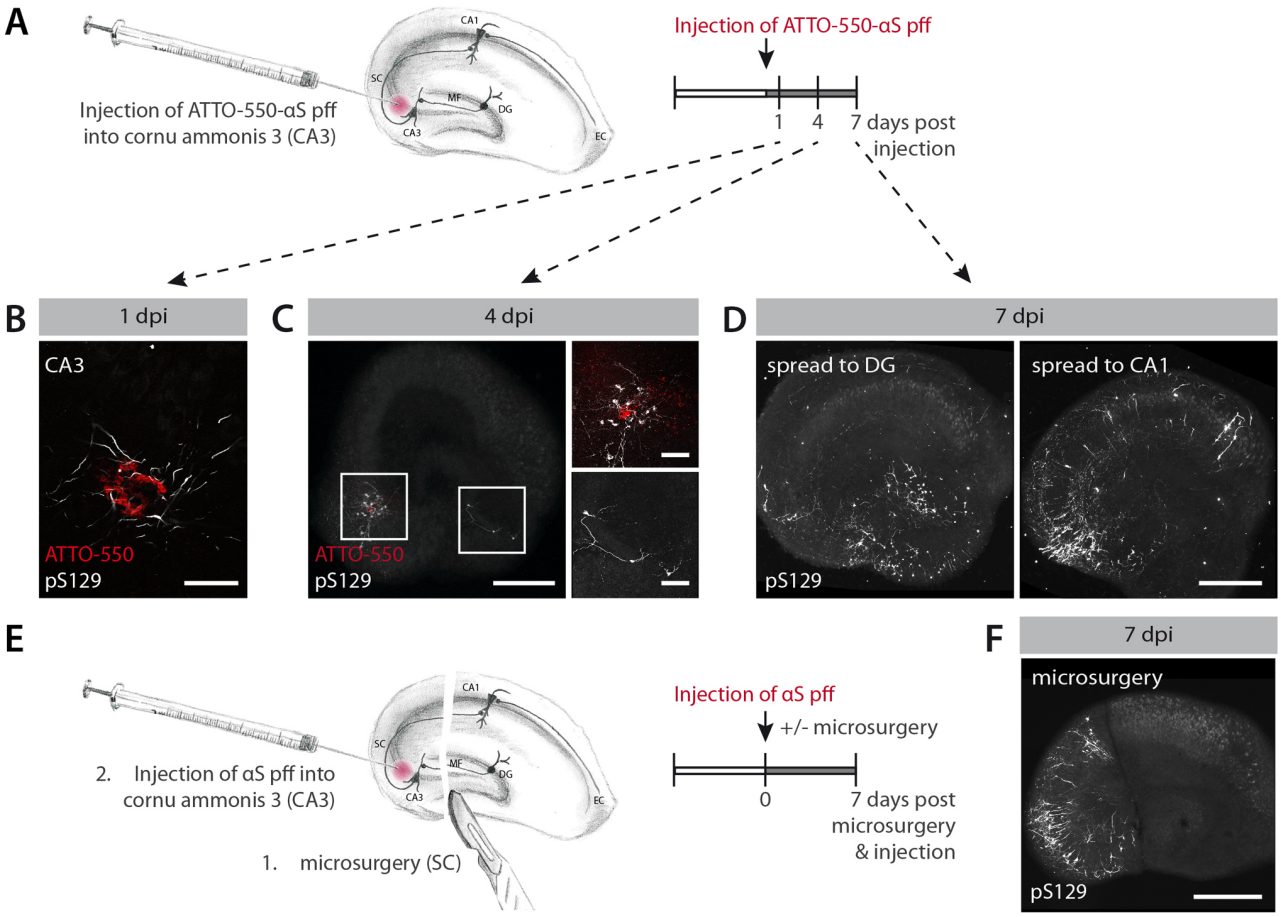


Figure 4: Spreading of αS lesions in mouse hippocampal slice cultures. (A) Schematic illustration of the local injection of ATTO-550-labelled αS pff into hippocampal CA3 region. **(B)** ATTO-550-αS pff (red) and the first neuritic pS129-positive inclusions (white) at 1 day post injection (dpi) around the injection site in CA3. Note that αS pff seeds are not phosphorylated and therefore are not stained by anti-pS129 antibody. Scale bars represent 50 µm. **(C)** pS129-positive inclusions at the injection site and in the dentate gyrus (DG) at 4 dpi. Scale bars represent 500 µm and 100 µm (inserts). **(D)** Apart from the injection site (CA3) and DG, pS129-positive inclusions also appeared in CA1 at 7 dpi. Scale bars represent 500 µm. **(E)** Schematic illustration depicting the microsurgery disconnecting CA3 from CA1 prior to regional injection of αS pff into the CA3 region. **(F)** Disconnecting CA3 from both CA1 and DG before injecting pff inhibited the spreading of the pathology at 7 dpi. Inclusions in microglia were rare at 7 dpi and appeared only after 3 weeks post seeding (see also Supplementary Fig. 4). Scale bars represent 500 µm.

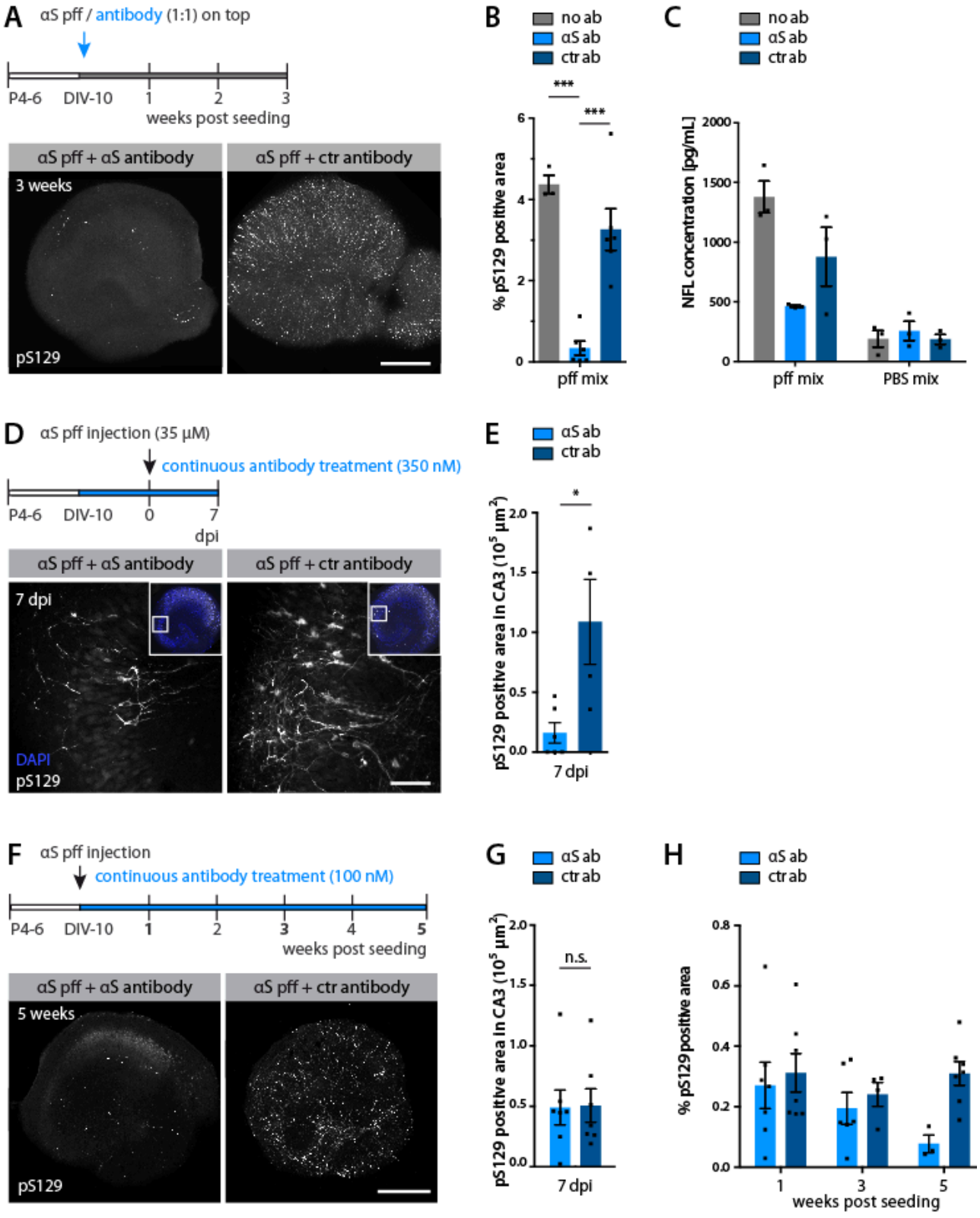


Figure 5: Immunotherapy targeting either induction or spreading of αS lesions. (A) Experimental scheme demonstrating the effect of anti-αS antibody HLu-3 on αS pff seed inactivation. 35 µM αS pff were mixed with 35 µM αS antibody (or ctr antibody) in a ration of 1:1 before applying 1 µl seed/antibody mix onto each tg HSC. After 3 weeks, αS pff / αS antibody-treated HSC showed very little pS129-positive inclusions compared to αS pff / ctr antibody treated HSC. Scale bars

represent 500 µm. **(B)** Quantification of pS129-positive inclusions in tg HSCs treated with αS pff alone (no antibody), αS pff / αS antibody mix, and αS pff / control antibody mix. Mean ± SEM and individual values are shown as circles; n(no ab) = 3; n(αS ab) = 6; n(ctr ab) = 6 HSCs per group; one-way-ANOVA (Treatment $F(2, 12) = 27.23$, $p < 0.0001$) with Sidak's correction for multiple comparisons, ***$p < 0.001$. **(C)** Measurements of NfL in culture medium of tg HSC treated with αS pff only (no antibody), αS pff / αS antibody mix, and αS pff / control antibody mix and corresponding PBS-treated cultures (i.e. no induction of αS lesions). Mixing αS pff with αS antibody inhibits NfL increase. Shown are the 2 weeks post-treatment time points with similar results at 3 weeks post treatment (not shown). Shown is mean ± SEM and individual values are shown as circles; n = 3 wells that contains the medium of 3 cultures per group; two-way- ANOVA revealed for treatment (pff vs PBS) $F(1, 12) = 47.43$, $p < 0.0001$; antibody $F(2, 12) = 5.933$, $p = 0.0162$; interaction $F(2,12) = 7.850$, $p = 0.0066$). Post-hoc Sidak's correction for multiple comparisons revealed $p = 0.0699$ for αS antibody vs control antibody. **(D)** Schematic illustration of experimental setup and immunofluorescence staining for pS129 of seeded and antibody-treated cultures. Antibodies were added to the medium 7 days prior to αS pff injection into CA3 (injection, see Fig. 4) and continuously added until 7 dpi. The antibody treatment largely prevented pS129-positive inclusions in CA3. Inserts show overview of HSC at low magnification. Scale bars represent 100 µm. **(E)** Quantification of pS129-positive inclusions in CA3 of HSCs injected with αS pff (35 µM stock concentration), and treated with either αS antibody (light blue) or ctr antibody (dark blue) in medium (350 nM) from -7 dpi up to 7 dpi. Mean ± SEM and individual values are shown as circles; n(αS ab) = 6; n(ctr ab) = 4 HSCs / group; unpaired two-tailed t-test $t(8) = 3.095$, $p = 0.0148$; *$p < 0.05$. **(F**) Schematic illustration of experimental setup and immunofluorescence staining for pS129 of seeded and antibody-treated cultures. Antibodies were applied 1 h after αS pff (35 µm) were injected into CA3 and continuously added until 5 weeks post-seeding. Analysis at 5 weeks post-seeding revealed that spreading of pS129-positive inclusions had been blocked by αS antibody. Scale bars represent 500 µm. **(G)** Quantification of pS129-positive inclusions in CA3 of HSCs injected with αS pff (35 µM stock concentration), and treated with either αS antibody (light blue) or ctr antibody (dark blue) in medium (100 nM each) from 1 hour after seeding up to 7 dpi. At 7 dpi, there was no apparent difference visible between the two treatment groups. Mean ± SEM are shown and individual values are shown as circles; n = 7 HSCs per group; unpaired two-tailed t-test, $t(12) = 0.07683$, $p = 0.94$. **(H)** Quantification of pS129-positive inclusions in the entire area of HSCs injected with αS pff (35 mM) and treated with αS antibody (light blue) or control antibody (dark blue) at 1, 3, and 5 weeks post injection. Mean ± SEM and individual values are shown as circles; n(1 week) = 9 HSCs per group, n(3 weeks) = 7-8 HSCs per group, n(5 weeks) = 3-6 HSCs per group; two-way-ANOVA (treatment $F(1, 31) = 6.630$, $p = 0.0150$; time $F(2, 31) = 2.447$, $p = 0.1031$) failed to show significance for the interaction but clearly reveled a trend $F(2, 31) = 2.801$, $p = 0.0761$).

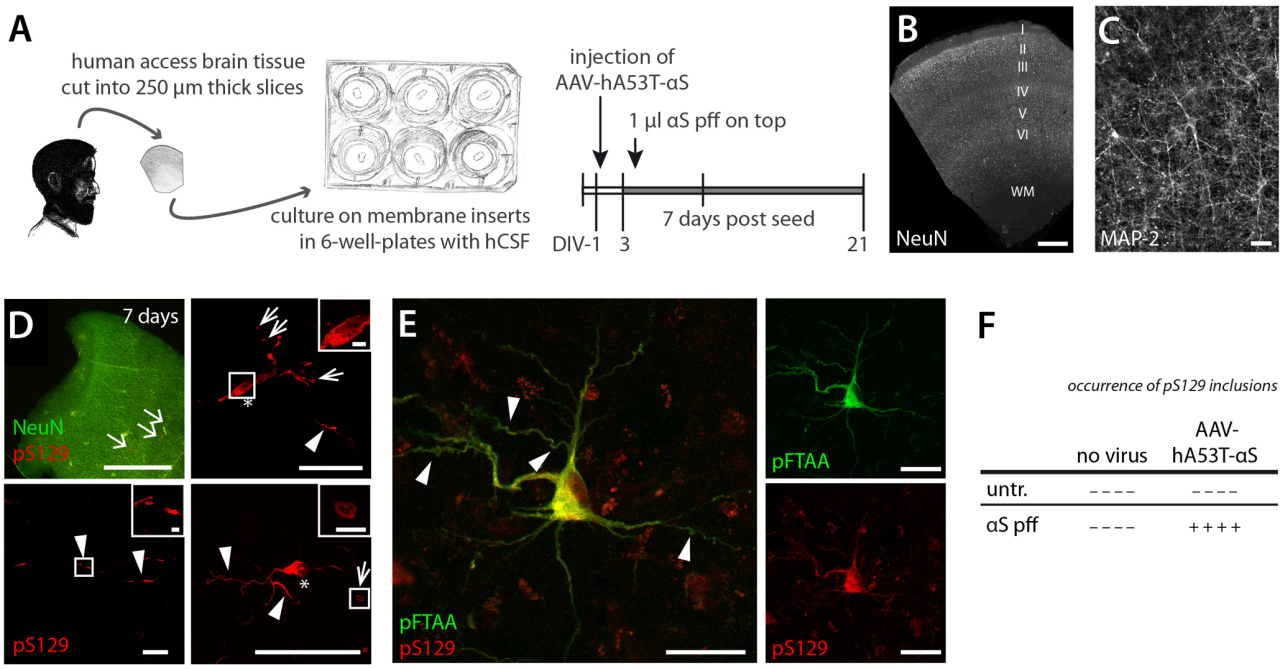
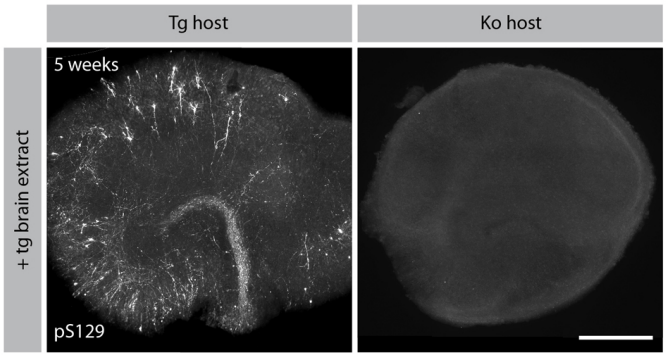
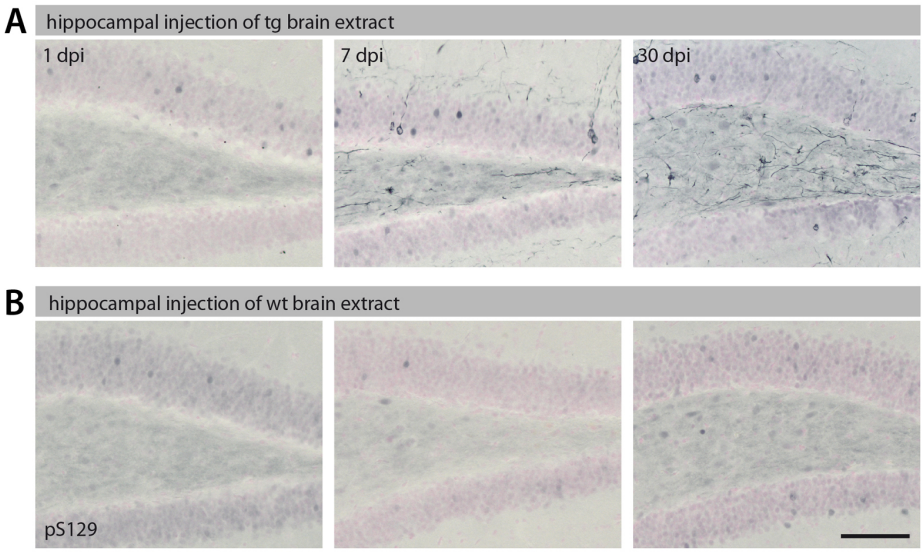


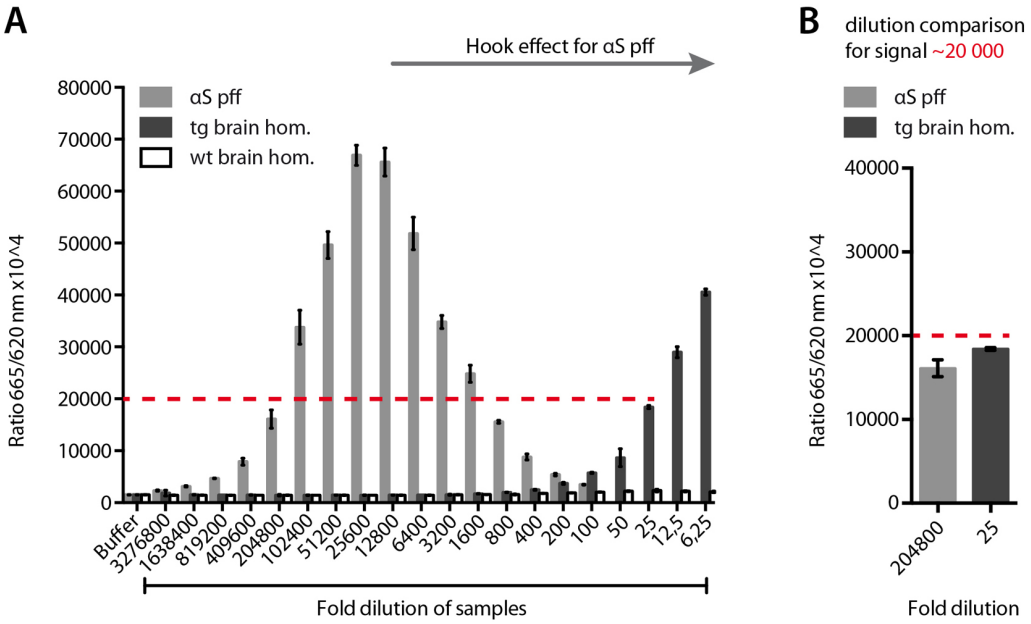
Figure 6: Induction of αS inclusion in adult human brain slice cultures. (A) Schematic of the preparation and treatment scheme of human neocortical slice cultures obtained from brain resection. Healthy access tissues of resected brain were used to prepare human slice cultures. Cortical tissue was sectioned into 250 µm slices perpendicular to the cortical surface. At day *in vitro* 1 (DIV-1) some cultures were injected with AAV-hA53T-αS to induce overexpression of αS. At DIV-3, 1 µl of αS pff (35 µM) was added on top of each culture. At DIV-10 the human slice cultures were fixed for histological analysis. Drawing shows fictional patient. **(B, C)** Immunofluorescence staining of neuronal nuclei (NeuN) show preserved neuronal layers I-VI at DIV-10, the time of fixation (WM = white matter) (B). Neuronal cell bodies with processes appear also intact (MAP-2-immunostaining; shown is DIV-12). Scale bars = 1 mm (B) and 50 µm (C). These observations are consistent with previous work demonstrating that resected human tissue can be cultured and maintain electrophysiological properties up to DIV-21 if the medium is replaced with human CSF (Schwarz et al. 2019). **(D)** Immunofluorescence staining for pS129 (red) in a human slice culture at 7 days post-seeding. Arrows in top left panel point to sites where neurons developed pS129-positive inclusions. Neuronal nuclei were stained by NeuN (green). Top right, bottom left and bottom right panels show single neurons with pS129-positive inclusions (from 3 different cultures). Note the diffuse and large inclusion in the neuronal soma (asterisks) and the filamentous inclusions in the processes (arrowheads) with often large spheroid-like inclusions (narrow arrows). Scale bars represent 1 mm (top left), 50 µm (top right, and bottom row) and 5 µm (inserts). **(E)** Immunofluorescence staining for pS129 antibody (red) and the amyloid-binding dye pFTAA (green). Note the sharp angles in neuronal processes, a clear sign for neuronal dystrophy (arrows). Also note that the red labelling in the background is lipofuscin autofluorescence. Scale bars represent 50 µm. **(F)** Table summarising the results from seeded αS aggregation in human cultures. The formation of pS129-positive inclusions is indicated (+) for each culture (n = 4 cultures; 3 patients with ages between 22 and 61 years old).



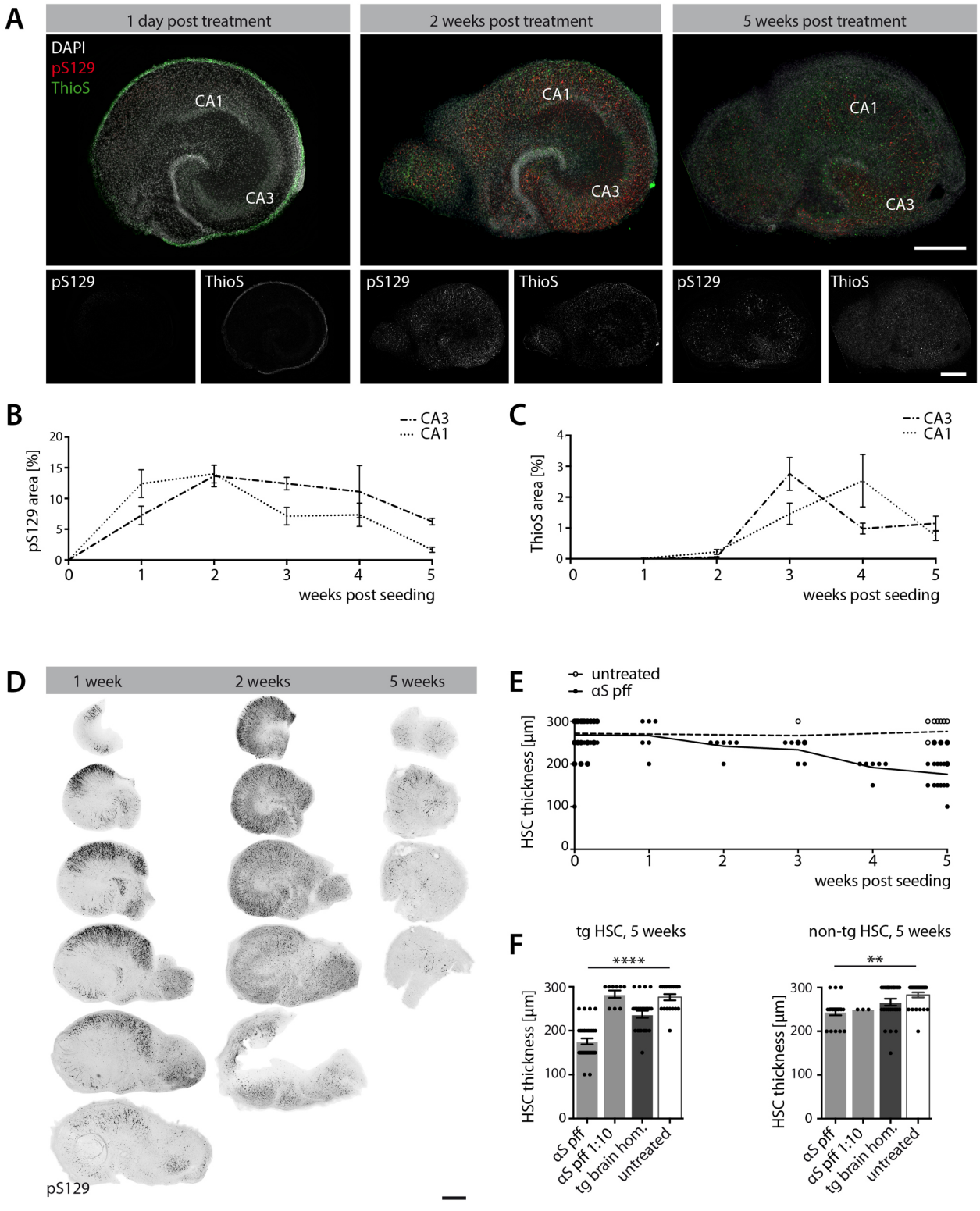
Supplementary Figure 1: Induction of αS aggregation is dependent on endogenous αS expression. Immunofluorescence staining for pS129 showed inclusions in tg HSC seeded with tg brain homogenate. In contrast, there was no induction of αS aggregation in *Snca*$^{-/-}$ HSC after seeding using the same brain homogenate a seed. Analysis was done 5 weeks after treatment. See Fig. 1 for methodological detail. The experiment was done twice with 3 cultures each. Scale bar represents 500 µm.



Supplementary Figure 2: Time course of seeded αS aggregation in A53T tg mice. (A, B) Immunohistochemistry for pS129 (blue) of DG from A53T tg mice injected at 3-4 months of age with tg brain homogenate (A) or wt brain homogenate (B) at one day post injection (dpi), 7 dpi or 30 dpi. For each group three mice were used. Sections were counter stained using nuclear fast red. Note that same brain homogenates were used for slice culture experiments. (A) First inclusions appeared around 7 dpi in all three mice and became more abundant by 30 dpi. (B) In contrast, no inclusions were for any time points analysed. Scale bar represents 100 µm.



Supplementary Figure 3: αS aggregates in αS pff and tg brain homogenate. (A, B) HTRF-FRET immunoassay analysis of aggregated αS in αS pff (light grey), tg brain homogenate (dark grey), and wt homogenate (white) at different sample dilutions. Data is reported as ratio of 665 nm / 620 nm x 10 000. Note that αS pff showed Hook effect at dilutions < 1: 51 200 (Hook effect: high aggregate concentrations will capture all antibodies leading to a plateau and to a decrease of the signal) (A). Dotted line (red) illustrates exemplary comparison of dilutions that resulted in similar aggregation signal (B). Aggregation levels that gave a signal ratio of ~20 000 for ratio of 665 nm / 620 nm x 10 000. αS pff (light grey) needed to be diluted approximately 204 800-fold, and tg brain homogenate (dark grey) 25-fold. Although the signal cannot be attributed to an absolute amount of aggregated αS given that the conformations of aggregated αS in pff and tg brain extract are likely to be different, results appear consistent with brain-derived αS seeds to be more seeding potent compared to αS pff seeds. Mean ± SEM; n = 3 triplicate measurements per dilution.



Supplementary Figure 4: Treatment of highly concentrated αS pff is neurotoxic to tg HSCs. (A) Distribution of pS129- and ThioS-positive aggregates in tg HSCs treated with 350 µM αS pff, and analysed at 1 day, 2 weeks, or 5 weeks post treatment. After 1 day, ThioS-positive αS pff (green) still surrounded the culture. At 2 weeks, pS129-positive neuronal inclusions (red) were abundant, but appeared to decline at 5 weeks post seeding. In contrast, ThioS-positive microglia inclusions increased from 2 to 5 weeks post-seeding. Scale bars represent 500 µm. **(B, C)** Quantification of pS129-positive (B) and ThioS-positive (C) αS inclusions over time. Graphs show percentage of positive area in CA3 (dashed line) and CA1

(dotted line) (C). The same is shown for the ThioS-positive largely microglia inclusion (percentage of ThioS positive area) in CA3 (dashed line) and CA1 (dotted line). Mean ± SEM are shown; n = 5 HSCs per group and timepoint. **(D)** Immunostaining of pS129-positive aggregates in 50 µm-horizontal sections from tg HSCs treated with 350 µM αS pff at 1 week (left column), 2 weeks (centre column), and 5 weeks (right column) post seeding. Scale bar represents 500 µm. **(E)** Measurement of culture thickness (in µm) over time at the time of fixation in αS pff treated (solid line) and untreated tg HSCs (dashed line). Mean ± SEM and individual values are shown as circles; n(untr., 0 weeks) = 32, n(untr., 3 weeks) = 3, n(untr., 5 weeks) = 19, n(pff, 0 weeks) = 47, n(pff, 1 week) = 6, n(pff, 2 weeks) = 6, n(pff, 3 weeks) = 6, n(pff, 4 weeks) = 6, n(pff, 5 weeks) = 35 HSCs per group. **(F)** Bar graphs show quantification of HSC thickness in tg and non-tg cultures at 5 weeks post treatment. Error bars mean ± SEM and individual values are shown as circles; n(tg, pff) = 35, n(tg, pff 1:10) = 9, n(tg, tg brain hom.) = 24, n(tg, untr.) = 19, n(wt, pff) = 18, n(wt, pff 1:10) = 3, n(wt, tg brain hom.) = 27, n(wt, untr.) = 24 HSCs per group; Kruskal-Wallis test: **p<0.01, ****p<0.0001 Dunn's multiple comparisons against untreated HSCs.

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3 PROMINENT MICROGLIAL INCLUSIONS IN TRANSGENIC MOUSE MODELS OF A-SYNUCLEINOPATHY THAT ARE DISTINCT FROM NEURONAL LESIONS

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Prominent microglial inclusions in transgenic mouse models of α-synucleinopathy that are distinct from neuronal lesions

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Abstract

Alpha-synucleinopathies are a group of progressive neurodegenerative disorders, characterized by intracellular deposits of aggregated α-synuclein (αS). The clinical heterogeneity of these diseases is thought to be attributed to conformers (or strains) of αS but the contribution of inclusions in various cell types is unclear. The aim of the present work was to study αS conformers among different transgenic (TG) mouse models of α-synucleinopathies. To this end, four different TG mouse models were studied (Prnp-h[A53T]αS; Thy1-h[A53T]αS; Thy1-h[A30P]αS; Thy1-mαS) that overexpress human or murine αS and differed in their age-of-symptom onset and subsequent disease progression. Postmortem analysis of end-stage brains revealed robust neuronal αS pathology as evidenced by accumulation of αS serine 129 (p-αS) phosphorylation in the brainstem of all four TG mouse lines. Overall appearance of the pathology was similar and only modest differences were observed among additionally affected brain regions. To study αS conformers in these mice, we used pentameric formyl thiophene acetic acid (pFTAA), a fluorescent dye with amyloid conformation-dependent spectral properties. Unexpectedly, besides the neuronal αS pathology, we also found abundant pFTAA-positive inclusions in microglia of all four TG mouse lines. These microglial inclusions were also positive for Thioflavin S and showed immunoreactivity with antibodies recognizing the N-terminus of αS, but were largely p-αS-negative. In all four lines, spectral pFTAA analysis revealed conformational differences between microglia and neuronal inclusions but not among the different mouse models. Concomitant with neuronal lesions, microglial inclusions were already present at presymptomatic stages and could also be induced by seeded αS aggregation. Although nature and significance of microglial inclusions for human α-synucleinopathies remain to be clarified, the previously overlooked abundance of microglial inclusions in TG mouse
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models of α-synucleinopathy bears importance for mechanistic and preclinical-translational studies.

Keywords: Synuclein, Microglia, Inclusion, Prion-like, Amyloid, Conformation, Parkinson's disease

Introduction

Accumulation of α-synuclein (αS) aggregates is a pathological hallmark of a group of neurodegenerative diseases called α-synucleinopathies. αS is the major component of Lewy bodies and Lewy neurites, which are intracellular inclusions found in neurons of patients with Parkinson's disease (PD) and dementia with Lewy bodies (DLB). Apart from the neuronal Lewy pathology, filamentous αS also accumulates in oligodendrocytes to form glial cytoplasmic inclusions (GCIs or Papp-Lantos bodies) found primarily in multiple system atrophy (MSA) [1]. Furthermore, αS-positive cytoplasmic aggregates have been reported in astroglial cells of PD and DLB as well as MSA [2]. Indirect evidence has suggested that the diverse nature of α-synucleinopathies may be characterized by distinct conformers (or strains) of αS aggregates [3–9]. What is more, structural analysis has revealed the presence of different filament structures of αS aggregates derived from MSA and DLB brains [10]. A majority of aggregated αS is phosphorylated at serine 129 (p-αS) [11], therefore antibodies directed against p-αS are commonly used as a surrogate marker of αS pathology.

αS is a 140 amino acid protein and is primarily expressed in neurons where it is enriched at the presynaptic terminal [1, 12]. Several missense mutations in the *SNCA* gene encoding αS have been linked to rare familial forms of PD and DLB. The amino acid substitutions alanine-to-threonine at codon 53 (A53T) and alanine-to-proline at codon 30 (A30P) both give rise to early-onset PD [13, 14]. Morphological differences between A53T- and A30P-mutated αS fibrils have been demonstrated in vitro [15–18], although their relevance for human disease pathogenesis remains uncertain. Subsequently, numerous transgenic (TG) mouse models overexpressing human A53T or A30P αS under various promoters have been generated that develop neuronal Lewy-like pathology and motor symptoms that resemble PD [19–21].

Here, we compare disease progression, as well as cellular and structural features of αS lesions in four TG lines: Prnp-h[A53T]αS (in the literature also referred to as 'M83') [22], Thy1-h[A53T]αS [23], Thy1-h[A30P]αS [24, 25], and Thy1-mαS TG mice [26]. While disease onset and progression differed among the TG lines, morphological appearance and regional distribution of αS lesions did not reveal robust differences. Intriguingly, however, in addition to the neuronal αS lesions, we found abundant αS-immunoreactive inclusions in microglia and this in all four TG mouse lines. Microglial inclusions differed from neuronal inclusions in morphological and conformational features.

Materials & methods

Mice

The following TG mouse lines were used: Prnp-h[A53T]αS [22], Thy1-h[A53T]αS [23], Thy1-h[A30P]αS [24], and Thy1-mαS [26]. The Prnp-h[A53T]αS line expresses human (h) αS with the A53T mutation under the control of the mouse prion protein promoter (Prnp) generated on the C57BL/6 x C3H background. Hemizygous Prnp-h[A53T]αS mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and bred to generate homozygous offspring for the study. The Thy1-h[A30P]αS line expresses human αS with the A30P mutation under the control of the neuron-specific murine Thy-1 promoter generated on the C57BL/6 J background. These mice are routinely maintained in our mouse facility and homozygous mice were produced by breeding homozygous pairs. The Thy1-h[A53T]αS line expresses the human αS transgene harboring the A53T mutation under the control of the murine Thy-1 promoter and the Thy1-mαS line is transgenic for an overexpression of the mouse (m) wildtype αS driven by the murine Thy-1 promoter, each of those lines was generated on the C57BL/6 J background. Both lines were obtained from Novartis (Basel) and transferred to our facility. All Thy1-h[A53T]αS and Thy1-mαS mice used in the studies were hemizygous and produced by breeding hemizygous males with C57BL/6 J females. Care was taken that both male and female mice were used at an equal proportion for all the experiments but their use was subjected to availability. All mice were kept under specific pathogen-free conditions and maintained on a 12 h light/dark cycle with food and water ad libitum. The experimental procedures were undertaken in compliance with the veterinary office regulations of Baden-Württemberg (Germany) and approved by the local Animal Care and Use Committees.

Determination of symptom onset, disease duration, and humane endpoint

A score sheet with a grading scale was used to evaluate and record the occurrence of motor signs in these mice. Rapid changes in body weight were used as clinical parameters to define the humane endpoints (i.e. loss of > 20% of the initial weight). For that purpose, mice were weighed weekly and checked for the onset of motor symptoms by using established criteria for neurodegenerative phenotypes in mice [27]. The behavioral assessment of mice was done first in open cages where the

general activity and movements were observed. Mice were then placed on a grid to check for motor impairment and to assess putative signs of ill health. The symptomatic phase typically comprised several stages of severity. Initially, the mice showed a disturbance in balance and gait, culminating in ataxia. As the movements became slower, tremor and rigidity were often seen. At the end-stage of the illness, partial paralysis of hind limbs occurred, at which the mice were sacrificed. With the appearance of the first symptoms, mice were provided with wet food pellets in the cage. Disease duration was determined as the days between the occurrence of the first symptoms and above defined the-end-stage of the illness.

Tissue processing

Brains were removed after the animals were deeply anesthetized and transcardially perfused with ice-cold PBS (0.1 M). For immunohistochemistry, one brain hemisphere was immersion-fixed for 48 h in 4% paraformaldehyde with PBS, then cryoprotected in 30% sucrose in PBS for an additional 2 days. After freezing, 25 μm-thick sagittal sections were serially cut through the entire hemisphere using a freezing-sliding microtome (Leica Microsystems). The sections were stored at − 20 °C in cryoprotection solution (35% ethylene glycol, 25% glycerol in PBS). For biochemical analysis, the other hemisphere was immediately snap frozen on dry ice and stored at − 80 °C.

Brain extracts

Extracts were prepared as described previously [28]. The A30P extract was derived from spontaneously ill Thy1-h[A30P]αS females (16–20 months). After removal of the forebrain and cerebellum, the brainstem was immediately fresh-frozen on dry ice and stored at − 80 °C until use. Tissue was then homogenized (Precellys®24, Bertin Technologies, France) at 10% (w/v) in sterile, phosphate-buffered saline (PBS, Lonza, Switzerland), vortexed and centrifuged at 3000 *x* g for 5 min. The supernatant was aliquoted and immediately frozen. For all following experiments, the 10% (w/v) extract was used. The wildtype extract was derived from aged C57BL/6 J mice (24–26 months old).

Stereotactic injection of brain extracts

αS host mice were anaesthetized with a mixture of ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight) in saline and administered carprofen (5 mg/kg body weight) prior to surgery. Stereotactic injections were performed manually with a Hamilton syringe bilateral (2.5 μl of brain extract per side) into the hippocampus / dentate gyrus (AP − 2.5 mm, ML ±2.0 mm, DV − 1.8 mm) of Thy1-h[A30P]αS mice. Injection speed was 1.25 μl/minute. The needle was kept in place for an additional 2 min before it was slowly withdrawn. The surgical area was cleaned with sterile saline, the incision was sutured, and the mice were monitored until recovery from anesthesia. Injections were performed at the age between 2 and 4 months.

Histology and immunohistochemistry

In preparation of immunolabeling, the brain sections were washed with Tris-buffered saline (TBS, 0.1 M, pH 7.4) and mounted onto microscopic glass slides (SuperFrost Plus, Langenbrinck, Germany). After treating the sections with 3% H_2O_2 (Applichem, Darmstadt, Germany) in TBS for 30 min to block the endogenous peroxidase, antigenicity was enhanced by boiling the sections in 10 mM citrate buffer (1.8 mM citric acid, 8.2 mM trisodium citrate, pH 6.0) at 90 °C for 35 min. Unspecific binding sites were blocked by using 5% normal goat serum in 0.3% Triton-X100 (Sigma-Aldrich, Steinheim, Germany) in TBS for 30 min at RT. To detect accumulating αS phosphorylated at serine 129 (p-αS), primary antibody rabbit monoclonal anti-p-αS (EP1536Y, Epitomics, Burlingame, CA, USA) was used at 1:750 dilution and incubated overnight at 4 °C. The following day, biotinylated secondary antibody (goat anti-rabbit biotinylated IgG, Vector laboratories, Burlingame, CA, USA) was added at 1:400 onto the sections and incubated for 45 min at RT. Antibody binding was detected after sections were incubated in avidin-biotin solution for 45 min (Vector Laboratories). To develop the staining, SG Blue kit (Vector laboratories, Burlingame, CA, USA) was used as the chromogenic substrate for horseradish peroxidase. After immunolabeling, sections were counterstained with nuclear fast red (Sigma-Aldrich, Steinheim, Germany). Coverslipping was done with Pertex mounting medium (Pertex, Medite, Burgdorf, Germany) on dehydrated sections using an ascending ethanol series (50 to 100%) and xylene. Bright-field imaging was done using a Zeiss Axioplan 2 microscope (Carl Zeiss, MicroImaging GmbH, Jena, Germany).

Pathology grading of p-αS-positive inclusions

Brain pathology was quantified in a set of every 12th serial, sagittal sections of one hemisphere by assessing both perikaryal and neuritic p-αS-labeling. The brain regions of each section were analyzed and the rater determined a mean pathological severity. A semi-quantitative severity score was used in a four-graded scale: Absent (−), mild (+), moderate (++), and severe (+++) p-αS-positive pathology. The person who performed the analysis was blinded towards the mouse genotypes.

Immunofluorescence, Thioflavin S and pFTAA staining

Brain sections were washed with PBS (3 × 10 min) and mounted on super frost slides. Sections were allowed to air dry for 2 h at room temperature (RT). Mounted and air-dried brain sections were subjected to antigen

retrieval by boiling in 10 mM citrate buffer (1.8 mM citric acid, 8.2 mM trisodium citrate, pH 6.0) at 90 °C for 35 min for p-αS, or 80% formic acid for 1 min at RT for epitope specific α-synuclein antibodies, and treated with 5% normal goat or donkey serum in 0.3% Triton-X100 in TBS for 1 h at RT to block unspecific binding. Sections were incubated with primary antibodies (p-αS 1:750, Abcam EP1536Y, Cambridge, United Kingdom; Iba1 1:500, ThermoFisherScientific, Waltham, MA, USA; NeuN 1:500, Millipore, Darmstadt, Germany; αS 34–45 1:200, αS 80–96 1:100, αS 117–122 1:100, BioLegend, San Diego, CA, USA) overnight at 4 °C. The following day, Alexa Fluor 488, 568, or 633 conjugated secondary antibodies (Invitrogen, Waltham, MA, USA, 1:250) were added and incubated for 2 h at RT. Subsequently, labeling with pentamer formyl thiophene acetic acid (pFTAA; stock solution of 1.5 mM in de-ionized water, diluted to a final concentration of 3 μM in PBS) was performed as previously described [29]. Sections were treated for autofluorescence with TrueBlack Lipofuscin Autofluorescence Quencher (Biotium, Fremont, CA, USA) for 30 s at RT. For Thioflavin S (ThioS, Sigma-Aldrich, Steinheim, Germany) staining, sections were incubated for 8 min with 1% w/v ThioS in ddH_2O. ThioS-stained sections were washed 2 x in 70% EtOH for 3 min and rinsed with ddH_2O. After air-drying, the sections were coverslipped with Dako Fluorescence mounting medium (Biozol Diagnostika, Cat# S3023). Images were captured on a Zeiss LSM 880 (Zeiss, Oberkochen, Germany) confocal microscope equipped with a spectral scanner.

Spectral analysis of pFTAA staining

Emission spectra were acquired from 470 to 695 nm and normalized to their respective maxima [30, 31]. Spectra were collected from selected neuronal or microglial cytoplasmic pFTAA-positive inclusions within the brainstem. The ratio of the intensity of emitted light at the red-shifted (584 nm) versus the green-shifted (513 nm) portion was used as a parameter for spectral distinction of different inclusions. These two wavelengths were selected because differences in pFTAA emission were most pronounced for different αS aggregates. Both for neuronal and microglial inclusions, at least three different ROIs per image were calculated. For each mouse, all ROIs from three images were averaged and the mean was taken for statistical analysis (n = number of mice; 5–8 mice were analyzed per TG mouse line).

Immunoassay for αS measurements in brain homogenates

Concentrations of total (human and mouse) αS were determined by a colorimetric HRP-linked immunoassay using the SensoLyte™ Anti-Alpha-Synuclein Quantitative ELISA Kit (AnaSpec, 55550, Fremont, CA, USA). Measurement was conducted according to the manufacturer's instructions. In brief, formic acid-soluble half-brains were used at 1:2000 (for non-tg mice) or 1:10000 (for tg mice) in dilution buffer (Component C, AnaSpec), added to 96-well plates and co-incubated with detection antibody (1 μg/ml) overnight at 4 °C. After washing, tetramethylbenzidine substrate solution was added and incubated at room temperature until the color was clearly observable. Stop solution was added to block the reaction and absorbance was read promptly on a Mithras LB 940 plate reader (Berthold Technologies, Bad Wildbad, Germany).

Statistics and image analysis

Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). Statistical significance was assessed using ANOVA followed by Bonferroni's post-hoc test. Data were expressed as indicated in the figure legends. For survival analysis, log-rank test was used. Multiple comparisons of Kaplan-Meier curves were performed with Bonferroni correction. Survival curves were expressed as median incubation times (days). There was no difference between males and females in all the statistical analysis carried out, thus males and females were combined. The grading of p-αS pathology was performed using a semi-quantitative scale ranging from absent (–), mild (+), moderate (++), to severe (+++) by analysis of various brain regions of spontaneously ill TG mice (n = 3–5). Percentage of pFTAA-positive inclusions was quantified using in-house written ImageJ macro. Three randomly selected animals from each line were used for the analysis. Three images were analyzed per animal. For spectra and ratios, data were collected from randomly selected n = 8 for Prnp-h[A53T]αS, n = 8 for Thy1-h[A53T]αS, n = 8 for Thy1-h[A30P]αS, and n = 5 for Thy1-mαS) mice. Statistical significance was assessed using two-way ANOVA.

Results

Symptom onset, life span, and lesions among αS TG mouse lines

Disease characteristics (i.e., life span and disease duration) were recorded in Prnp-h[A53T]αS, Thy1-h[A53T]αS, Thy1-h[A30P]αS and Thy1-mαS mice. To this end, 15 animals per mouse line were aged and sacrificed when they displayed the characteristic end-stage neurological signs, i.e. progressive gait instability and/or partial paralysis of the hind limbs (see methods). Results revealed that the Thy1-h[A30P]αS mice are the most long-lived with a median life span of 580 days, followed by Prnp-h[A53T]αS mice (447 days) (Fig. 1a). By contrast, Thy1-h[A53T]αS and Thy1-mαS mice all showed symptoms at an earlier age (221 and 242 days, respectively) (Fig. 1a). The time interval between occurrence of first disease symptoms (slight disturbance in balance, jerky movements) and

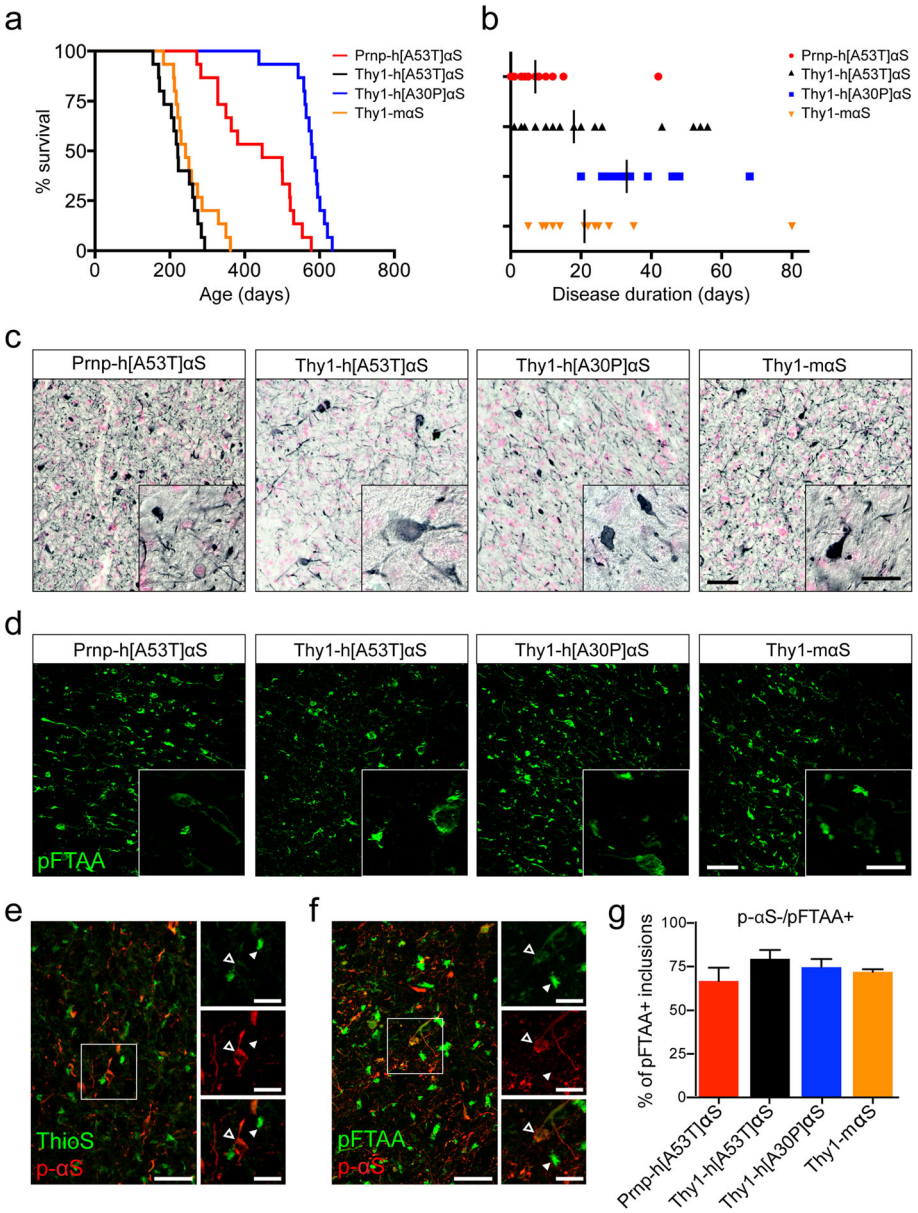

Fig. 1 Life span, disease duration, and end-stage αS lesions of different αS TG mouse lines. **(a)** Kaplan-Meier curves for the appearance of clinical end-stage motor signs in Prnp-h[A53T]αS (red curve, median 447 days, $n = 15$), Thy1-h[A53T]αS (black curve, median 221 days, $n = 15$), Thy1-h[A30P]αS (blue curve, median 580 days, $n = 15$), and Thy1-mαS (orange curve, median 242 days, $n = 15$). When survival times of TG lines were compared to each other pair-wise, statistically significant differences were found (Log-rank test, $p < 0.0001$) except for Thy1-h[A53T]αS vs. Thy1-mαS. **(b)** Disease duration starting from onset of motor signs until end-stage phenotype in Prnp-h[A53T]αS (red, median 7 days), Thy1-h[A53T]αS (black, median 18 days), Thy1-h[A30P]αS (blue, median 33 days), and Thy1-mαS (orange, median 21 days). When disease durations of TG lines were compared to each other pair-wise, only Prnp-h[A53T]αS and Thy1-h[30P]αS lines had a statistical difference in their disease duration (One-way ANOVA, Bonferroni's multiple comparison test, $p < 0.0001$). **(c)** Immunostaining of inclusions labeled with the p-αS antibody, which recognizes phosphorylated αS at serine 129, in Prnp-h[A53T]αS, Thy1-h[A53T]αS, Thy1-h[A30P]αS, and Thy1-mαS mice. Nuclear fast red was used as counterstain. Representative sagittal sections of the midbrain from 12-, 7.3-, 20.8-, and 8.3-month-old mice, respectively, are shown. Scale bars, 50 μm and 20 μm (insert). **(d)** Representative images of pFTAA-positive inclusions in the brainstem of terminally ill Prnp-h[A53T]αS, Thy1-h[A53T]αS, Thy1-h[A30P]αS, and Thy1-mαS mice. Scale bars, 50 μm and 20 μm (insert). **(e)** Fluorescence double-staining for p-αS (red) and ThioS (green) of brainstem pathology in Thy1-h[A30P]αS. Examples of p-αS-positive inclusion (arrowhead outlines) and ThioS-positive aggregate (white arrowheads) are shown in high magnification (inserts). **(f)** Fluorescence double-staining for p-αS (red) and pFTAA (green) of brainstem pathology in Thy1-h[A30P]αS. Note that many of the pFTAA-positive inclusions are not co-labeled with the p-αS antibody. Examples of a p-αS/pFTAA-double-positive inclusion (arrowhead outline) and a pFTAA-positive deposit in absence of p-αS signal (white arrowhead) are in high magnification (inserts). **(g)** Percentage of pFTAA-positive inclusions that are lacking p-αS signal. A similar proportion of non-overlapping pFTAA signal among the lines was found ($n = 3$ mice per mouse line). Results are expressed as mean ± SEM

symptom-related time of sacrifice was defined as the disease duration (Fig. 1b). Of note, the shortest symptomatic phase was observed in Prnp-h[A53T]αS mice and lasted only 7 days.

Brain pathology of the four αS TG mouse lines was examined after the mice were sacrificed. All four lines revealed neuronal p-αS-positive inclusions in both cell soma and neurites. The lesions were most prominent in the brainstem (Fig. 1c). Other regions, including zona incerta, deep cerebellar nuclei showed moderate amounts of αS inclusions, while only sparse αS inclusions were found in the frontal cortex (Supplementary Fig. 1, Supplementary Table 1). Of note, the hippocampus was devoid of any αS pathology in all four lines (Supplementary Fig. 1, Supplementary Table 1). Overall, end-stage αS lesions did not reveal major differences in morphological appearance and regional distribution between the mouse lines (despite some differences in αS levels, Supplementary Fig. 2).

Luminescent conjugated oligothiophenes (LCOs) are dyes that bind to cross-β-sheet structures. These dyes have a flexible backbone that allows changes in their spectral properties depending on the amyloid conformation [32–34]. Recently, pFTAA has been used to detect aggregated αS species in vitro and in vivo [35–37]. pFTAA-staining was performed in all four TG lines and pFTAA-positive inclusions were found most robustly in the brainstem, as was the case for p-αS staining (Fig. 1d) but also in all other brain regions with p-αS-positive inclusions. However, double-staining for p-αS and pFTAA was only partially overlapping, and in absence of the p-αS signal pFTAA-positive inclusions appeared much brighter than inclusions that were also p-αS-positive (Fig. 1f). Similar results were observed when stained for p-αS and ThioS (Fig. 1e). Moreover, pFTAA-positive/p-αS-negative inclusions appeared as a compact "ball of threads" and are henceforward referred to as "wool-like inclusions" (Fig. 1f, inserts). In all four lines between 70 and 75% of pFTAA-positive inclusions were p-αS negative (Fig. 1g). No pFTAA-positive staining was found in aged C57BL/6 J wild type (WT) mice (Supplementary Fig. 3).

pFTAA-positive inclusions are found in microglia and are distinct from neuronal αS aggregates

To study the cellular association of the pFTAA-positive inclusions, co-staining for pFTAA and for either the neuronal marker NeuN, the microglia marker Iba1 (Fig. 2), or the astrocytic marker GFAP (data not shown) was performed. As expected, pFTAA-signal that co-localized with NeuN-positive cells appeared morphologically similar to the p-αS staining (Fig. 2a). The pFTAA-positive inclusions that co-localized with Iba1 again had a bright wool-like appearance (Fig. 2b). Some pFTAA-positive structures were observed in astrocytes however by far less abundant than in neurons or microglia (data not shown). Quantification revealed that approximately 25% of total microglia contained pFTAA-positive inclusions and this was similar for all four TG lines (Fig. 2c).

To study conformational differences of inclusions between cell types, spectral analysis (pFTAA/NeuN vs pFTAA/Iba1) was performed (Fig. 2d, e). Spectra were obtained from perikaryal neuronal or microglial inclusions. For all TG mouse lines, there were robust spectral differences between the aggregates in microglia and neurons. However, there was no difference in the spectral signature of the neuronal or microglial inclusions between the lines. These results indicate that microglial inclusions are part of the pathophysiology observed in TG mice and that they are conformationally distinct from neuronal aggregates.

Microglial inclusions comprise C-terminally truncated αS

To assess whether the pFTAA-positive inclusions found in microglia contain αS, double-labeling of the microglial marker Iba1 together with a panel of commercially available anti-αS antibodies specific for both termini and the non-amyloid component (NAC) domain was performed (Fig. 3). Antibodies specific for the N-terminus (epitope between amino acids 34–45) and the NAC region (amino acids 80–96) abundantly co-localized with Iba1-positive microglia, whereas antibodies specific for the C-terminus (amino acids 117–122) and p-αS did not co-localize (Fig. 3). These observations suggest that microglial inclusions contain C-terminally truncated αS. Notably, on some occasions, αS C-terminal-positive structures were found to be associated with Iba1-positive microglia. However, in these instances, these inclusions had a different appearance that was reminiscent of microglial engulfment of αS inclusion-positive structures (Fig. 3d and e, enlarged images).

Microglial inclusions are already present in presymptomatic TG mice

To investigate whether αS inclusions in microglia are a feature of end-stage pathology or if they develop alongside neuronal αS lesions before the first motor signs occur, mice were analysed at presymptomatic stage (Fig. 4a, b). Due to its extended survival time, the Thy1-h[A30P]αS mouse line was initially chosen for this analysis (Fig. 1a). Intriguingly, at the time of the first p-αS-positive neuronal inclusions of around 15 months of age there were also scarce pFTAA-positive microglial inclusions present (Fig. 4a, b). Presymptomatic Thy1-h[A53T]αS also showed similar co-occurrence (Supplementary Fig. 4). This indicates that neuronal and microglial inclusions may develop around the same time, which further highlights a potential role of microglia in pathology.

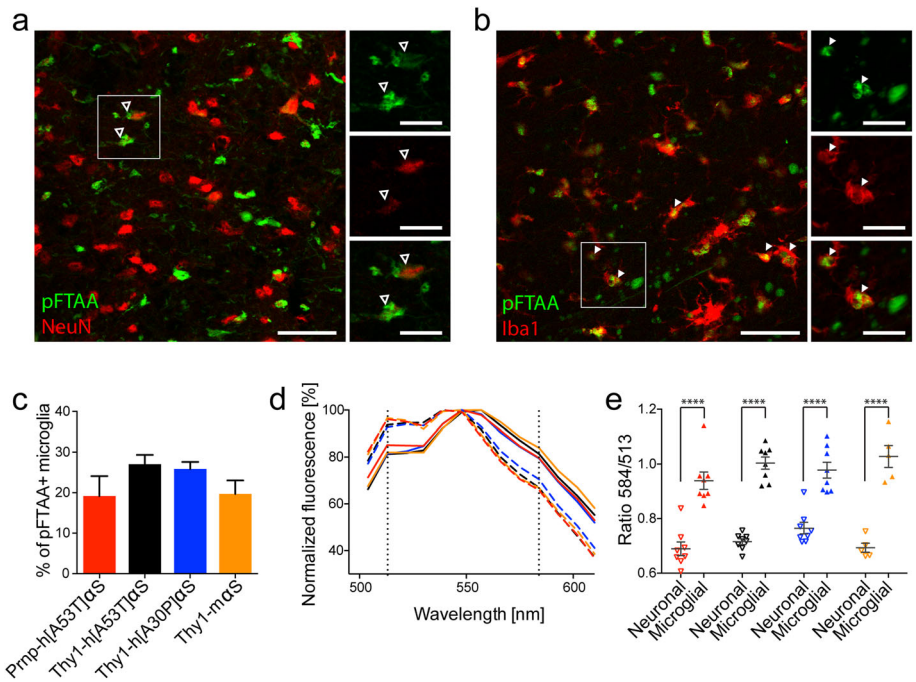

Fig. 2 Distinct pFTAA-positive inclusions in neurons and microglia of symptomatic αS TG mice. **(a)** Fluorescence double-staining for pFTAA (green) and NeuN (red) of brainstem pathology in Thy1-h[A30P]αS. Examples of neuronal pFTAA-positive inclusions around the nuclei are shown (arrowhead outlines). Scale bars, 50 μm and 20 μm (inserts). **(b)** Fluorescence double-staining for pFTAA (green) and Iba1 (red) of brainstem pathology in Thy1-h[A30P]αS. Examples of microglial pFTAA-positive inclusions are shown (arrowheads). Scale bars, 50 μm and 20 μm (inserts). **(c)** The percentage of microglia containing pFTAA-positive inclusions for all mouse lines. The proportion of pFTAA-/Iba1-double-positive cells was not significantly different between the lines ($n = 3$ mice per line). Results are expressed as mean ± SEM. (**d**, **e**) Spectral analysis of pFTAA-positive inclusions in neurons and microglia. **(d)** Mean emission spectra of NeuN-positive (dotted lines) and Iba1-positive (solid lines) deposits in Prnp-h[A53T]αS (red, $n = 8$), Thy1-h[A53T]αS (black, $n = 8$), Thy1-h[A30P]αS (blue, $n = 8$), and Thy1-mαS (orange, $n = 5$). Vertical black dotted lines represent the first peak and the shoulder of pFTAA spectra at wavelengths of 513 and 584 nm, respectively. **(e)** The ratio of emission intensity at wavelengths 513 and 584 nm calculated to show the spectral shift of pFTAA upon binding to neuronal (empty triangles) and microglial (solid triangles) inclusions in Prnp-h[A53T]αS (red, $n = 8$), Thy1-h[A53T]αS (black, $n = 8$), Thy1-h[A30P]αS (blue, $n = 8$), and Thy1-mαS (orange, $n = 5$). Two-way ANOVA (cell type x mouse line) revealed a significant effect for cell type [$F(1,50) = 218$, $^{****}P < 0.0001$], but not for mouse line [$F(3,50) = 2.156$, $P = 0.1049$] or interaction between cell type and mouse line [$F(3,50) = 1.842$, $P = 0.1516$]. The results are expressed as mean ± SEM

Seeded induction of neuronal αS inclusions is also accompanied by microglial inclusions

To further study the link between neuronal and microglial αS inclusions, neuronal αS pathology was induced in young, presymptomatic Thy1-h[A30P]αS mice by seeding [28]. To this end, brainstem extract from end-stage Thy1-h[A30P]αS TG mice or brain extract from WT mice were injected into the hippocampus of 2–4-month-old Thy1-h[A30P]αS mice (Fig. 4c-e). As expected, 30 days post-injection, mice inoculated with TG brainstem homogenate revealed p-αS-positive neuronal inclusions around the injection site (Fig. 4c). In addition, pFTAA-positive microglial inclusions were also present in vicinity of the injection site (Fig. 4e). In contrast, mice injected with control WT brain homogenate did not develop any neuronal or microglial lesions (Fig. 4d).

Discussion

The initial aim of the present work was to study disease progression and features of αS lesions among TG mouse models of α-synucleinopathies and their correlation with αS conformers. The mouse lines revealed major differences in age-of-symptom onset and disease progression. Postmortem analysis though revealed an overall very similar appearance and distribution of the αS lesions in all the lines. However, strikingly, in addition to neuronal lesions, we found αS-positive inclusions in microglia in all four lines. Although it had not been reported with such an abundance before, previous studies made note of apparent αS aggregates in microglia in viral vector-based and TG αS-overexpressing mouse models after seeding [38–41].

This unexpected finding of robust inclusions in microglia in αS TG mice was initially made through the analysis with the amyloid-binding dye pFTAA and was subsequently confirmed through ThioS-positive labeling. LCOs have previously been reported to bind and discriminate structural variants of PrP [32], Aβ [33, 42], and tau aggregates [29, 43]. More recently, studies also showed that αS aggregates can be detected in solution and in an

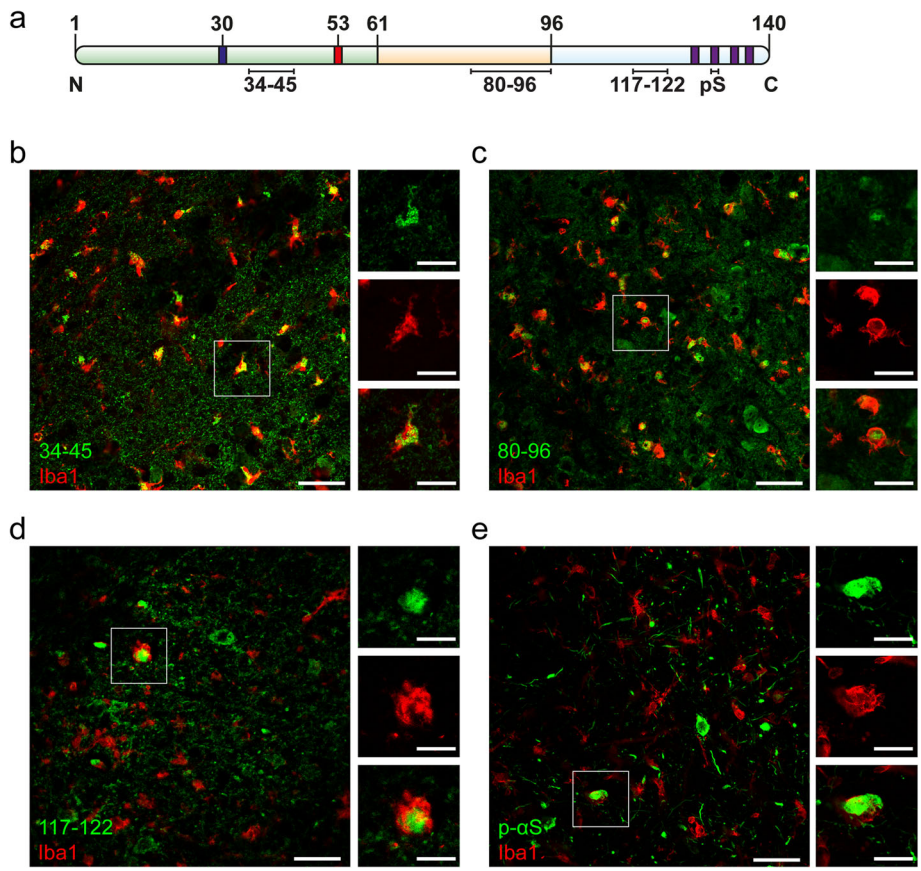

Fig. 3 Characterization of microglial αS inclusions in symptomatic αS TG mice. **(a)** Schematic of αS showing the N-terminal region (light green) with PD-linked mutations A30P (blue) and A53T (red), NAC domain (light orange) and C-terminus (light blue) with four phosphorylation sites (purple). Black lines indicate the epitopes of antibodies specific for the N-terminus (34–45), NAC domain (80–96), C-terminus (177–122), and p-αS (phosphorylated αS at serine 129) that were used for immunofluorescence staining. **(b-e)** Co-immunofluorescence staining for Iba1 (red) and epitope-specific αS antibodies (green) in the brainstem of terminally ill Thy1-h[A30P]αS. (**b**) Section of Iba1-positive microglia with 34–45-positive αS aggregates. Note that most Iba1-positive cells are also labeled with anti-αS antibody. Complete overlapping signal of Iba1 and anti-αS 34–45 antibodies in the enlarged images. (**c**) Section of Iba1-positive microglia with 80–96-positive αS inclusions. Note that most Iba1-positive cells are co-localized with anti-αS antibody. Complete overlapping signal of Iba1 and anti-αS 80–96 antibodies in the enlarged images. (**d**) Section of Iba1-positive microglia and 117–122 anti-αS antibody. Note that most Iba1-positive cells are devoid of 117–122 anti-αS antibody staining. A rare occasion with partial overlap of Iba1 and 117–122 anti-αS signal is demonstrated in the enlarged images suggesting microglia phagocytosing αS-positive structure. (**e**) Section of Iba1-positive cells labeled with p-αS antibody. Note that most of the Iba1-positive cells are p-αS-negative. Similar to (d), a rare occasion with partial overlap of Iba1 and anti-p-αS signal is demonstrated in the enlarged images suggesting microglia phagocytosing αS-positive structure. Scale bars, 50 μm and 20 μm (enlarged images)

in vitro seeding assay using pFTAA [35, 37] and in disease samples using other LCOs [7, 8, 44]. Although we did not succeed to distinguish A53T from A30P αS aggregates readily with pFTAA, we found that spectral analysis using pFTAA could clearly distinguish the neuronal from the microglial inclusions suggesting conformational differences of the inclusions between these cell types.

Differences in structural features between neuronal and microglial inclusions are also in line with stainings using different αS antibodies. While N-terminal- and NAC domain-specific αS antibodies also labeled microglial inclusions, the C-terminal-specific antibody did not detect microglial inclusions (including p-αS at serine 129). Therefore, microglial inclusions are easily overlooked when using p-αS antibodies. Notably, in rare cases, we also observed microglia-associated inclusions that were p-αS-positive. However, in most such instances, inclusions appeared to be part of a neuronal element that appeared to be engulfed actively by a microglial cell [39]. This observation is reminiscent of microglial phagocytosis of neurons filled with tau filaments [45].

Our data suggest that there is a link between the neuronal and microglial inclusions in αS TG mice, since they always co-occur and we never observed only neuronal or only microglial αS inclusions. Also, at early presymptomatic stages or upon seeded induction of αS inclusions, neuronal inclusions were always accompanied by

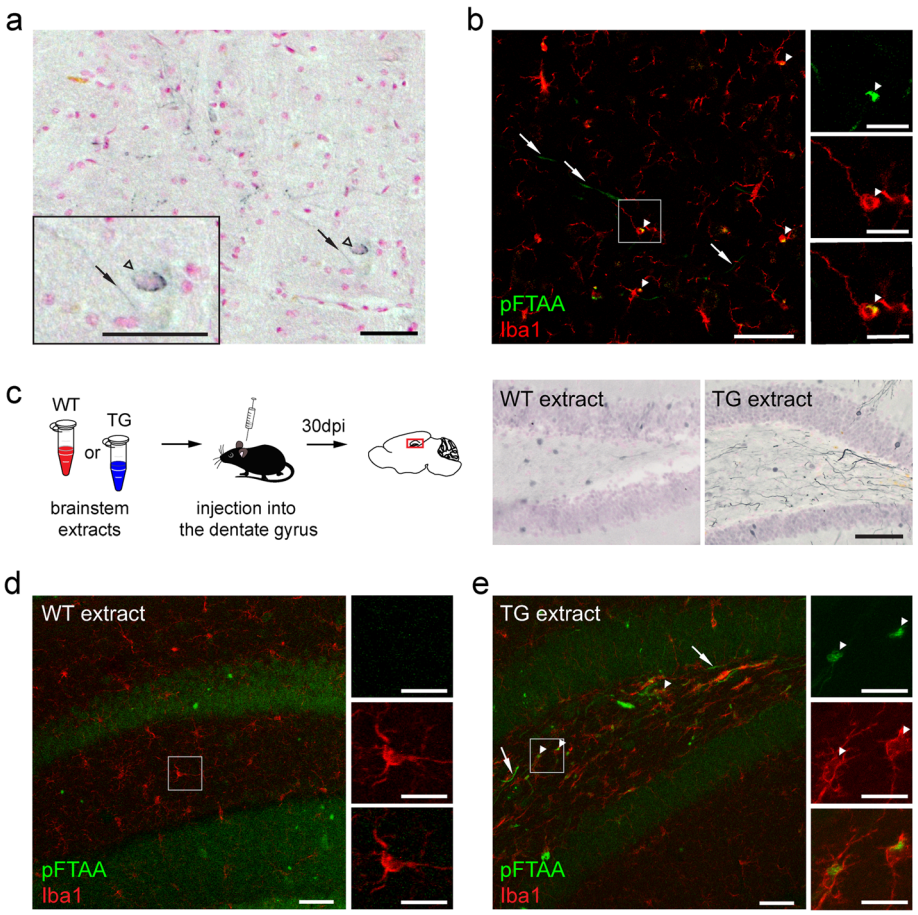

Fig. 4 pFTAA-positive microglia in presymptomatic αS TG mice and seeded αS induction model. **(a, b)** Representative brainstem sections of a 15-month-old presymptomatic Thy1-h[A30P]αS mouse, presumed 4–6 weeks before the first symptoms occur. (**a**) Immunostaining of p-αS-positive aggregates (black). Perikaryal (arrowhead outlines) and neuritic (arrows) inclusions are highlighted. Section was counterstained using nuclear fast red. Scale bars, 50 μm and 20 μm (insert). **(b)** Fluorescence double staining of Iba1-positive microglia (red) and pFTAA-positive inclusions (green), showing neuritic pFTAA-positive inclusions (arrows) and pFTAA-positive aggregates in Iba1-positive cells (arrowheads). Example of a pFTAA-positive microglia is shown in high magnification (inserts). Scale bars, 50 μm and 20 μm (inserts). **(c)** Schematic illustration of the intracerebral injection paradigm (left) and sagittal sections of dentate gyrus (DG, site of injection) stained with p-αS antibody (blue) from Thy1-h[A30P]αS mice that have been injected 30 days prior with either brainstem extract from end-stage TG mice (TG extract) or brain extract from WT mice (WT extract). In TG extract-injected mice, abundant p-αS-positive aggregates were detected. In contrast, WT extract did not induce any inclusion. Sections were counterstained using nuclear fast red. Scale bar, 100 μm. **(d, e)** Representative sagittal sections from either WT extract- (**d**) or TG extract-injected (**e**) Thy1-h[A30P]αS mice stained with pFTAA (green) and Iba1 (red). (**d**) No pFTAA-positive inclusions can be found in microglia of WT extract-injected mice. Scale bars, 50 μm and 20 μm (enlarged images). **(e)** In contrast, pFTAA-positive inclusions are found in Iba1-positive microglia in DG from Thy1-h[A30P]αS mice. Examples of neuritic pFTAA-positive inclusions (arrows) and pFTAA-positive deposits in microglia (arrowheads) are highlighted. Scale bars, 50 μm and 20 μm (enlarged images)

microglial inclusions in close vicinity of the neuronal inclusions. It is possible that microglial inclusions are the results of phagocytosed neuronal elements or uptake of neuron-released αS [39, 41] with subsequent removal of the C-terminus, which leads to structural rearrangement and changes in pFTAA emission spectra. It is also conceivable that microglia take up neuronally-secreted soluble oligomeric αS species [46], which then assemble within the microglia to filamentous αS aggregates. It is known that different cellular environments influence the composition and conformation of proteopathic seeds [47], exemplified by αS aggregates in oligodendrocytes that are more compact and reveal higher seeding potency than their neuronal counterparts [6]. Finally, αS is expressed at low levels in microglia under homeostatic conditions [48]. It is therefore plausible that activated microglia upregulate αS expression and that microglial inclusions are partly formed by aggregation of microglia-generated αS.

An abundance of microglial αS inclusions described here has not been reported in humans [1], albeit very recently, microglia αS inclusions in the human olfactory bulb of PD patients have been described [49].

Furthermore, seeding-prone αS species were detected in human microglial exosomes isolated from CSF of sporadic PD and MSA patients [50] and microglia are involved in the spreading of αS lesions [51, 52]. These studies raise the possibility that αS aggregates in microglia in α-synucleinopathies are more common than previously thought and that they may also contribute to disease progression. If, however, abundant microglial αS inclusions turn out to be restricted to αS overexpressing TG mouse models, this knowledge is important when αS TG models are utilized in preclinical-translation studies.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s40478-020-00993-8.

Additional file 1: Figure S1 Histopathology in αS TG mouse lines. **Figure S2.** Total brain αS levels in TG and WT mice. **Figure S3.** Negative controls for p-αS immunohistochemistry and pFTAA staining. **Figure S4.** p-αS immunohistochemistry and pFTAA staining in presymptomatic Thy1-h[A53T]αS mice. **Table S1.** Grading of p-αS-positive αS pathology in TG mice

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Authors' contributions

GT, M. Bacioglu, MS, DK-V, and YSE designed and performed mouse experiments; M. Bacioglu and MS designed and performed intracerebral injections; GT, M.Bacioglu, MS, JM, UO, and M.Barth performed histology and immunochemistry. GT, M. Bacioglu, MS, JM, BMW-B, and AS. analysed data; KPRN provided the amyloid-binding dye pFTAA; DRS provided Thy1-h[A53T]αS and Thy1-mαS mice; PJK provided Thy1-h[A30P]αS mice; GT, M. Bacioglu, MS., YSE, and MJ designed the overall study and wrote the manuscript. All other co-authors edited the manuscript. The author(s) read and approved the final manuscript.

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Availability of data and materials

The authors will provide upon request raw data and material (some of them via material transfer agreement).

Competing interests

The authors declare no conflict of interest.

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4 MICROGLIA DEPLETION AND THE DIFFERENTIAL EFFECT ON A-SYNUCLEIN SEEDING IN LONG-TERM MURINE HIPPOCAMPAL SLICE CULTURES

III. Microglia depletion and the differential effect on α-synuclein seeding in long-term murine hippocampal slice cultures

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ABSTRACT

Microglia, the immune cells of the brain, play an important role in regulating brain homeostasis and the failure of this function is closely related to neurodegenerative diseases. A subgroup of neurodegenerative diseases, called synucleinopathies for their characteristic intracellular deposits of aggregated α-synuclein (αS), does not remain unaffected by their failure. The recent discovery of aggregate-laden microglia in transgenic (tg) mouse models of synucleinopathies draws the attention of the field to the contribution of various brain cells to the process of αS aggregate formation and the underlying mechanisms. Our study focuses on elucidating the role of microglia in neuronal αS pathology using a seeding model of murine hippocampal slice cultures (HSCs) as well as microglial depletion and repopulation. Preliminary results indicate that reversible depletion of microglia with PLX5622 before seeding did not have an effect on the development of neuronal aggregates. However, when the seeded cultures were depleted of microglia during the expansion of pathology, fewer neurons showed αS inclusions. This work suggests that microglia do not interfere with the initial seeding of the first affected neurons, but might play a role in the inter-cellular spreading of the pathology.

INTRODUCTION

The pathological hallmarks of synucleinopathies, including Parkinson's disease (PD), dementia with Lewy bodies (DLB), and multiple systems atrophy (MSA) are the accumulation of misfolded α-synuclein (αS) into intraneuronal Lewy bodies (LB) and Lewy neurites (LN) in PD and DLB, and Papp-Lantos bodies in oligodendroglia in MSA (Goedert et al. 2017). In addition to the abundance of αS assemblies, missense mutations in the αS gene, SNCA, are known to cause familial PD (Polymeropoulos et al. 1997; Zarranz et al. 2004; Appel-Cresswell et al. 2013; Proukakis et al. 2013; Kiely et al. 2013; Lesage et al. 2013; Martikainen et al. 2015; Pasanen et al. 2014; Yoshino et al. 2017; Krüger et al. 1998), which support the notion that αS plays a key role in the pathogenesis. In experimental models, αS has been shown to spread intercellularly and from one brain region to another (Danzer et al. 2009; Desplats et al. 2009), reminiscent of the pathological spreading pattern identified in the majority of PD brains (Braak et al. 2003b). Numerous studies have demonstrated a prion-like mechanism of αS pathology by templated amplification and propagation of the misfolded protein (Ma et al. 2019; Luk et al. 2012a; Masuda-Suzukake et al. 2013; Rey et al. 2013; Freundt et al. 2012; Bieri et al. 2018).

In a recent study, we found abundant microglial inclusions, in addition to neuronal αS pathology, in αS transgenic mice (Tanriöver et al. 2020). These inclusions were structurally different from neuronal inclusions, possibly resulting from C-terminal truncation of αS. Therefore, microglial inclusions could be identified by the strong binding of amyloid-dye Thioflavin S (ThioS), as opposed to neuronal inclusions characterised by the phosphorylation of αS at serine 129 (pS129). Interestingly, we microglial inclusions to be present already at pre-symptomatic stages of pathology. These results left us wondering if microglia might contribute to pathogenesis, e.g. by releasing their own aggregated αS species and therefore amplify the prion-like propagation. To investigate the impact of microglia on αS seeding, we designed an experimental setup in an αS seeding model of hippocampal slice cultures (Barth et al., *submitted*), that allowed us to deplete microglia reversibly with the CSF1R inhibitor PLX5622, and examine the effect on αS pathology at different time points of microglial and neuronal inclusion formation.

RESULTS & DISCUSSION

Microglial inclusions appear secondarily to the development of abundant neuronal inclusions

Murine hippocampal slice cultures (HSCs) were prepared from new-born Thy1-hA53TαS tg mice and grown in culture for 10 days to stabilize. The formation of αS inclusions was induced by one-time seed application of αS pre-formed fibrils (αS pff) to HSCs, as described previously (Barth et al, *submitted*). To determine the temporal order of the development of neuronal and microglia αS inclusions, we analysed the time course of inclusion appearance (Figure 1 A). Already after 1 week post αS seeding, perikaryal and neuritic αS lesions had been induced that were phosphorylated at serine 129 (pS129), a common marker of aggregated αS (Fujiwara et al. 2002). At this time point, very few Thioflavin S (ThioS) positive microglial inclusions were visible. The amount of neuronal inclusions increased until its apex at 3 weeks post treatment, but was reduced after 5 weeks (Figure 1B, C), possibly because of the degeneration of αS filled neurons (Osterberg et al. 2015). On the other hand, ThioS positive amyloid structures appeared with a delay to the emergence of neuronal inclusions, starting to develop from week 2 on, particularly in hippocampal subregion CA1. Moreover, they did not seem to diminish within the investigated time frame, with the highest level of inclusions at 5 weeks post seeding (Figure 1 B, C).

PLX5622 treatment depletes microglia reversibly

To investigate the time course of microglial depletion and repopulation with the CSF1R inhibitor PLX5622, we treated HSCs with PLX5622 for 7 days and counted microglia at different time points after treatment end (Figure 2 A). Immediately after treatment, nearly all microglia were depleted, with only few amoeboid shaped cells left (Figure 2 B, insert). To repopulate the slice cultures, microglia required 2 weeks regeneration time with PLX5622 free medium (Figure 2 B, C), which is in line with other studies (Coleman et al. 2020). After 2 weeks regeneration, many, but not all, cultures showed a microglia population density similar to the DMSO-treated control HSCs, whose microglial population naturally declines by 30% after 2 weeks in culture (Figure 2 C).

Microglia depletion at different time points with respect to seeding might influence development of neuronal and microglial αS inclusions

Next, we depleted microglia population at different timepoints before or after seeding with αS pff to study the differential effects on the time course of hyperphosphorylated neuronal, and microglial αS inclusion development (Figure 3 A, B), and compared with seeded cultures that were permanently depleted of microglia (Figure 3 C) or kept under control conditions without depletion (Figure 3 D). At 3 weeks post seeding, activated microglia had repopulated in pre-seed PLX5622 treated cultures (Figure 3 A), but were reduced in the post-seed treatment group (Figure 3 B). Notably, the development of ThioS positive microglial inclusions at 3 weeks post seeding is strikingly reduced in both pre-seed and post-seed PLX5622 treated cultures, (Figure 3 A, B). Strikingly, in permanently with PLX5622 treated cultures, microglia were completely depleted for the entire time (Figure 3 C), highlighting that these aggregates are exclusively located in microglia, as suggested in an earlier study (Tanriöver et al. 2020). Moreover, while post-seed PLX5622 treatment lead to a short-term

reduction of microglial αS inclusions, 2 weeks later, after fully recovery, the phagocytic cell population was again teeming with ThioS positive inclusion (Figure 3 B). This could be explained by a continuation of αS uptake from neurons, which would suggest that microglial αS inclusion formation is dependent on the release of neuronal αS.

Next, we wondered if microglia might also be crucial for the development of neuronal inclusions. Therefore, we examined the amount of phosphorylated αS at 3 weeks post seeding, the apex of neuronal inclusion formation. Preliminary results suggest that pre-seed microglia depletion does not lead to a decrease of phosphorylated αS inclusions (Figure 3 A), but might rather result in a temporary increase, possibly due to a reduced competition for seed uptake by neurons (George et al. 2019). Conversely, microglial depletion just before the apex of neuronal αS inclusions appears to reduce the amount of neuronal inclusions at 3 weeks post seeding (Figure 3 B), which can also be observed after permanent depletion (Figure 3 C). This result could indicate that microglia contribute to the development of neuronal pathology.

Taken together, this work implies that microglial presence plays a role in the pathology spreading of αS. There is evidence that microglia affect neuron-to-neuron transfer of αS by changing the extracellular environment to an inflammatory state (George et al. 2019). On the other hand, it has been suggested that microglia are able to contribute directly to αS transmission via exosomal αS release (Xia et al. 2019; Guo et al. 2020). Further experiments need to be conducted to evaluate which of the two scenarios is causative, or if both have an impact. In the future, this experimental setup is hopefully going to answer some questions regarding the importance of microglia and their effect on seeding and spreading of neuronal inclusions.

METHODS

Mice

Heterozygous Thy1-hA53TαS tg (van der Putten et al. 2000) pups were used for preparation of hippocampal slice cultures (HSCs). Thy1-hA53TαS tg mice overexpress human mutant (A53T) αS under control of the Thy1-promoter. Experimental procedures were carried out in accordance with the veterinary office regulations of Baden-Wuerttemberg (Germany) and were approved by the local Animal Care and Use Committees.

Preparation of mouse hippocampal slice cultures (HSCs)

HSCs were prepared from murine pups at postnatal day 4-6 (P4-6) according to previously published protocols (Mayer et al. 2005; Novotny et al. 2016). In brief, pups were decapitated, brains were aseptically removed, the hippocampi were dissected and cut perpendicular to the longitudinal axis into 350 µm sections with a McIllwain tissue chopper. Carefully selected intact hippocampal sections were transferred into petri dishes containing an ice-cold buffer solution (minimum essential medium (MEM) supplemented with 2 mM GlutaMAX^{TM} at pH 7.3). Three sections were placed onto a humidified porous polyethylene (PTFE) membrane insert (Merck Millipore, PICM0RG50) and into a 6-well plate with 1.2 ml culture medium (20 % heat-inactivated horse serum in 1x MEM complemented with GlutaMax^{TM} (1 mM), ascorbic acid (0.00125 %), insulin (1 µg/ml), $CaCl_2$ (1 mM), $MgSO_4$ (2 mM) and D-glucose (13 mM) adjusted to pH 7.3) per well. HSCs were kept at 37°C in humidified 5% CO_2-enriched atmosphere. The culture medium was completely changed three times per week.

αS pre-formed fibrils

Expression in *E. coli*, purification and quality control of human recombinant monomeric wt αS was done as previously described (Bousset et al. 2013). For fibril formation, soluble wt αS was incubated in Tris-HCl buffer (50 mM Tris-HCl, pH 7.5, 150 mM KCl) at 37 °C under continuous shaking for 5 days and formation of fibrils was assessed with Thioflavin T. The fibrils were quality checked by transmission electron microscopy after negative staining before and after fragmentation. Their limited proteolytic pattern was also assessed (Bousset et al. 2013). The average size of the fibrils after fragmentation 47 ± 5 nm was derived from length distribution measurements and their average molecular weight (16 200 kDa) was derived from analytical ultracentrifugation sedimentation velocity measurements. The fibrils (350 µM) were aliquoted (6 µl per tube), flash frozen in liquid nitrogen and stored at -80°C.

Seeding of the cultures

Murine HSCs were kept for 10 days *in vitro* (DIV-10) without any experimental treatment. At DIV-10, 1 µl of αS pff (35 µM) was pipetted on top of each culture.

PLX5622 treatment

PLX5622 wasused for HSC microglia depletion. PLX5622 diluted in DMSO and added to the medium at a final concentration of 10 µM PLX6522 and 0.2 % DMSO. In control groups, culture medium was only supplemented with 0.2% DMSO. Cultures were treated with PLX5622 for 7 days (3 medium changes) at different time points, as indicated in Figures 2 and 3.

Histological analysis of cultures

Cultures were fixed with 4 % PFA in PB at pH 7.4 for 2 h. After fixation, HSCs were rinsed 3 times with 0.1 M PBS for 10 min and stored in PBS at 4°C for up to 1 month until sectioning. The membrane carrying the fixed HSCs was cut out and mounted onto a planar agar block. With a vibratome (Leica VT 1000S Vibratome, Leica Bio-systems), the cultures were sliced into 50 µm sections. Typically, 5-6 intact sections per HSC were obtained and collected in PBS to be stained within one week.

For immunohistochemical staining of microglia, sections were treated with 3 % H_2O_2 (Applichem, Darmstadt, Germany) in PBS for 30 min to quench the endogenous peroxidase. Unspecific binding sites were blocked by using 5 % normal goat serum (NGS), 5 % horse serum (HS) and 0.3 % PBST for 2 h at room temperature. To detect microglia, a rabbit monoclonal iba1 antibody (Wako Chemicals GmbH, Cat# 019-19741, 1:500) was used and incubated overnight at 4°C. The following day, secondary antibodies (goat-anti-rabbit biotinylated IgG, Vector laboratories, Burlingame, CA, USA; 1:250) were added onto the sections and incubated for 2 h at room temperature. Antibody binding was detected after sections were incubated in avidin-biotin solution for 45 min (Vector Laboratories). To develop the staining, SG Blue kit (Vector laboratories) was used as the chromogenic substrate for horseradish peroxidase. After immunolabelling, sections were counterstained with nuclear fast red (Sigma-Aldrich, Steinheim, Germany). Coverslipping was performed with Pertex mounting medium (Pertex, Medite, Burgdorf, Germany) on dehydrated sections using an ascending ethanol series (50% - 100%) and xylene.

For phosphorylated αS (pS129) detection, antigen retrieval was performed by heating the sections in 10 mM citrate buffer at 90°C for 35 min. Sections were blocked for 2 h at room temperature with 5 % NGS, 5 % HS and 0.3 % PBST. Rabbit monoclonal pS129 antibody (Abcam, EP1536Y, Cat# ab51253, 1:1000) was applied overnight at 4°C. For microglia detection rabbit monoclonal iba1 (Wako Chemicals GmbH, Cat# 019-19741, 1:250). Subsequently, goat-anti-rabbit Alexa-568 (Thermo Fisher, Cat# A11011) secondary antibodies were applied in a concentration of 1:250. DAPI counterstaining was performed at a concentration of 1:500. For staining with amyloid binding dye thioflavin S (ThioS), sections were incubated for 1 h with ThioS (Sigma-Aldrich, Cat# T1892; 1 % w/v ThioS in milliQ H_2O). ThioS-stained sections were washed 2 x in 70 % EtOH and for 10 min. Slices were transferred on glass slides and coverslipped with Dako Fluorescence mounting medium (Biozol Diagnostika, Cat# S3023).

Sections were analyzed using an Axioplan2 imaging microscope (Zeiss, Jena, Germany) and digitised with an AxioCam HRm black and white camera (Zeiss) using AxioVision 4.8 software (Zeiss). With a Plan Neofluar 10x/0.50 objective lens (Zeiss), 16-bit RGB mosaics of the whole culture were obtained with a resolution of 170 pixels / µm. High resolution images were acquired using a Zeiss LSM 510 META (Axiovert 200M) confocal microscope with an oil immersion 20×/0.8 Plan Apochromat objective and LSM software 4.2 (Carl Zeiss). Sequential excitation of fluorophores was performed using lasers with the wavelength 405 nm (DAPI), 488 nm

(ThioS), and 543 nm (Alexa-568 coupled secondary antibodies). Stacks of 5 µm were taken and maximum intensity projections (MIP) images produced.

Quantification of immunohistochemical stainings

For microglia quantification, mosaic images of iba1 stained cultures were blinded, microglia were manually counted, and the total culture area was measured using FIJI ImageJ (version 2.0.0-rc-64/1.51s). The ratio of microglia counts and total area in $(100\ \mu m)^2$ was obtained (AU, figure 1).

For the quantification of both pS129 and ThioS in hippocampal subregions images were acquired using an LSM 510 META (Axiovert 200M) confocal microscope with a 20×/0.8 Plan Apochromat objective and LSM software 4.2 (Carl Zeiss), using sequential excitation of fluorophores, as described above. Images were blinded, colour channels of MIP images were split using FIJI and a fixed intensity threshold was applied to both red and green channel. To exclude unspecific staining of ThioS, the particle size of signal in the green channel was limited to $20 – 200\ \mu m^2$. On each image, the selected area over the total image area was calculated.

Statistical Analysis.

GraphPad Prism (v.5) was used for statistical analyses.

FIGURES

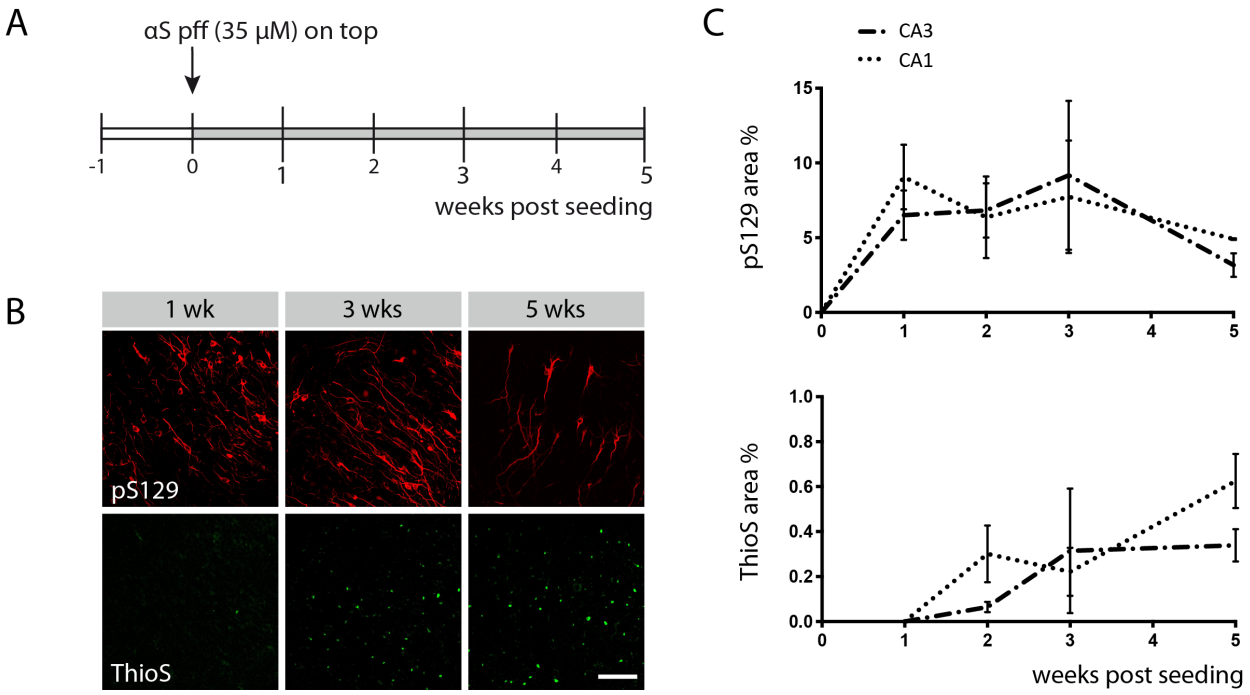

Figure 1: Microglial inclusions appear after the development of abundant neuronal inclusions. (A). Treatment scheme for seeded hippocampal slice cultures (HSCs). Hippocampi of 4-6-day old Tg-A53TαS (tg) mice were treated with 1 µl of αS preformed fibrils (αS pff) at 35 µM on top of each culture. All three cultures within one well received the same treatment. 1-5 weeks later, the cultures were collected for analysis. **(B, C)** Quantification of pS129-positive (B) and ThioS-positive (C) αS inclusions over time. Graphs show percentage of positive area in CA3 (dashed line) and CA1 (dotted line) (C). Mean ± SEM are shown; n = 3 HSCs per group and timepoint.

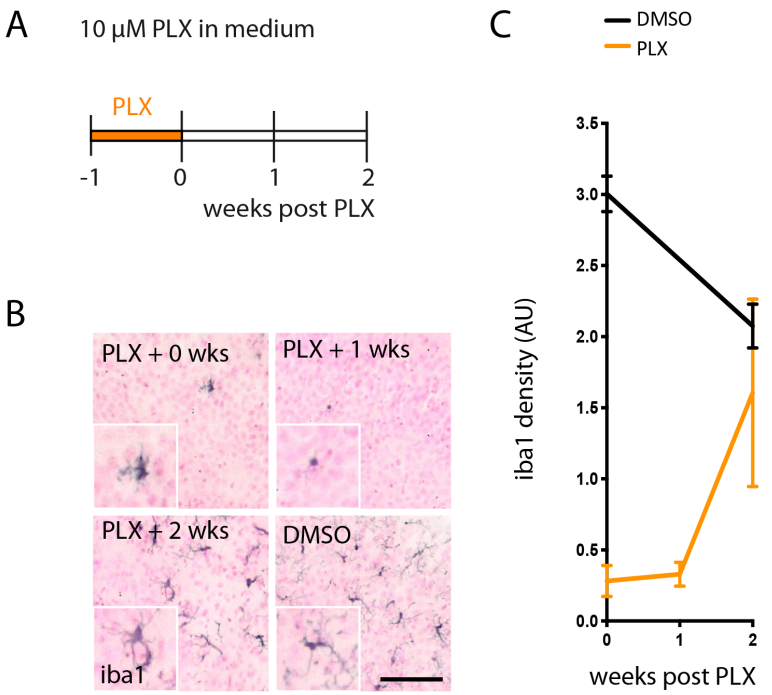

Figure 2: PLX5622 treatment depletes microglial population reversibly. (A) Treatment scheme for microglial depletion in HSCs. Culture medium was supplied with 10 µM PLX5622 for 1 week, and cultures were collected for analysis at day of treatment stop (0 weeks), 1 week and 2 weeks post treatment. **(B)** Immunohistochemical staining against microglia (iba1) of PLX5622 (PLX) treated cultures at 0, 1, and 2 weeks post PLX treatment, and control treated cultures (DMSO). Scale bars = 500 µm. **(C)** Quantification of microglia density in response to PLX5622 treatment. Graphs show density of iba1 positive cells per $(100\ \mu m)^2$. Mean ± SEM are shown; n = 15 (DMSO, 0 weeks), n = 6 (DMSO, 2 weeks), n = 5 (PLX, 0 weeks), n = 3 (PLX, 1 week; PLX, 2 weeks) HSCs.

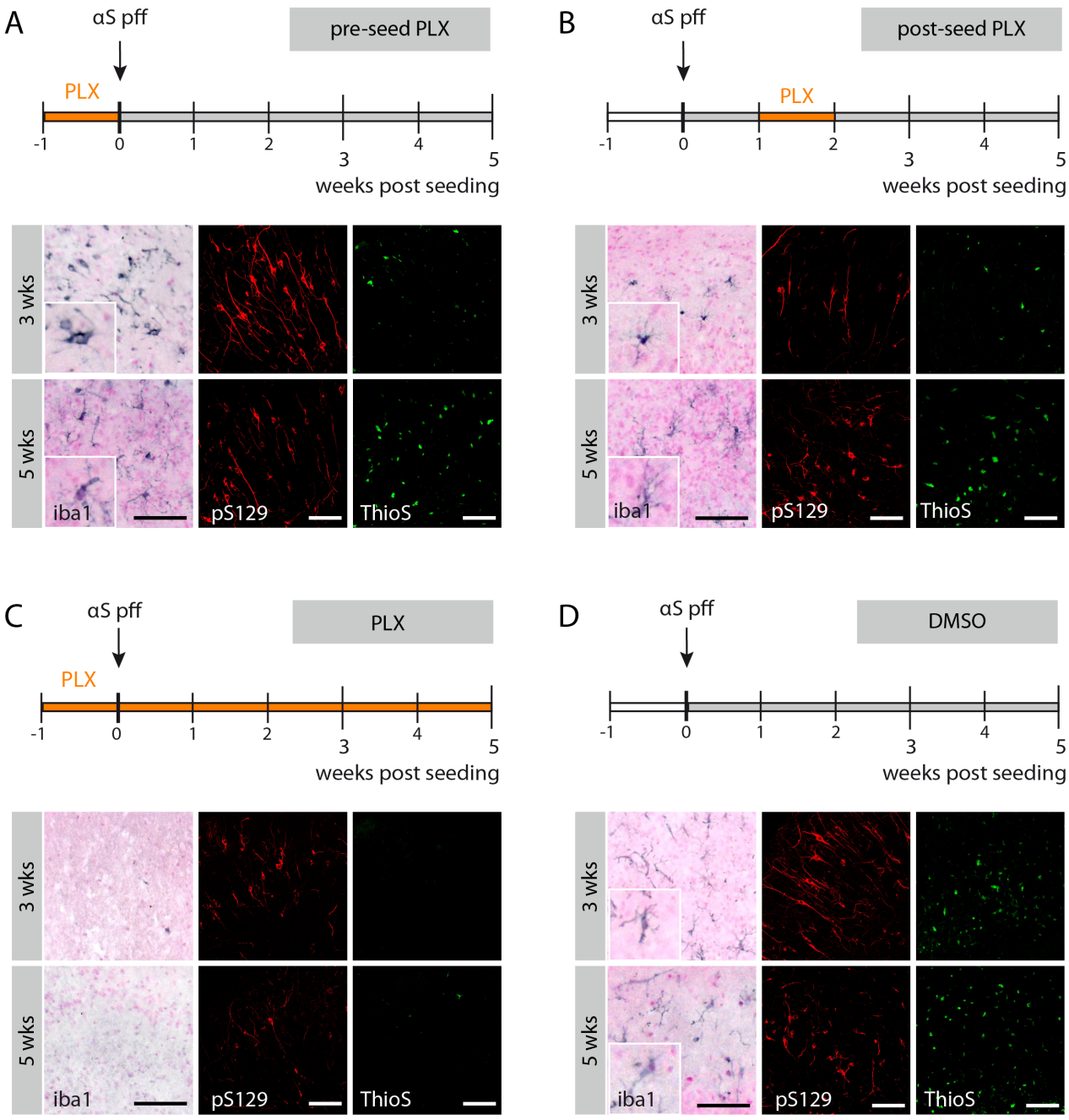

Figure 3: Microglia depletion prior to and post αS seeding might influence induced pathology. Treatment scheme for pre-seed PLX treatment and immunohistochemical staining against iba1, immunofluorescent staining against pS129 (red), and staining with amyloid-binding dye ThioS (green) at 3 weeks and 5 weeks post seeding. **(A)** 7 days prior to the application of 35 µM αS pff, HSCs were treated with 10 µM PLX in the culture medium. **(B)** 7 days after seed application, HSCs were treated for 7 days with PLX. **(C)** HSCs were permanently treated with PLX in the culture medium. **(D)** DMSO treated controls. Note the difference in microglia amount and shape (insert) in response to PLX treatment, and the abundance of pS129 and ThioS positive neuronal and microglia αS inclusions, respectively. n = 5-6 HSCs per group and timepoint. Scale bars = 100 µm.

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V. APPENDIX

ABBREVIATIONS

2D	2-dimensional
3-NT	3-nitro-tyrosine
3D	3-dimensional
3R	reduce, refine, replace - principle of animal protection policies
α3-NKA	Na+/K+ transporting subunit α3
αS	α-synuclein
αS pff	αS pre-formed fibrils
A30P	point mutation of alanine 30 to proline
A53E	point mutation of alanine 53 to glutamic acid
A53T	point mutation of alanine 53 to threonine; also: abbreviation for Thy1-hA53TαS tg
A53V	point mutation of alanine 53 to valine
aa	amino acids
AAV	adeno-associated virus
AD	Alzheimer's disease
ADP	adenosine diphosphate
AFM	atomic force microscopy
AIF	apoptosis inducing factor
ATP	adenosine triphosphate
Aβ	amyloid-β
Aβ40	amyloid-β peptide with 40 residues
BBB	blood-brain barrier
CA[1-4]	cornu ammonis [1-4]
CJD	Creutzfeld-Jacob's disease
CNS	central nervous system
CR	Congo red
CRISPR	clustered regularly interspaced short palindromic repeats interference
cryo-EM	cryogenic electron microscopy
CSF	cerebrospinal fluid
CSF1	colony-stimulating factor 1
CSF1R	colony-stimulating factor 1-receptor
CTD	C-terminal domain

DG	dentate gyrus
DLB	dementia with Lewy bodies
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
E36K	point mutation of glutamic acid 36 to lysine
EC	entorhinal cortex
ECS	extracellular space
ER	endoplasmic reticulum
ESC	embryonic stem cell
Fab	antibody binding fragment
Fc	fragment crystallisable region (tail of an antibody)
FcγR	Fc receptor for antibody type IgG
FTD	frontotemporal dementia
G51D	point mutation of glycine 51 to aspartic acid
GCI	glial cytoplasmic inclusions
h[A30P]αS	human αS with an A30P mutation
h[A53T]αS	human αS with an A53T mutation
H4	human brain neuroglioma cell line 4
H50Q	point mutation of histidine 50 to glutamine
HEK-293	human embryonic kidney cell line 293
HSC	hippocampal slice culture
HSPG	heparan sulfate proteoglycan
i.c.	intracerebral
i.m.	intramuscular
i.v.	intravenous
iPSCs	induced pluripotent stem cells
LAG3	lymphocyte activating gene-3
LB	Lewy bodies
LCO	luminescent conjugated oligothiophenes
LN	Lewy neurites
LPS	lipopolysaccharides
MF	mossy fibres
MMP	matric metalloprotease
mRNA	messenger RNA
MSA	multiple systems atrophy
MTS	mitochondrial targeting sequence
mαS	murine αS
NAC	non-amyloid-beta component
NAD+	nicotinamide adenine dinucleotide
NCI	neuronal cytoplasmic inclusions

NEDD4	neuronally expressed developmentally down-regulated gene 4
NeuN	neuronal nuclei (staining)
NfL	neurofilament light
NFT	neurofibrillary tangles
NLS	nuclear localisation sequence
NOS	nitric oxide synthase
NSF	N-ethylmaleimide sensitive fusion protein
NTD	N-terminal domain
O^{-}	superoxide
OB	olfactory bulb
$ONOO^{-}$	peroxy-nitrite
p-FTAA	pentamer formyl thiophene acetic acid
P[number]	postnatal day [number]
p62	cytosolic protein of 62 kDa; also known as sequestosome 1
PAF	pure autonomic failure
PAR	poly(ADP-ribosyl)
PARP-1	poly(ADP-ribose)polymerase-1
PD	Parkinson's disease
pH	potential of hydrogen ion
PTM	post-translational modification
PNS	peripheral nervous system
PP	perforant path
PP2A	protein phosphatase 2A
PrP	prion protein
PrP^{Sc}	scrapie variant of the prion protein
pS129	phosphorylation at serine 129
pS87	phosphorylation at serine 87
PsP^{C}	cellular prion protein
pT125	phosphorylation at tyrosine 125
RNA	ribonucleic acid
ROS	reactive oxygen species
SC	Schaffer collaterals
SH-SY5Y	sub-clone of human bone marrow neuroblastoma cell line
SIAH	seven in absentia homolog
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
str.	*stratum*
sub	subiculum
SN	*substantia nigra*
SNpc	*substantia nigra pars compacta*
SV	synaptic vesicles

TDP-43	transactive response DNA binding protein 43
TEM	transmission electron microscopy
TFEB	transcription factor EB
TH	tyrosine hydroxylase
ThioS	thioflavin S
ThioT	thioflavin T
TLR	toll-like receptor
TNT	tunnelling nanotube
UAS	upstream activation sequence