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Investigation of the protective efficacy of α-synuclein-specific antibodies in a novel neuronal cell model for α-synucleinopathies

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2 List of abbreviations

AA	Amino acid
AB	Antibody
AMP	Adenosine monophosphate
ANOVA	One-way analysis of variance
AV	Adenovirus
CT	C-terminus
Ctrl	Control
DAPI	4’,6-diamidino-2-phenylindole
DLB	Dementia with Lewy bodies
DM	Differentiation medium
ECL	Enhanced chemiluminescence
FCS	Fetal calf serum
FL	Full length
GFP	Green fluorescent protein
GM	Growth medium
HRP	Horseradish peroxidase
IP	Immunoprecipitation
kDa	Kilodalton
LDH	Lactate dehydrogenase
LN	Lewy neurites
LUHMES	Lund human mesencephalic cells
MOI	Multiplicity of infection
MSA	Multiple system atrophy
NAC	Non-amyloid-β component
NT	N-terminus
PBS	Phosphate buffered saline
PD	Parkinson’s disease
PFA	Paraformaldehyde
rpm	Revolutions per minute
RT	Room temperature
SEM	Standard error of the mean
TBS-T	Tris buffered saline (+ 0.1% Tween)
WT	Wild type
αSyn	α-synuclein

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4 Abstract

Parkinson's disease (PD) is the second most common neurodegenerative disease. One key feature of PD is the abnormal accumulation of α-synuclein (αSyn) in different regions of the brain. The pathological mechanism behind the progression of the disease is the cell-to-cell transmission of αSyn in the patient's brain. Disease-modifying therapies for αSyn-related diseases must either alter the formation of αSyn aggregates or inhibit the spreading of the protein deposits. Different αSyn species have been identified in patients, even though the precise nature of the transmissible species has not yet been revealed. Over the last decade a variety of immunotherapeutic approaches to PD have been investigated. By treating patients with monoclonal antibodies directed against αSyn, the progression of the disease could be influenced.

In this thesis, an *in vitro* model was established to investigate αSyn spreading from affected to previously healthy Lund human mesencephalic (LUHMES) cells. In this model, aSyn-overexpressing LUHMES cells were co-cultured with GFP-expressing LUHMES cells. Cell death in the GFP-labelled healthy control cell population was measured optically by fluorescence microscopy. These co-cultures were subsequently treated with αSyn-specific monoclonal tool antibodies in order to assess the antibodies' protective abilities against αSyn-induced toxicity in the GFP^+ subpopulation. Furthermore, the binding sensitivity to recombinant αSyn monomers and their epitope were tested using dot blots, while their binding properties to cell-derived αSyn were studied in Western blots and by immunoprecipitation. A subset of these tool antibodies was able to reduce αSyn-mediated toxicity in our model. Despite not sharing the same epitope, all tested anti-αSyn antibodies showed a similar sensitivity towards recombinant αSyn. However, the affinity index, resulting in high antibody-antigen-complex stability, was significantly different between protective and unprotective antibodies. Furthermore, the protective antibodies bound cell-derived αSyn to a greater extent. This thesis presents a useful model and reliable approach to test a variety of potential therapeutics for α-synucleinopathies and sheds light on the mechanisms behind immunotherapy targeting αSyn. By investigation of other αSyn-specific antibodies in this model further information about mechanisms behind αSyn spreading could be obtained in the future.

5 Zusammenfassung

Die Parkinson Erkrankung (PD) ist die zweithäufigste neurodegenerative Krankheit. Das Hauptmerkmal von PD ist die vermehrte Anreicherung von α-Synuclein (αSyn) in verschiedenen Teilen des Gehirns. Der wichtigste Mechanismus für das Fortschreiten und die Ausbreitung der Krankheit im Gehirn des Patienten ist die Übertragung von αSyn von Zelle zu Zelle. Krankheitsmodifizierende Therapien für αSyn assoziierte Krankheiten müssen die Ausbreitung der αSyn-Aggregate positiv beeinflussen. In post-mortem Proben von Patienten konnten verschiedene αSyn-Spezies identifiziert werden. Trotzdem konnte bis heute die übertragene Spezies nicht genau charakterisiert werden. In den letzten zehn Jahren wurden verschiedene immuntherapeutische Ansätze für PD untersucht. Durch die Behandlung von Patienten mit spezifischen monoklonalen Antikörpern könnte möglicherweise das Fortschreiten der Erkrankung positiv beeinflusst werden.

In dieser Studie wurde ein *in vitro* Modell entwickelt, um die Ausbreitung von αSyn von betroffenen auf ehemals gesunde menschliche mesenzephalen Lund-Zellen (LUHMES) abzubilden. Hierfür wurden αSyn überexprimierende und GFP exprimierende LUHMES Zellen in einer Co-Kultur gemeinsam kultiviert. Der Zelltod in der GFP-markierten gesunden Kontrollzellpopulation wurde optisch mittels Fluoreszenzmikroskopie gemessen. In einem nächsten Schritt wurden Co-Kulturen mit αSyn-spezifischen monoklonalen Testantikörpern behandelt und die Verringerung der αSyn-induzierten Toxizität in der gesunden Subpopulation untersucht. Darüber hinaus wurden diese Antikörper mittels Dot Blots auf ihre Bindungsaffinität gegenüber rekombinanten αSyn-Monomeren und auf ihre Epitope hin untersucht. In Western Blots und durch Immunpräzipitation wurde zudem die Bindungseigenschaften der Antikörper an von Zellen stammendes αSyn getestet. Eine Untergruppe dieser Antikörper konnte, die von αSyn induzierte Toxizität reduzieren. Obwohl die getesteten Antikörper nicht dasselbe Epitop teilen, zeigten alle getesteten anti-αSyn Antikörper eine vergleichbare Sensitivität für rekombinantes αSyn. Besonders in Bezug auf den Affinitätsindex, ein Maß für die Antikörper-Antigen-Komplex-Stabilität, konnten signifikante Unterschiede zwischen den protektiven und den nicht protektiven Antikörpern beobachtet werden. Des Weiteren banden die protektiven Antikörper aus Neuronen stammendes αSyn im größeren Maße. Diese Arbeit stellt ein neues, zellbasiertes Modell zur Untersuchung verschiedener potenzielle Therapeutika für α-Synucleinopathien vor und liefert wichtige Einblicke in die Mechanismen der Antikörpertherapien gegen αSyn. Auf dieser Basis können Untersuchungen weiterer αSyn-spezifischer Antikörper Beitrag zur Identifikation der, für die Ausbreitung von αSyn im Köper verantwortlichen Spezies leisten.

6 Introduction

Parkinson's disease (PD) affects roughly 1% of world's population above the age of 60 and thus has an enormous economic impact all around the globe. It is the second most common neurodegenerative disease (Obeso et al. 2017). A central feature of the disease is the accumulation of protein deposits in patients' brains, referred to as Lewy bodies (LB) and Lewy neurites (LN). In 1912, when F. H. Lewy first published these findings, the composition and molecular structure of the deposits was unknown. The key player in the pathogenesis of PD, α-synuclein (αSyn), was first identified in 1997. Polymeropoulos et al. (1997) linked the gene SNCA, which encodes for αSyn, to an increased risk for developing PD and Spillantini et al. (1997) were able to show that LBs contain large amounts of αSyn. The interest in the properties, the behavior, and the role of αSyn in the development and progress of PD has risen steadily ever since.

6.1 α-Synucleinopathies

The age of onset, features, and progress vary considerably among cases of PD. The classic motor symptoms include bradykinesia, rigor, postural instability, and resting tremor. Furthermore, preceding symptoms such as depression, REM sleep behavior disorders, and olfactory dysfunction occur (Obeso et al. 2017). The present classification of Parkinson's syndromes includes the idiopathic Parkinson's syndrome, also known as Parkinson's disease, genetic Parkinson's syndromes, symptomatic (secondary) Parkinson's syndromes, and atypical Parkinson's syndromes (Oertel et al. 2012). Corticobasal degeneration, dementia with Lewy-bodies (DLB), progressive supranuclear palsy, and multiple system atrophy (MSA) are commonly known as atypical Parkinson's syndromes (Levin et al. 2016). A distinguishing feature of PD, DLB, and MSA is the abnormal accumulation of αSyn (Valera et al. 2016) and thus are collectively referred to by the term α-synucleinopathies (Spillantini et al. 2000). Pathological similarity is the occurrence of LB and LN in distinct areas of the brain. LB and LN are mainly intracellular inclusions consisting of αSyn in various aggregation forms (Stefanis 2012; Ingelsson 2016; Spillantini et al. 1997). The various manifestations of the diseases differ in the exact cell types involved and the specific locations where the pathology is present (Valera et al. 2016). In PD, dopaminergic neurons in the substantia nigra, the mid brain, the nucleus basalis of Meynert, and the brain stem undergo cell death. In DLB, dopaminergic and cholinergic neurons in the nucleus basalis of Meynert and the limbic system are affected. In MSA, neurons and oligodendrocytes in the putamen, the middle

cerebellar peduncle, the pons, and the cerebellum undergo degradation. Together with ß- and γ-synuclein, αSyn belongs to the synuclein family (George 2001).

6.2 α-Synuclein as a key player in pathogenesis of α-synuncleinopathies

αSyn plays an important role in the pathogenesis of α-synculeinopathies. Elevated αSyn levels, for example in patients with αSyn gene duplications or triplications, have been associated with inherited Parkinson's syndromes (Singleton et al. 2003; Ibáñez et al. 2004; Chartier-Harlin et al. 2004). αSyn is highly abundant in neurons and is mainly found in the cytoplasm, but can also be localized in the nucleus and the presynaptic terminus (Grozdanov et al. 2018). Monomeric αSyn consist of 140 amino acids (AA), which can be subdivided into three domains, each of which exhibiting a specific behavior. The amino-terminus (NT, AA 1-60) contains characteristic 11-residue repeats, which can form amphipathic α-helices when bound to a membrane (Burré et al. 2018; George et al. 1995). AA 61-94, also known as the non-amyloid-ß component (NAC), is a conformationally highly flexible and hydrophobic region, which tends to aggregate via hydrophobic interactions. The carboxy-terminus (CT, AA 96-140) is naturally disordered but seems to play a role in the physiological function of αSyn (Gallegos et al. 2015). Like many cytosolic proteins αSyn can undergo a multitude of posttranslational modifications with unknown physiological relevance (Burré et al. 2018). There is evidence that αSyn is intrinsically unfolded or helical if associated with membranes (Burré et al. 2013; Burré et al. 2018; Fauvet et al. 2012). On the other hand, a soluble tetrameric form of αSyn can be found in human erythrocytes (Bartels et al. 2011). Therefore, it is still unknown which form is predominant in mammalian cells (Grozdanov et al. 2018). Recent results indicated that monomeric αSyn adopts compact conformations by NT acetylation, which seems to protect the NAC region from the cytoplasm and thus prevents spontaneous aggregation (Theillet et al. 2016). Many cellular functions of αSyn have been described, for example in membrane biogenesis, chaperone activity, vesicle trafficking, dopamine synthesis, and neurotransmitter release (as reviewed by Burré et al. (2018)). Furthermore, αSyn can attach to the endoplasmic reticulum, the Golgi complex, mitochondria, and the cytoskeleton (Burré et al. 2018; Grozdanov et al. 2018). To this day, the main cellular function of αSyn remains unknown. The pathogenic properties of the protein for the individual cell, αSyn's aggregation, the release, and the uptake are currently investigated.

6.2.1 Aggregation and α-synuclein's toxic effect

Not only the finding that LB and LN contain large amounts of αSyn but also the aggregation-prone properties of the NAC domain drove the current opinion towards the importance of aggregation of αSyn for the pathophysiology of associated diseases. Starting from the monomer, αSyn assembles to different intermediate, soluble oligomeric forms, which are generally termed protofibrils. Protofibrils are considered to be toxic for neurons (Burré et al. 2018). Further on, aggregation progresses and more insoluble species (fibrils) form (reviewed in Giráldez-Pérez et al. 2014; Lindström et al. 2014; Stefanis 2012; Wong et al. 2017; Burré et al. 2018; Marques et al. 2012). By the inhibition of αSyn aggregation the αSyn-related toxicity could be altered (Hashimoto et al. 2001; Periquet et al. 2007). However, the precise mechanisms of αSyn-related toxicity have not yet been revealed. Gallegos et al. (2015) reviewed various potential pathways influenced by αSyn accumulation and aggregation. Possible effects on cell organelles such as proteasomes, mitochondria, and the endoplasmic reticulum have been studied. Furthermore, as well as αSyn's membrane binding capacity, its disruptive effect on cell membranes is currently a topic of investigation. It was shown that αSyn can aggregate within the membrane and build pore-like structures. These result in increased membrane permeability and calcium influx that could possibly harm cells (Danzer et al. 2007; Tsigelny et al. 2012). Aggregates of αSyn activate microglia and drive an inflammatory response, which enhances dopaminergic neurodegeneration (Zhang et al. 2005).

6.2.2 Release and uptake of α-synuclein

Typically, clinical features progress over the course of PD. Postmortem analyses of PD patients' tissue have revealed αSyn deposits in various regions of the body and the brain. In 2003, Braak et al. (2003) postulated that αSyn-related pathology could start elsewhere in the body, but ultimately propagates into the brain. Starting from the olfactory bulb and in the gut, the pathology spreads into the central nervous system (Braak et al. 2003a; Braak et al. 2003b; Hawkes et al. 2007; Hawkes et al. 2009). Further evidence towards a dispersion through the body and the brain is based on the finding that the LB pathology can spread from the brain of PD patients into grafted brain tissue. LB-like αSyn inclusions were found in dopaminergic neurons formerly grafted to PD patients' brains as a therapeutic approach against dopaminergic neuron loss in the substantia nigra (Kordower et al. 2008a; Kordower et al. 2008b; Li et al. 2008; Li et al. 2010). Similar to the PrPSc protein in Creutzfeldt-Jakob disease, αSyn seems to spread stereotypically from one brain region to the next. Misfolded αSyn can subsequently

recruit endogenous αSyn for further assembly (Luk et al., 2009; Volpicelli-Daley et al. 2014). This led to the concept of α-synucleinopathies as prion-like disorders. The pathophysiology behind the neuron-to-neuron transmission of αSyn has been investigated intensively over the last two decades (Steiner et al 2011; Steiner et al. 2018; Gallegos et al. 2015; Grozdanov et al. 2018; Peelaerts et al. 2018; Rietdijk et al. 2017). The release of αSyn into the extracellular space was shown to occur via diffusion through intact or defective cell membranes, by conventional and Golgi- or endoplasmic-reticulum-independent exocytosis (Lee et al. 2005) or by exosomes (Fussi et al. 2018). Possible mechanisms for the uptake of αSyn include passive transport by diffusion or active transport by endocytosis and phagocytosis. There is strong evidence suggesting that αSyn is internalized either by assembling with Na^+/K^+-ATPase (Shrivastava et al. 2015) or lymphocyte-activation gene 3 (Mao et al. 2016). Exosomes containing αSyn oligomers are taken up and have a toxic effect on the recipient cell (Danzer et al. 2012). Recognizably, uptake mechanisms may vary from cell-type to cell-type. αSyn has also been shown to propagate via tunneling nanotubes and actin-based membranous bridges between non-neuron cells (Dieriks et al. 2017).

6.3 Immunotherapy against α-synuclein

Since developing a disease-modifying therapy for PD is an unmet goal, PD is treated mainly symptomatically in current practice. Many promising therapies either targeting αSyn directly or reducing αSyn-induced pathology are under current investigation (Lang et al. 2018). The exact species of extracellular αSyn crucial for the spreading of the disease remain unknown. Reducing the amount of αSyn within the extracellular space could have a positive effect on the progression of the disease (Lee et al. 2016). One possible way to accomplish this would be by means of immunotherapy. The act of interfering with the formation of potentially toxic species, opsonizing of already existing forms, or physically shielding against hazardous species could possibly induce beneficial effects in patients (Bergström et al. 2016). Currently, there are two types of immunotherapeutic approaches for α-synucleinopathies: active and passive. In active immunotherapy, an immune response is induced in the patient, which leads to the production of specific antibodies (AB) directed against the target. Active vaccination would be both less costly and less time-consuming compared to passive immunotherapy. In passive immunotherapy, preformed ABs are administered to act against the target. This approach is more expensive, needs frequent administration, and well-characterized effective ABs. Monitoring of serum levels and restricted half-lives of the ABs in the body would allow physicians to control side effects and adjust the therapy individually (Bergström et al. 2016).

6.3.1 Active immunotherapy

In active immunization, an antigen is administered leading to an immune response towards the antigen (Schneeberger et al. 2016). It has been shown for a MSA transgenic mouse model (MPB-αSyn) that active vaccination with a short αSyn peptide AFF1 induced the formation of specific antibodies that also crossed the blood-brain barrier. This led to increased clearance of αSyn by astroglia and reduced neuronal loss in different parts of the brain (Mandler et al. 2015). Interestingly, mice that produced antibodies against the C-terminal region of αSyn (AA 85-99, 109-123, 112-126, and 126-138) benefitted the most (Masliah et al. 2005). Approaches for the perfect vaccine include synthetic peptides, DNA, the utilization of dendritic cells or other regulatory mechanisms (reviewed by Schneeberger et al. (2016)). Recent approaches have used nanoparticle-linked epitopes (Rockenstein et al. 2017) or virus-linked particles (Doucet et al. 2017). Even though increased neuroinflammation has not been reported in studies, Valera et al. (2013) reviewed cases of autoimmune responses and vasculitis. In an immunization study against amyloid-β, another protein involved in neurodegenerative diseases, cases of meningoencephalitis led to a cancellation of the trial (Gilman et al. 2005). This suggests that the immune system, in particular its regulatory players, play an important role in the pathogenesis of neurodegenerative diseases (Christiansen et al. 2016). Schneeberger et al. (2016) discussed a possible disadvantage of vaccination, i.e. only 0.1% to 1.0% of peripherally produced ABs penetrate through the blood-brain-barrier, potentially reducing the positive effect. In passive immunotherapy, the ABs' ability to migrate into the brain can be optimized by altering the ABs' structure (Spencer et al. 2014). Hultqvist et al. (2017) found that the uptake across the blood-brain barrier can be increased by a factor of 80 if a monoclonal antibody is fused to two single-chain variable fragments of the transferrin receptor.

6.3.2 Passive immunotherapy

A variety of studies have addressed the idea to overcome the aforementioned problems with active immunotherapy by treatment with preformed ABs. Sahin et al. (2017) showed in a cell-free assay that ABs were capable of inhibiting fibrillation of αSyn and membrane permeability induced by αSyn. Different groups have demonstrated the effectiveness in reduction of aggregation / oligomerization of αSyn in various cell models by using ABs (e.g. B103 cells, H4 neuroglioma cells, etc.) (Näsström et al. 2011; Games et al. 2014; Gustafsson et al. 2017; Tran et al. 2014). *In vivo* studies of the effectiveness of ABs were mainly performed in transgenic mouse models (Masliah et al. 2011; Games et al. 2014;

Bae et al. 2012; Lindström et al. 2014) and rats (Shahaduzzaman et al. 2015). For this purpose, the pathology was induced by overexpressing mutated human or wild type (WT) αSyn by viral vectors. While overexpression of αSyn led to an accumulation of endogenous αSyn, Tran et al. (2014) induced αSyn aggregation via injecting recombinant preformed fibrils into the striatum of non-transgenic mice. Bergström et al. (2016) stated that the most promising effects of ABs might happen by two mechanisms: clearance and blocking (see Figure 1).

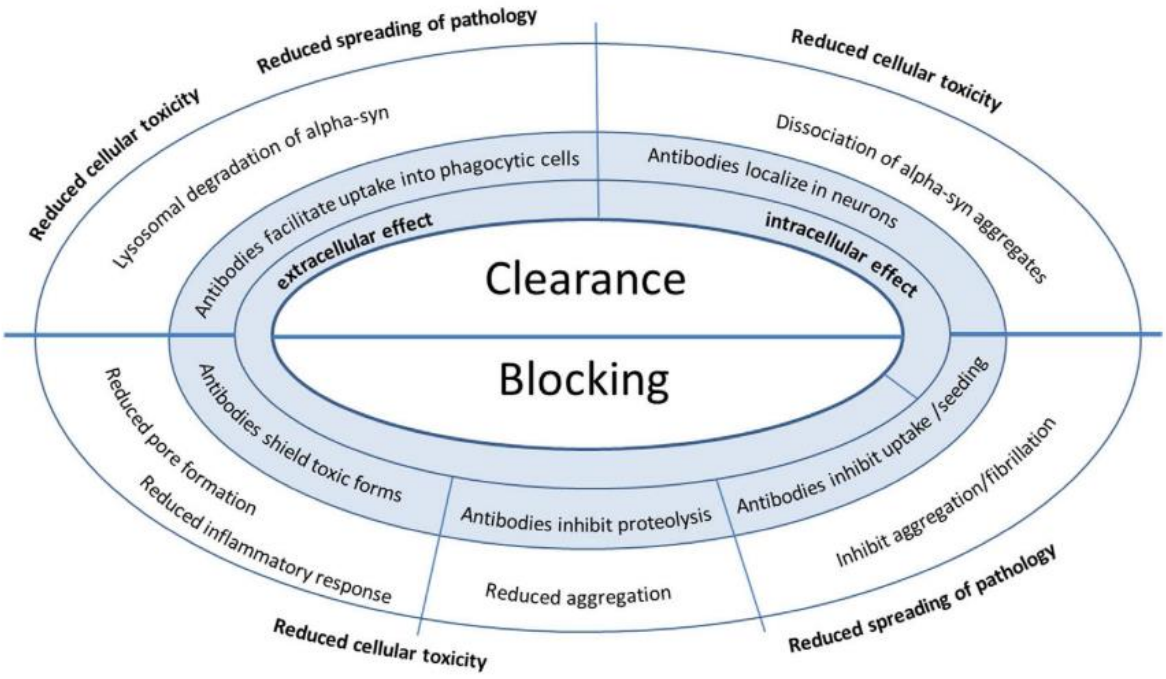

Figure 1 **Proposed mechanisms of αSyn-specific immunotherapy.** The exact mechanisms behind immunotherapy in α-synucleinopathies are not yet well understood. Theoretically, the pathology could be altered either through clearance of exciting toxic species or by blocking formation or propagation of named species (adapted from Bergström et al. 2016).

The clearance of αSyn could take place in three compartments: intracellular, membrane-bound and extracellular. ABs against αSyn have been found inside neurons (Masliah et al. 2005, 2011). Labeled ABs against αSyn activated the autophagic pathway in the rat neuroblastoma cell line B103 (Masliah et al. 2011). Microglia are capable of internalizing αSyn fibrils and oligomers bound to ABs mediated by Fc-receptors (Bae et al. 2012). The amount of ABs taken up into human neuroglioma H4 cells can be augmented by the presence of αSyn in the medium (Gustafsson et al. 2017). The amount of αSyn in the medium and in cell lysates decreased significantly under passive immunization in a H4 neuroglioma cell culture (Nässtrom et al. 2011). Cell membrane-bound αSyn could also be recognized by ABs since the NT of αSyn interacts with the membrane and the CT of αSyn is exposed to the outside

(Tsigelny et al. 2008a, 2008b). ABs could interfere with αSyn by blocking the harmful activation of the immune system, by shielding against toxic species of αSyn, and by reducing the formation and the processing of these species. While active vaccination activates microglia and induces the production of anti-inflammatory cytokines, passive immunotherapy reduced the activation of microglia in a rat model (Shahaduzzaman et al. 2015). Preventing the uptake of αSyn preformed fibrils by monoclonal ABs reduced the formation of LB/LN and reduced synaptic loss as well as spreading of the pathology (Tran et al. 2014). Interestingly, ABs reduced αSyn propagation not only within the medium (Games et al. 2014) but also when the propagation occurred via axons in a micro fluidic chamber (Tran et al. 2014). Furthermore, ABs reduced the propagation of αSyn into the contra-lateral hemisphere in a transgenic mouse model (Spencer et al. 2017). Blocking of calpain-1-mediated cleavage of αSyn oligomers has been discussed by Masliah et al. (2011) and Games et al. (2014) as a possible AB effect. Calpain-1 is known to cleave αSyn fibrils in the CT (AA 114-122), which induces a further assembly of αSyn (Mishizen-Eberz et al. 2005, 2003). The spreading of αSyn oligomers could be reduced by preventing CT-truncation by ABs that have their epitope in or directly adjacent to the calpain-1 cleavage site (Games et al. 2014).

Summarizing the preclinical data, the use of ABs against αSyn is a promising treatment for αSyn-related diseases. Various ABs have shown to be effective, with some targeting the CT, some the NT and some protofibrils and oligomeric species (Bergström et al. 2016). Recently, potential immunotherapies have been subjected to clinical testing. PRX002, an AB which derives from the previously tested CT-AB 9E4 (Games et al. 2014; Masliah et al. 2011), was able to reduce the level of αSyn in the serum (Schenk et al. 2017; Jankovic et al. 2018). Furthermore, the efficacy of PRX002 is currently under investigation in the PASADENA study (NCT03100149) since June 2017. BIIB054 (NCT02459886, NCT03318523), a N-terminal, oligomer-specific anti-αSyn-antibody showed favorable tolerability in a phase-I-trail (Brys et al. 2019). Furthermore, BAN0805 (m8A57), an oligomer- / protofibril-selective AB, recently entered clinical testing (Lindström et al. 2014; Gustafsson, Eriksson, et al. 2017). Overall, these preclinical and clinical data provide growing evidence that immunotherapy is a suitable and promising approach, which has the ability to alter the progression of α-synculeinopathies.

7 Aim of the study

Three main aims were addressed in this study:

- Establishment of a neuron-based cell model of αSyn-spreading
- Investigation of the therapeutic efficacy of three tool ABs targeting αSyn
- Characterization of the ABs' properties

It has been demonstrated previously that mild overexpression of WT αSyn by adenoviral vectors leads to cell death in postmitotic dopaminergic Lund human mesencephalic (LUHMES) neurons (Höllerhage et al. 2014, 2017). In this model, αSyn is released into the medium (Fussi et al. 2018; Chakroun et al. 2020) and degraded by autophagy (Höllerhage et al. 2017). By suppressing the autophagy-pathway, the cells were prevented from the αSyn-induced cell death but the cells increased the secretion of αSyn by exosomes into the medium (Fussi et al. 2018). This provides evidence that αSyn-overexpression has a toxic effect on LUHMES neurons and leads to an increase of αSyn in the medium. The released αSyn possibly spreads to previously healthy cells and induces pathology. Therefore, a co-culture model was designed, in which pathologically affected cells, which overexpressed αSyn, were cultured next to healthy control cells, which expressed green fluorescing protein (GFP). Cell death was expected in the control population (GFP^+), which we monitored optically by fluorescence imaging. For this purpose, the DNA dye DRAQ7 was used. DRAQ7 penetrates impaired cell membranes and therefore exclusively stains dying cells. It has been shown that αSyn species spread from one cell to another and colocalize with endogenous αSyn. This effect can be reduced by immunotherapy (Games et al. 2014). In the present study we aimed to investigate the effects of three potentially therapeutic ABs to reduce αSyn-related cell death in the control population. A deeper understanding of the mechanisms behind passive immunotherapy against αSyn-mediated diseases was expected by investigating the selected ABs binding to αSyn species in the medium and by testing for sensitivity towards recombinant αSyn. Furthermore, the tested antibodies were characterized by mapping their epitope and by investigating the stability of the αSyn-antibody-complex. Implications on future test models and treatment in PD patients were expected.

8 Methods

8.1 Cell culture

8.1.1 Dishes and coating

For the preparation of the proliferation phase, T75 flasks (EasYFlasks, Nunclon surface, NUNC A/S, Roskilde, Denmark) were coated with 50 µg/ml poly-L-ornithine (PLO; Sigma-Aldrich, St. Louis, MO, USA) at 4 °C overnight and washed three times with phosphate buffered saline (PBS; Life Technologies, Carlsbad, CA, USA). For the differentiation phase, either T25 flasks, 6-well plates, or 48-well plates (Nunclon surface, NUNC A/S) were coated with PLO at 4 °C overnight and washed three times with PBS before incubation with 5 µg/ml fibronectin (Sigma-Aldrich) at 37 °C overnight. Before seeding of the cells, the plates or flasks were washed once with PBS.

8.1.2 Medium

During the proliferation phase, LUHMES cells were cultivated in a growth medium (GM) consisting of DMEM/F12 (Sigma-Aldrich) with 1% (v/v) N2-supplement (Life Technologies) and 0.04 µg/ml basic fibroblast growth factor (PeproTech, Rocky Hill, CT, USA). The differentiation medium (DM) consisted of DMEM/F12 with 1% N2-supplement, 1 µg/ml tetracycline (Sigma-Aldrich), 0.49 µg/ml dibutyryl cyclic AMP (Sigma-Aldrich), and 2 ng/ml glial cell-derived neurotrophic factor (R&D Systems, Minneapolis, MN, USA).

8.1.3 Cultivation of LUHMES cells

LUHMES cells were cultivated at 37 °C and 5% CO_2. Initially seeded at an approximate density of 3 x 10^6 per T75 flask in 14 ml GM. At a density of approximately 25 x 10^6 cells per flask, the cells were ready for use for the actual experiments. For differentiation, the cells were reseeded – the old GM was removed, centrifuged to separate cell debris (1,200 rpm, 5 min), and the supernatant saved for the resuspension of the detached cells. The cells were detached by incubation with 2 ml trypsin (1x; Sigma-Aldrich) at 37 °C for 5 min. The trypsin was then inhibited by 1 ml fetal calf serum (FCS; Merck Millipore,

Darmstadt, Germany) and diluted using the GM retained from the earlier step. After the detachment, the single-cell suspension was then centrifuged at 1,200 rpm for 7 min, the supernatant was collected, and the cells were resuspended in DM at the desired concentration. In T25 flaks, the cells were seeded at a density of 2.7 x 10^6 cells per flask, in a 6-well format at 1 x 10^6 cells per well and in a 48-well format at 120,000 cells per well.

8.1.4 Adenoviral transduction

The cells were seeded as described above. The adenoviral (AV) transduction was performed on the first day after differentiation for the co-culture experiments and on the second day after differentiation for the Western blots sample preparation. On the designated day, the cells were transduced by adenoviral vectors encoding either WT human αSyn or GFP under the cytomegalovirus promoter control (BioFocus DPI, Leiden, Netherlands) at a multiplicity of infection (MOI) of 2. This was performed by exchanging parts of the medium with the DM containing the adenoviral vectors (for 6-well plates 500 µl, for T25 flasks 1 ml). Control cells received fresh DM not containing the vectors. After incubation for 24 h, the cells were washed three times with PBS and received fresh DM.

8.2 Co-culture of α-synuclein-overexpressing and GFP-expressing LUHMES cells

All cell culture experiments were performed in triplicates (N = 3). For the process of co-culturing, the cells were seeded in a 6-well format and transduced 24 h later (see Figure 2). One day following the transduction, the cells were washed three times with PBS. To minimize the possibility of their harm, the transduced cells were carefully detached by incubating with Accutase (BD Biosciences, Franklin Lakes, NJ, USA) at 37 °C for 1 h. Thereafter, the cells were suspended in the DM containing 10% FCS. After centrifugation at 1,200 rpm for 7 min, the supernatant was discarded, and the cells were resuspended in the DM.

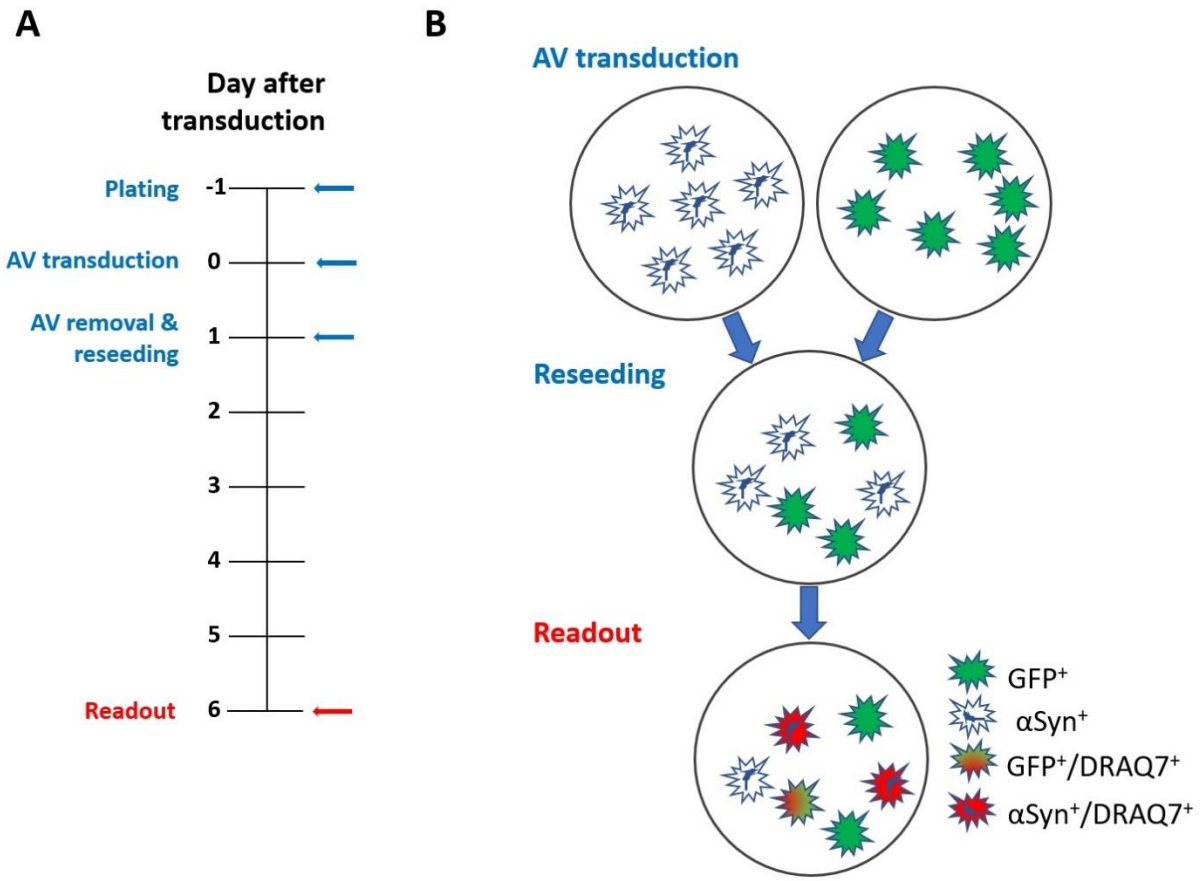

Figure 2 **A Timeline of the co-culture protocol.** LUHMES were plated as described in section 8.1.3. On the following day, the adenoviral (AV) transduction was performed. Twenty-four hours later, the AV vectors were removed, cells were washed and reseeded. **B Scheme of the co-culture protocol.** GFP and αSyn-transduced cells were cultivated separately. After removing the AV, the cells were detached and reseeded into a co-culture.

Testing for toxicity in the GFP subpopulation

The co-cultures were plated in a 48-well plate format at a density of 120,000 cells per well in a total volume of 300 µl. Each well received either only GFP^+ cells, or a mixture of GFP^+ and αSyn^+ in ratios of 1:3, 1:1, or 3:1. The edge wells were filled with PBS without cells in order to protect the inner 24 wells from evaporation. The readout was carried out on day six after the transduction.

Antibody treatment

The antibody treatment was similarly performed in a 48-well plate format at a density of 120,000 cells per well in a total volume of 300 µl. GFP^+ and αSyn^+ cells were seeded in a ratio of 1:1 in a volume of 250 µl. The antibodies EG27/1, 23E8, 5D12, and 8A5 were diluted in 50 µl DM and were added to the co-cultures. The protectiveness was assessed at an antibody concentration of 25 nM. The control cells received 50 µl DM without the antibodies. The edge wells were filled with PBS without cells in order

to protect the inner 24 wells from evaporation. The readout was carried out on day six following the transduction.

8.3 Fixation and staining

To determine apoptotic cells, the entire population of cells was stained with the fluorescent dye DRAQ7 (Abcam, Cambridge, United Kingdom) at a final concentration of 1.5 µM (1:200) at 37 °C for 5 min. DRAQ7 does not enter living cells. If the membrane integrity is affected, this probe enters the cell and binds to the DNA (Wlodkowic et al. 2013). Therefore, only dying cells are stained by DRAQ7. After washing with 300 µl PBS, the cells were fixed by using 150 µl of 3.75% paraformaldehyde solution (PFA, Sigma-Aldrich) in PBS at 37 °C for 10 min. The PFA solution was then collected and the cells were incubated with 250 µl 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) 1:1,000 in PBS at RT for 10 min. After removing the DAPI, the wells were filled with 600 µl PBS and were kept refrigerated in the dark at 4 °C for microscopy.

8.4 Microscopy

Pictures of the cell culture were taken using an inverted microscope (DMI6000, Leica Microsystems, Wetzlar, Germany) with the Leica Application Suite Advanced Fluorescence 2.6 software. A 20x lens was used to take 5 pictures per well. Pictures were randomly renamed with Ant Renamer 2.12 (Antoine Potten, Brussels, Belgium) to achieve blinding for treatment conditions prior to counting the cells. Counting was performed using the ImageJ 1.52e counting plugin (Wayne Rasband, National Institute of Health, Bethesda, MD, USA).

8.5 Western blot

Sample preparation

Samples of cell-free DM and samples of the medium from untreated control cells, GFP-transduced cells, and αSyn-transduced cells were collected on day four and six post transduction. To separate any present cell debris from the medium, the samples were centrifuged at a speed of 5,000 rpm for 10 min. The medium was collected and stored at -80 °C. Before Western blotting the medium was

concentrated. This was achieved by centrifugation at 4,000 x g at 4 °C in 3 kDa molecular weight cut-off filters (Vivaspin 6; Sartorius, Göttingen, Germany) for 3 h. To measure the protein concentration in the sample, the Pierce 660 nm Protein Assay kit (Thermo Fischer Scientific, Waltham, MA, USA) was performed according to the manufacturer's instructions. A bovine serum albumin standard (Thermo Fischer Scientific) was used as the reference protein. 10 µl of the medium or the reference protein were incubated with 150 µl of the Pierce 660 nm Protein Assay solution for 5 min and the absorption was measured using a FLUOstar plate-reader (BMG Labtech, Ortenberg, Germany). The protein concentrations were calculated by reference to absorbances obtained for a series of bovine serum albumin dilutions.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

For Western blots of the medium and the immunoprecipitation samples, Criterion 12% Bis-Tris gels (Bio-Rad Laboratories) were used. 7 µl of PrecisionPlus ladder (Bio-Rad Laboratories), as well as 5 µl of Page-Ruler ladder (Thermo Fischer Scientific) were used as size references. 100 µg of each sample were suspended in XT sample buffer (4x, Bio-Rad Laboratories) containing XT reducing agent (20x, Bio-Rad Laboratories). The proteins in the sample were denatured at 95 °C for 5 min (500 rpm). After loading into the gel, the proteins were separated electrophoretically using a constant voltage of 125 V. Proteins were then transferred to a polyvinylidenfluoride membrane (Bio-Rad Laboratories) using semi-dry transfer and fixed to the membrane using 0.4% PFA (Sigma-Aldrich) in PBS for 30 min. The membranes were then washed three times in PBS for 10 min. To minimize the unspecific binding of the antibodies to the membrane, a 3x Roti-Block blocking solution (10x; Carl Roth, Karlsruhe, Germany) was applied for 2 h. The primary AB solution was prepared in 1x Roti-Block blocking solution in Tris buffered saline containing 0.05% Tween-20 (TBS-T, pH 7.4) and the membrane was incubated overnight at 4 °C, followed by 1 h at RT. The membrane was washed again three times with TBS-T for 5 min. Afterwards, the membrane was incubated in the horseradish peroxidase(HRP)-conjugated secondary AB solution (1:2,500 in 1x Roti-Block; Vector Laboratories, Burlingame, CA, USA) at RT for 2 h and washed again three times in TBS-T. The membrane was then incubated in Clarity Western ECL substrate (Bio-Rad Laboratories) for 10 min and the ECL signal was visualized using the Odyssey Fc imaging system (LI-COR Biotechnology, Lincoln, NE, USA).

Antibodies

EG27/1, 23E8, 5D12, and 8A5 diluted to 100 nM were used as primary antibodies. A C-terminal rabbit anti-αSyn AB (1:500 [#2642]; Cell Signalling Technologies, Danvers, MA, USA) and a N-terminal rabbit

anti-αSyn AB (1:500 [EP1646Y]; Abcam) were used for Western blots after immunoprecipitation. As secondary antibodies, HRP-conjugated IgG (anti-mouse: 1:2,500 [PI-2000]; anti-rabbit: 1:5,000 [PI-1000]; Vector Laboratories) were used.

8.6 Dot blot

Prior to all dot blot experiments, nitrocellulose membranes (0.45 µm pore size; GE Healthcare Life Science, Freiburg, Germany) were wetted in TBS-T, then put on Whatman paper (GE Healthcare Life Science) to dry the liquid film.

Epitope Mapping

αSyn is a protein consisting of a length of 140 amino acids (AA). Three distinct regions can be identified: the amino-terminus (NT; AA 1-60), the non-amyloid-component (NAC; AA 61-95), and the carboxy-terminus (CT; AA 96-140). To determine the antibodies' epitope, their affinity to certain αSyn fragments and to full-length (FL) αSyn was assessed. Specifically, the following fragments were used:

- Full-length αSyn (AA 1-140) (rPeptide, Bogart, GA, USA)
- N-terminal fragment of αSyn (AA 1-60) (rPeptide)
- N-terminal + NAC domain fragment of αSyn (AA 1-95) (rPeptide)
- C-terminal (AA 96-140) fragment of αSyn (rPeptide)
- C-terminal + NAC domain fragment of αSyn (AA 61-140) (rPeptide)
- NAC domain (AA 61-95) fragment of αSyn (JPT Peptide Technologies, Berlin, Germany)

For each antibody (EG27/1, 23E8, 5D12, and 8A5), a single membrane was loaded with 1 µg of recombinant αSyn and 1 µg of each fragment in 2 µl of milliQ water. The NAC domain fragment solution was sonicated three times for 30 seconds before loading to disengage any possible aggregates since NAC domains tend to aggregate easily. The membranes with the loaded protein were air-dried at RT for 5 min. To fix the protein to the membranes, the membranes were incubated in 0.4% PFA in PBS for 30 min. After they were washed three times in PBS for 10 min, the membranes were then blocked for 2 h in 3x Roti-Block blocking solution to minimize unspecific binding of the antibodies to the membranes. Solutions of the tested antibodies (100 nM) were prepared in 1x Roti-Block. The membranes were incubated in the AB solutions overnight at 4 °C followed by 1 h at RT. The membranes were washed three times for 5 min with TBS-T, before a secondary HRP-conjugated anti-mouse AB (Vector Laboratories) was applied at RT for 2 h. The membranes were washed again in TBS-T, then

were incubated in Clarity Western ECL substrate (Bio-Rad Laboratories) for 10 min and the ECL signal was visualized using the Odyssey Fc imaging system (LI-COR Biotechnology).

Sensitivity Testing

To determine the binding sensitivity of the four antibodies EG27/1, 23E8, 5D12, and 8A5 to recombinant αSyn, a dot blot was performed. Samples of 0.25 µg of recombinant full-length (AA 1-140) αSyn were loaded on membranes. The proteins were fixed to the membranes using a 0.4% PFA solution. As a loading control, the Revert 700 total protein stain (LI-COR Biotechnology) was performed according to the manufacturer's instructions. The membranes were incubated in the Revert 700 total protein staining solution at RT for 5 min. Afterwards, the membranes were washed two times with the provided Revert 700 wash solution. Images were taken using the Odyssey Fc imaging system (LI-COR Biotechnology). The membrane was subsequently washed again in milliQ water and proceeded to blocking in 3x Roti-Block for 2 h. Serial dilutions of the four tool antibodies were prepared in 1x Roti-Block. For each antibody (EG27/1, 23E8, 5D12, and 8A5), the sequence contained 12 dilution steps (1:2) starting at 25 nM. The membranes were incubated in these AB solutions at 4 °C overnight and at RT for 1 h. After three washing steps (10 min) with TBS-T, a fluorescence-coupled anti-mouse antibody (1:10,000; IRDye 800CW; LI-COR Biotechnology) was applied at RT for 1 h. The membranes were washed again three times with TBS-T for 10 min and the fluorescence signal was visualized using an Odyssey Fc imaging system (LI-COR Biotechnology).

Determination of the binding stability of the antibody-antigen-complex

A measure for the stability of the antibody-antigen-complex is the so-called affinity index of the antibody (Svobodova 2013). This index can be measured by challenging the binding between antibody and antigen using a chaotropic agent and it correlates with the likelihood of the antibody to dissociate from the antigen into solution, respectively the binding strength. For the estimation of the affinity indices of the antibodies, dot blots were performed as described above. Monomeric αSyn was loaded at an amount of 0.25 µg, and incubated in 25 nM antibody solutions (EG27/1, 23E8, 5D12, 8A5) overnight at 4° C. The following day, the membranes were washed three times with TBS-T. Afterwards, the antibodies binding to αSyn, respectively the membrane, was challenged using the chaotropic agent ammonium thiocynate (HN_4SCN; Sigma-Aldrich) in increasing concentrations (0 – 2 M) for 5 min at room temperature. Thereafter, the membranes were washed with TBS-T, incubated with a fluorescence-coupled anti-mouse antibody (1:10,000; IRDye 800CW; LI-COR Biotechnology), and the signal was visualized using the Odyssey Fc imaging system (LI-COR Biotechnology).

8.7 Immunoprecipitation and ELISA

αSyn-overexpressing cells were cultivated in T25 flasks as described before. On day six after αSyn transduction, the medium was harvested. The protein in the medium was concentrated to an amount of 75 µg in 0.5 ml as described. 30 µg of the tested antibodies were added. This antibody-medium-solution was incubated at 4 °C overnight and at RT for 1 h to establish antibody-protein-complexes. The following day, 60 µl of magnetic beads (Pierce Protein G Magnetic Beads, Thermo Fischer Scientific) were washed using 1 ml of freshly prepared TBS-T (Tris 25 mM; NaCl 0.5 M; Tween 0.05% in milliQ water) followed by washing with 1 ml of milliQ water according to the manufacturer's instruction. Between each washing step the beads were collected using a magnetic stand (Merck Millipore). The AB-medium-solution was then added to the beads. This AB-medium-beads-solution was incubated at RT for 1 h and at 4 °C overnight to establish AB-beads-complexes. The antibodies, bound to the beads, were then collected using a magnetic stand. The AB-beads-complexes were washed three times with 500 µl TBS-T, followed by one time washing with 500 µl milliQ water in order to diminish any unspecific signal from unbound proteins. After the last washing step, 100 µl of XT sample buffer (1x; 4x diluted in water; Bio-Rad Laboratories) was added to the AB-beads-complexes. To denature the proteins and detach the ABs from the beads, the solution was heated to 95 °C for 10 min at 1,400 rpm. Finally, the beads were collected using a magnetic stand, but with no antibodies bound to them. The supernatant, containing antibodies and any previously bound proteins, was collected.

To quantify the amount of αSyn the antibodies scavenged, the antibodies (50 nM) were added to unconcentrated conditioned medium from αSyn-overexpressing cells and the IP was performed as described. The alpha Synuclein Human ELISA Kit (ThermoFisher Scientific) was used to measure the amounts of αSyn remaining in the medium after IP according to the manufacturer's instructions.

8.8 Statistical analysis

GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis. To compare the experimental groups, one-way ANOVA with post hoc Tukey's multiple comparison tests (Tukey's test) were performed. Data are presented as the mean ± standard error of the mean (SEM). A p-value < 0.05 was considered to be statistically significant. For the co-culture experiment, the percentage of $DRAQ7^+$ cells in the GFP^+ subpopulation of each well was normalized to the mean of all corresponding

populations within the 48-well plate. For an optimal display of the data, each normalized value was multiplied by the mean of the entirety of the 50% αSyn^+ / 50% GFP^+ populations.

9 Results

9.1 α-Synuclein-mediated toxic effect on GFP-expressing control cells

In order to investigate the toxic effect of extracellular αSyn released from αSyn-overexpressing cells, co-cultures of αSyn-overexpressing and GFP-expressing LUHMES cells were produced. Cultures of 100% GFP^+ cells, of 75% GFP^+ and 25% $αSyn^+$ cells, of 50% GFP^+ and 50% $αSyn^+$ cells, and of 25% GFP^+ and 75% $αSyn^+$ cells were seeded in a 48-well format. On the fifth day after reseeding into co-cultures, DRAQ7 was used to stain the cells. DRAQ7 penetrates impaired cell membranes and therefore exclusively stains dying cells. By this means, the αSyn-mediated toxic effect on the GFP-expressing cells was monitored (see Figure 3).

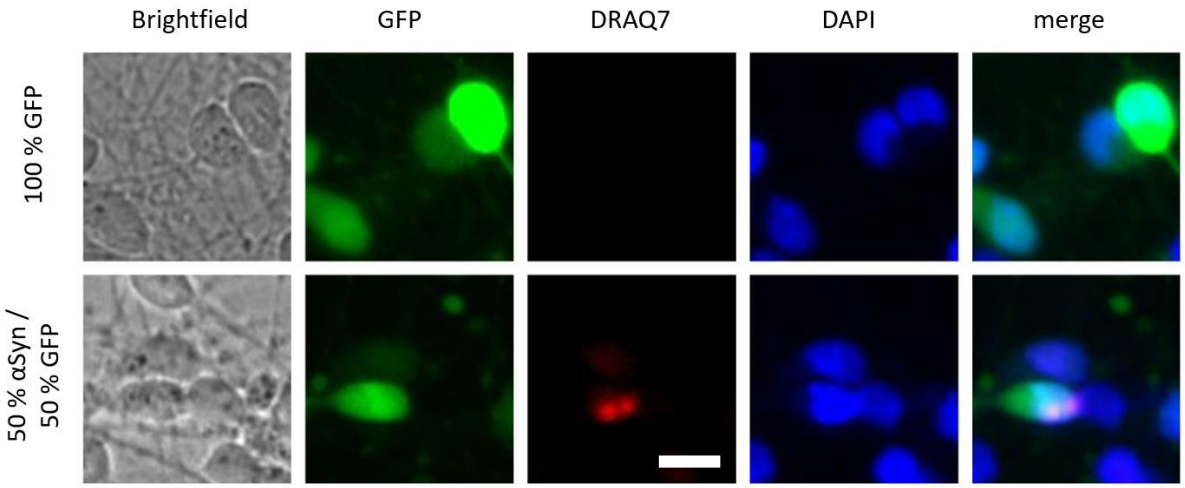

Figure 3 **Representative set of pictures of the co-culture.** The toxicity in the culture was measured using DRAQ7. Cells with co-localization of DRAQ7 and GFP signal were counted as positive. Cells only containing GFP without DRAQ7 were assumed to be healthy. The ratio of positive (affected) to healthy cells was calculated. DAPI was used to stain the nuclei. Following the process of staining and fixation, images were taken using a Leica Microscopy system and the cells were counted manually. The displayed images are magnified for optimal display. Scale bar: 10 µm.

We observed a toxic effect on the GFP^+ cells by increasing the ratio of αSyn-overexpressing to GFP-expressing cells in the co-culture. As shown in Figure 4, GFP-expression in LUHMES cells resulted in a baseline toxicity within the 100% GFP^+ population of 4.9 ± 0.5%. By co-seeding 75% GFP^+ and 25% $αSyn^+$ cells, we observed a not significant increase in toxicity in comparison to GFP-induced baseline toxicity (5.8 ± 0.5%; p = 0.8 vs. 100% GFP^+ culture). In a co-culture of 50% $αSyn^+$ and 50% GFP^+ neurons, the GFP^+ cells were affected significantly and the percentage of $DRAQ7^+$ GFP^+ cells increased to 9.2 ±

0.4% ($p = 0.0004$ vs. 100% GFP^+ culture). An even greater effect on the GFP^+ population could be shown when 75% of the cells were transduced with αSyn (15.2 ± 1.1%; $p < 0.0001$ vs. 100% GFP^+ culture). This finding indicates a harmful effect of αSyn-overexpressing cells on previously healthy cells in proximity, most likely due to the release of αSyn into the medium.

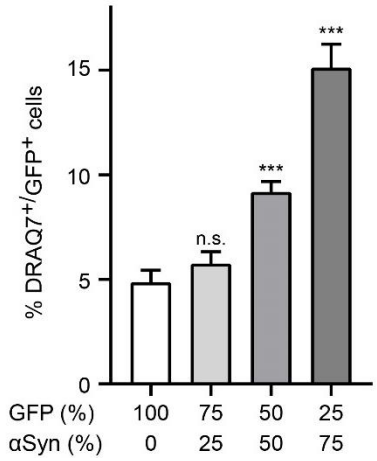

Figure 4: **α-Synuclein-mediated toxic effect on GFP-expressing control cells.** Co-cultures of αSyn-overexpressing and GFP-expressing cells were prepared, and the cells were stained with DRAQ7 to monitor cell death. The localization of DRAQ7 in GFP^+ cells was counted and divided by the total number of GFP^+ cells in the culture. We observed an increased cell death in the GFP^+ population with an increasing number of αSyn-overexpressing cells in the co-culture. The data were normalized to the 50% $αSyn^+$ / 50% GFP^+ population of each plate. Values are presented as mean ± SEM. ***$p < 0.001$ vs. 100% GFP^+ (one-way ANOVA, post hoc Tukey's test).

9.2 Antibody-aided protection against α-synuclein mediated toxicity

To study the protective abilities of three αSyn-specific tool antibodies, 50% $αSyn^+$ / 50% GFP^+ co-cultures were treated with the antibodies on the day of reseeding into co-cultures. A baseline toxicity of 5.4 ± 0.6% was observed in the 100% GFP^+ control population. In comparison, the fraction of dead GFP^+ cells in the untreated 50% $αSyn^+$ / 50% GFP^+ positive control co-culture was 10.2 ± 0.2% ($p < 0.0001$ vs. 100% GFP^+ control culture). Four different antibodies were investigated:

- EG27/1: Control antibody.
- 23E8: Anti-αSyn tool antibody.
- 5D12: Anti-αSyn tool antibody.
- 8A5: Anti-αSyn tool antibody.

Each antibody was tested in a concentration of 25 nM for a potential protective efficacy. Toxicity was assessed five days after reseeding. As DRAQ7 exclusively stains dying cells, the ratio of $DRAQ7^+$ GFP^+ cells to the total number of GFP^+ cells was calculated as a measure for the toxic effects in the culture.

Two of the four ABs tested decreased the αSyn-induced toxicity significantly compared to the control co-culture without treatment. Control antibody EG27/1 did not reduce the number of $DRAQ7^+$ cells compared to the untreated control co-culture (10.0 ± 1.1%; $p > 0.99$ vs. untreated control), indicating no unspecific biological effect due to antibody treatment. Additionally, anti-αSyn AB 5D12 also showed no ability to reduce the amount of $DRAQ7^+/GFP^+$ cells in the culture (12.2 ± 1.5%; $p = 0.23$ vs. untreated control). 23E8 reduced the αSyn-mediated toxicity to 6.0 ± 0.4% ($p < 0.0001$ vs. untreated control) (see Figure 5). In addition, it was found that also 8A5 reduced the toxicity significantly (6.6 ± 0.7%; $p = 0.0006$ vs. untreated control). Both anti-αSyn antibodies 23E8 and 8A5 also demonstrated protective efficacy compared to the control antibody EG27/1 ($p = 0.005$ vs. 23E8, $p = 0.03$ vs. 8A5).

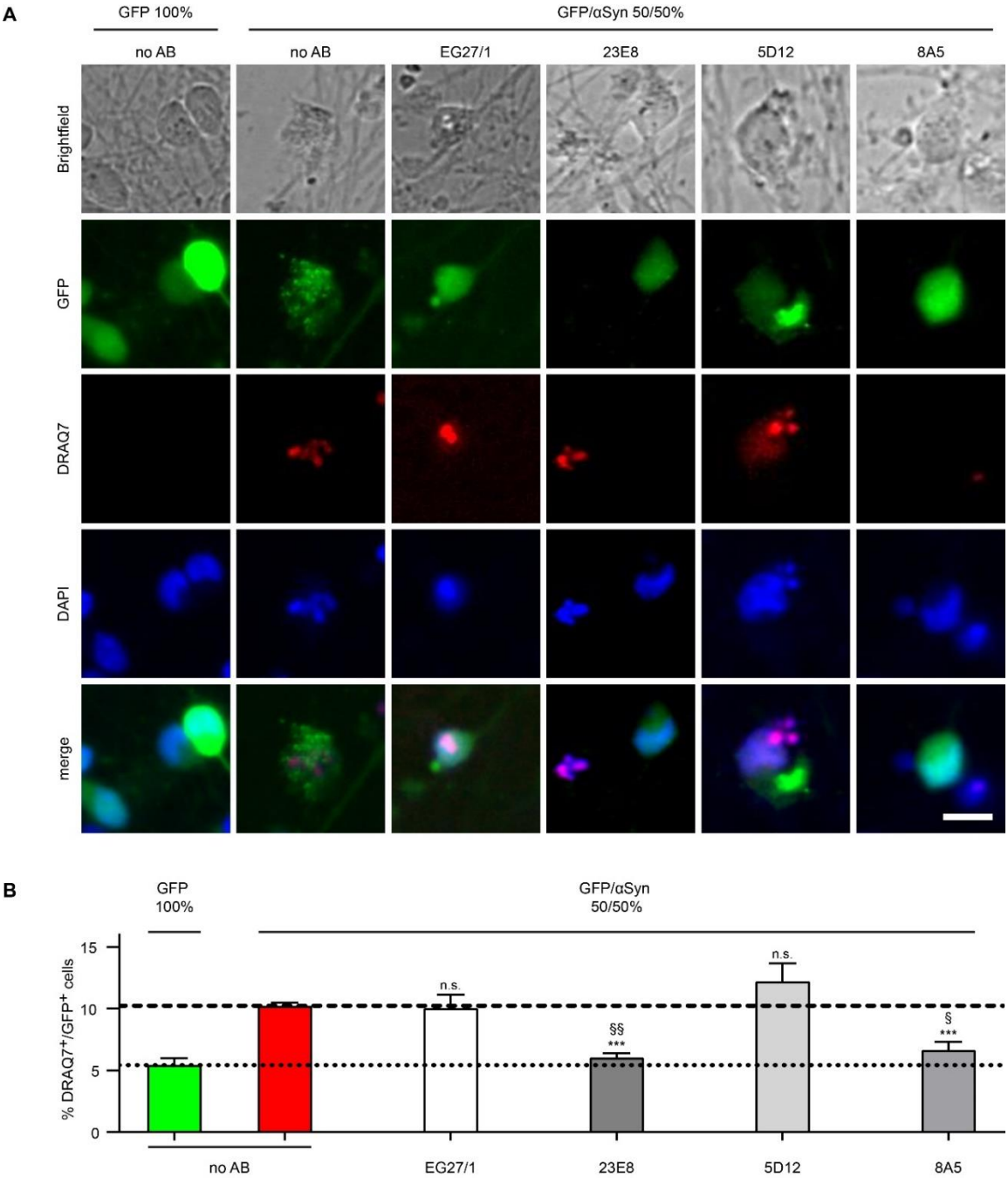

Figure 5 A **Representative microscopic images of the antibody-treated co-culture.** To measure the protective effect against αSyn-mediated toxicity, the four antibodies were tested in a 50% αSyn$^+$ / 50% GFP$^+$ co-cultures. The toxic effect in each culture was measured using DRAQ7. The cells with a colocalization of DRAQ7 (red) and GFP (green) signal were counted as positive. Cells which only contained GFP, but without DRAQ7 were considered to be healthy. A ratio of DRAQ7$^+$ GFP$^+$ cells to healthy GFP$^+$ cells was calculated. DAPI (blue) was used to stain the nucleus. Scale bar: 10 µm. ***B*** **Investigation of the protective efficacy of αSyn-specific antibodies against αSyn-mediated toxicity.** On day six after the antibody treatment, the cells were stained with DRAQ7. The number of DRAQ7$^+$ / GFP$^+$ cells was counted. 23E8 and 8A5 were able to significantly reduce the αSyn-mediated toxicity. The data were normalized to the 50% αSyn$^+$ / 50% GFP$^+$ population, and values are presented as mean ± SEM. § $p < 0.05$, §§ $p < 0.01$ vs. control antibody EG27/1; *** $p < 0.001$ vs. untreated control co-culture (one-way ANOVA, post hoc Tukey's test).

9.3 α-Synuclein bound by the antibodies in the cell culture medium

9.3.1 α-Synuclein detected by Western blots

Two of three of the tested tool antibodies protected against αSyn-related toxicity in our cell model. One possible mechanism of action could be shielding against pathogenic extracellular αSyn. To identify the αSyn species bound by the antibodies in the medium, Western blots were performed. On the fourth and sixth day after seeding, medium of αSyn-overexpressing LUHMES cells was collected. After the separation of the proteins in the medium by gel electrophoresis and protein transfer to membranes, the membranes were incubated in solutions of the antibodies EG27/1, 23E8, 5D12, and 8A5 (see Figure 6).

Control antibody EG27/1 showed no intrinsic affinity to αSyn. All anti-αSyn antibodies 23E8, 5D12, and 8A5 bound proteins at the size of αSyn in its monomeric form (15 kDa) at day six in the medium from αSyn-overexpressing cells. They did not display any preference for distinct αSyn species i.e. monomers, oligomers, or fragments. Additionally, the protective antibodies 23E8 and 8A5 also bound αSyn in the medium four days after transduction (blue arrows). Since the abundancy of αSyn in this medium was lower compared to medium from day six, we concluded that 23E8 and 8A5 have a higher affinity to cell-derived αSyn compared to 5D12.

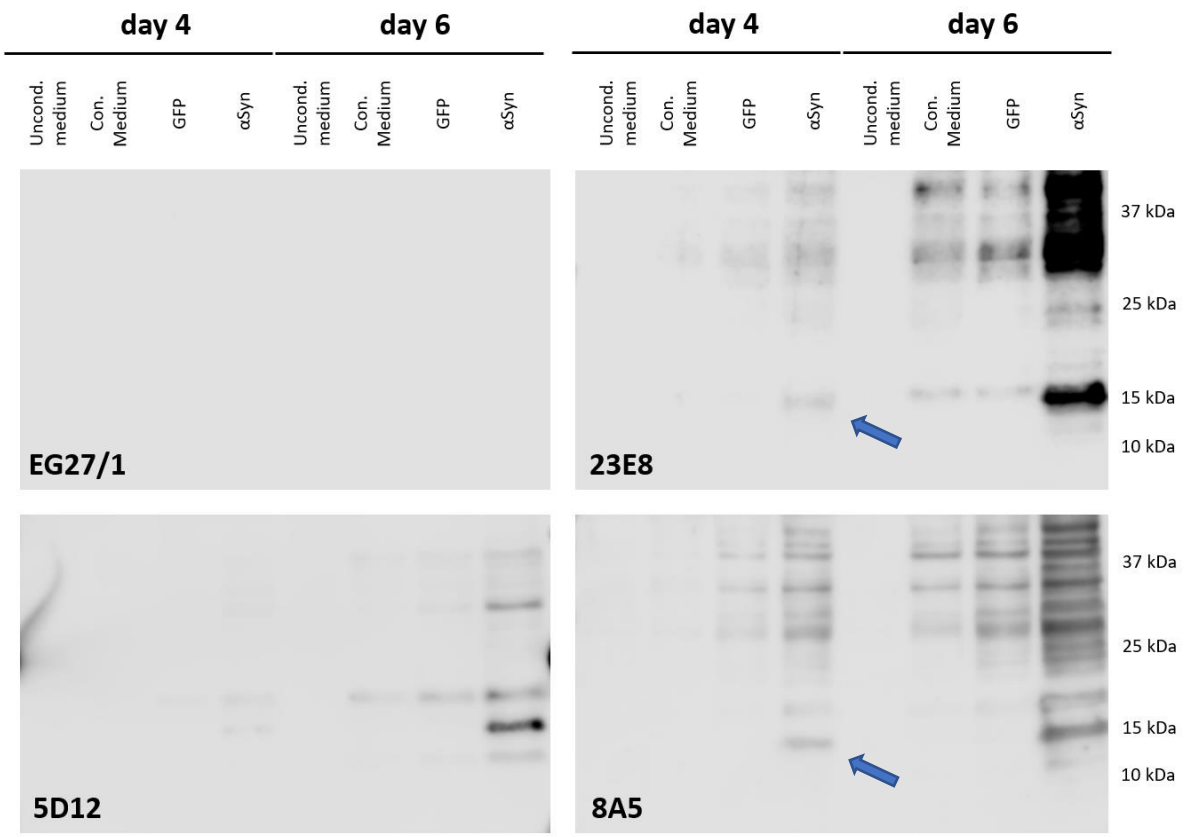

Figure 6 **Binding pattern of the antibodies in the cell culture medium.** Western blot analysis of the antibodies' binding pattern in the medium. The unconditioned medium without cells, the conditioned medium from untransduced LUHMES cells, the medium from GFP-expressing LUHMES cells, and the medium from αSyn-overexpressing LUHMES cells were all harvested at two time points (day 4 and day 6 after transduction). Anti-αSyn antibodies 23E8, 5D12, and 8A5 bound to monomeric αSyn (15 kDa) at day 6. In addition, 23E8 and 8A5 detected αSyn also at day 4. EG27/1 did not show any binding.

9.3.2 α-Synuclein detected by immunoprecipitation

In the preceding section, we demonstrated that the tested tool antibodies bind to a protein at the size of monomeric αSyn in the cell culture medium. To further investigate the amount of αSyn bound by the tested tool antibodies, an immunoprecipitation was performed. Figure 7a displays the electrophoretically separated αSyn species bound by the tested antibodies in the immunoprecipitation (IP). Two different commercial αSyn antibodies were used to visualize the αSyn bound by the antibodies. Monomeric αSyn (ca. 15 kDa) was effectively scavenged by antibodies 23E8 and 8A5 from the medium. Control antibody EG27/1 did not scavenge the αSyn monomer. Compared to 23E8 and 8A5, 5D12 only bound small amounts of αSyn in the medium, resulting only in a faint band in the Western blots (Figure 7a). Additionally, we used an ELISA to quantify the amount of αSyn remaining in the corresponding cleared medium after immunoprecipitation (Figure 7b). Correspondingly, as

visualized in the performed ELISA, 23E8, and 8A5 scavenged high amounts of αSyn from the conditioned medium (23E8: 79 ± 1.5% of control AB EG27/1; 8A5: 71 ± 2.2% of control AB EG27/1). Antibody 5D12, however, reduced the total extracellular αSyn only by 27 ± 2.6% compared to control AB EG27/1 (Figure 7b).

Taken together, this indicates that sufficient amounts of extracellular αSyn need to be captured by ABs for them to show effectiveness.

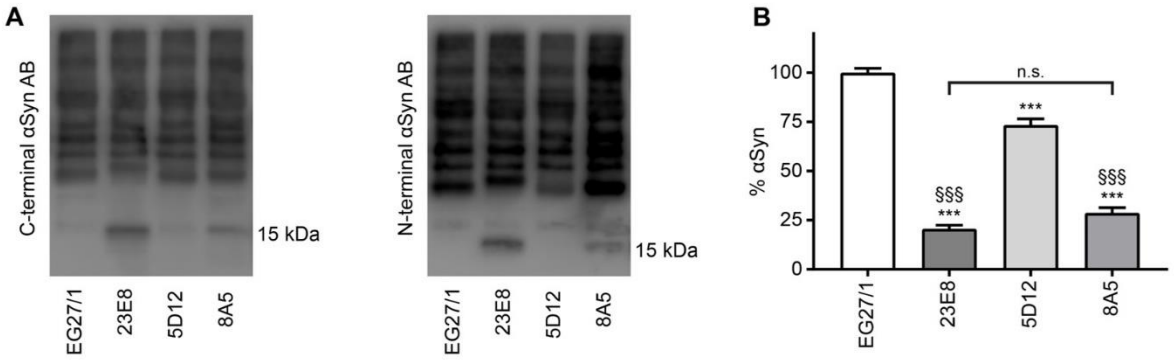

Figure 7 A **Western blot analysis of the αSyn-species separated by immunoprecipitation (IP).** Samples from the IP were separated electrophoretically. The four tested antibodies were used for the immunoprecipitation. After breaking up the antigen-antibody-bead complex, the Western blot was performed. Commercially available anti-αSyn antibodies (N-terminal and C-terminal) were used to visualize the αSyn species in the samples. 23E8 and 8A5 bound the αSyn monomer (15 kDa) to a greater extent compared to 5D12. The bands above the monomer were residues from the ABs that remained in the cleared medium. ***B*** **Anti-αSyn ELISA of the cleared medium after the IP.** 23E8 and 8A5 significantly reduced the amount of αSyn remaining in the medium after IP compared to 5D12. Values are presented as mean ± SEM. *** $p < 0.001$ vs. EG27/1, §§§ $p < 0.001$ vs. 5D12 (one-way ANOVA, post hoc Tukey's test).

9.4 Epitope mapping of the potentially therapeutic antibodies

The epitope specificity could be important for the antibodies' effectiveness as proposed by some authors (e.g. Games et al. 2014). To determine the epitopes that the antibodies bind to, a dot blot analysis was performed. In order to do this, recombinant full-length (FL) and fragmented αSyn was loaded on membranes. The antibodies' binding capacities to commercially available fragments of αSyn (the N-terminus (NT), the NT and NAC domain (NT+NAC), the C-terminus (CT), the CT and NAC domain (CT+NAC), and the NAC domain alone) were investigated (see Figure 8). The secondary anti-mouse antibody displayed no spurious unspecific signals (No AB).

While EG27/1 showed neither an intrinsic affinity to the FL αSyn nor to the αSyn fragments, all AB against αSyn detected the FL. As seen in Figure 8b, 23E8 showed clear signals from the NT, and the

NT+NAC, but lacked any signal from the CT. This indicates an epitope in the NT region (AA 1-60). 5D12 demonstrated strong signals to the CT+NAC fragment and weak signals from the CT alone. This indicates an epitope in the CT. 8A5 was the only antibody to strongly detect the CT. Since there was also signal from the CT+NAC, this is strong evidence for an epitope in the CT region (AA 91-120). It did not bind to the NT. All four antibody showed week signals in the NAC domain which we therefore considered as unspecific signals.

Interestingly, the two protective antibodies (23E8 and 8A5) did not share the same epitope. Therefore, we concluded that epitope specificity was not sufficient to explain the differences in the protective efficacies between the antibodies.

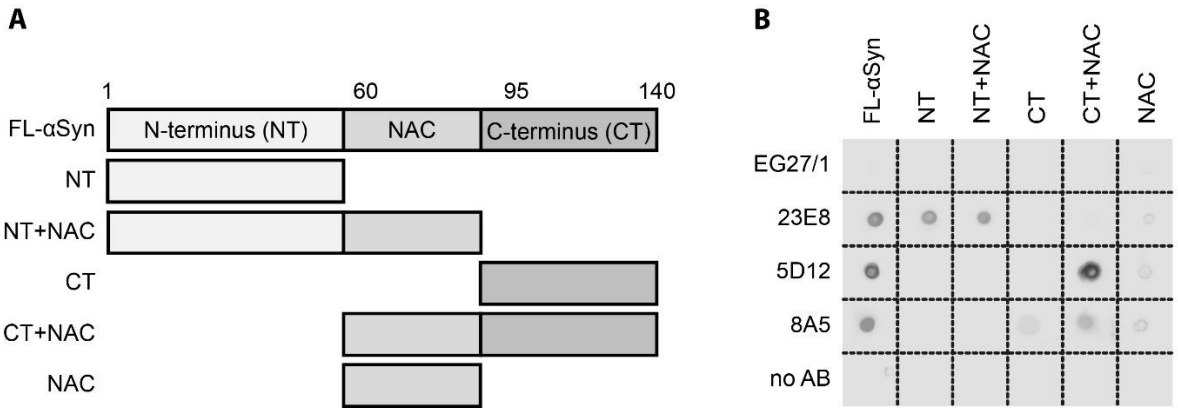

Figure 8 A **Fragments of αSyn used for epitope mapping.** Commercially available fragments of the N-terminus (NT), the NT and NAC domain (NT+NAC), the C-terminus (CT), the CT and NAC domain (CT+NAC), and the NAC domain alone were used. ***B*** **Epitope mapping of the four tested antibodies.** 1 µg recombinant full-length or fragmented αSyn was loaded on a membrane, followed by incubation with four tested ABs and only the secondary antibody as control (No AB). The signal was acquired by a HRP-labelled anti-mouse AB. All anti-αSyn ABs bound the full length αSyn. 23E8 displayed affinity to the NT, while 5D12 and 8A5 bound C-terminally.

9.5 Sensitivity of the antibodies to the recombinant α-synuclein monomer

In order to study the sensitivity of the four tested ABs to full-length αSyn, recombinant full-length αSyn was loaded on membranes. The membranes were incubated in dilutions of the tested antibodies, starting from 25 nM with 1:2 dilution steps. The amount of antibody bound to the recombinant full-length αSyn was visualized by the use of a fluorescence-coupled secondary anti-mouse antibody. The signal from the secondary antibody was proportional to the sensitivity of the tested antibodies to the recombinant full-length αSyn.

As displayed in Figure 9, all tool antibodies 23E8, 5D12, and 8A5 bound to αSyn. The control antibody EG27/1 did not bind to the recombinant full-length αSyn ($p < 0.0001$ vs. 23E8, 5D12, 8A5). Overall, 8A5 yielded the strongest absolute signal. 23E8 showed notably weaker signals in dilution steps 1 ($p = 0.021$), 5 ($p = 0.032$), and 7 ($p = 0.013$) compared to 8A5. 5D12 displayed a sensitivity to αSyn between the two protective antibodies 23E8 and 8A5, however, not statistically significant.

This indicates that sensitivity to recombinant αSyn was not sufficient to explain the differences in the antibodies' protective abilities.

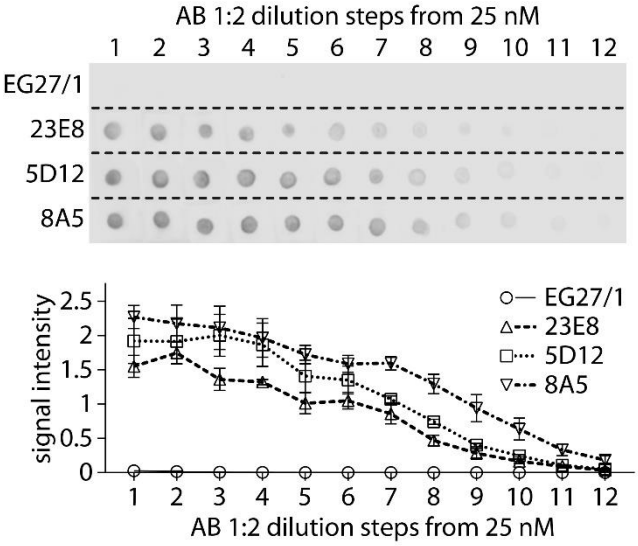

Figure 9 **The antibodies' sensitivity to the recombinant α-synuclein monomer.** 0.25 µg of recombinant full-length αSyn were loaded on a membrane. The membrane was incubated in dilutions of the antibodies, starting from 25 nM with 1:2 dilution steps. The signal was then normalized to the total protein amount. 5D12 displayed a sensitivity to αSyn between the protective antibodies 8A5 (higher sensitivity) and 23E8 (lower sensitivity).

9.6 Binding stability of the antibody-antigen-complex

For effective scavenging of the antigen, an antibody needs to form a stable complex with the antigen. The stability of the complex can be estimated by measuring the affinity index of the antibody (Svobodova 2013). Therefore, we challenged the antibody-antigen binding with ammonium thiocyanate (HN_4SCN), a chaotropic agent, in increasing concentrations. We observed no differences in the resistance to the chaotropic agent between the protective antibodies 23E8 and 8A5 (see Figure 10). Despite the fact that 5D12 displayed comparable sensitivity towards αSyn, it demonstrated a lower affinity index in comparison to 23E8 and 8A5. From this, we concluded that the complex of 5D12 and αSyn was more likely to dissociate, leading to a lower scavenging ability.

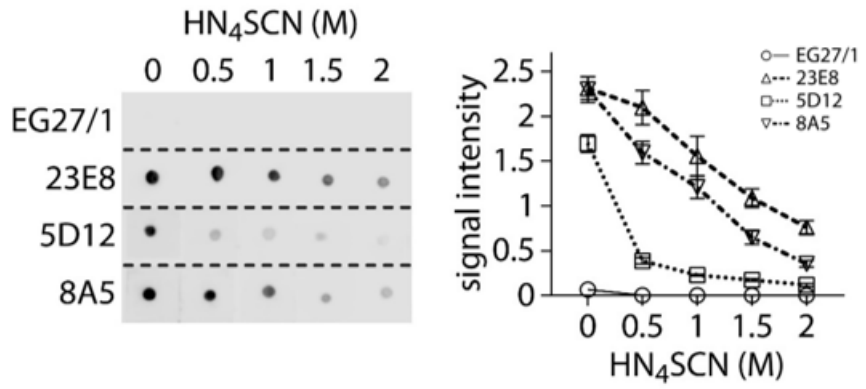

Figure 10 **Binding stability of the antibody-antigen-complex.** The antibody-antigen-complex of the antibodies EG27/1, 23E8, 5D12, and 8A5 were challenged using a chaotropic agent (HN_4SCN) in increasing concentrations. We observed that the complex of 5D12 and αSyn dissociated easier compared to the complexes of αSyn and 23E8/8A5.

10 Discussion

The main objectives of this thesis were the establishment of a cell-based model of αSyn spreading and the investigation of the protective properties of three anti-αSyn antibodies. All antibodies were investigated in order to identify common properties. An *in vitro* model of co-cultured αSyn-overexpressing and GFP-expressing LUHMES neurons was established. The co-culture model of GFP^+ and $αSyn^+$ LUHMES cells led to toxicity in the GFP^+ subpopulation. This co-culture model was subsequently treated with anti-αSyn antibodies. Two of the tested tool antibodies (23E8 and 8A5) significantly reduced the level of αSyn-mediated toxicity. Even though the two protective antibodies did not share the same epitope, they demonstrated a resemblance in the manner in which they bound to cell-derived αSyn. Furthermore, we were able to demonstrate that the protective antibodies compared to the unprotective antibody formed more stable complexes with αSyn and therefore scavenged extracellular αSyn in significantly higher amounts.

10.1 A novel cell culture model of α-synuclein spreading to investigate potential therapeutics

In order to reduce αSyn-mediated neurodegeneration, various disease-modifying drugs, including immunotherapeutic antibodies, have been investigated over the last decade. Especially potent cell models can be used to screen large drug libraries and can additionally deliver relevant insights into the underlying mechanisms behind immunotherapy. Even though different cell lines were used in previous studies to study passive immunotherapy against αSyn, no study was conducted in human dopaminergic neurons so far. Our newly designed model is based on LUHMES cells, an immortalized cell line, which in its differentiated state, displays a neuronal phenotype and shows markers of dopaminergic neurons (Scholz et al. 2011; Höllerhage et al. 2014). Lotharius et al. (2002) demonstrated that these LUHMES neurons express tyrosine hydroxylase, which is the rate-limiting enzyme in the dopamine biosynthesis. Furthermore, LUHMES cells show electric activity, while they also synthesize and release dopamine upon stimulation (Scholz et al. 2011). Previously, researchers from our group demonstrated that adenoviral overexpression of wild-type αSyn led to the accumulation of αSyn in LUHMES cells, which resulted in increased cell death (Höllerhage et al. 2014). Additionally, it has been shown that adenoviral-overexpression led to increased αSyn release by these cells into the cell medium (Fussi et al. 2018; Chakroun et al. 2020). In the present study, we demonstrated that αSyn-overexpression not only affected transduced cells but also led to the spreading of the pathology to

previously healthy cells. It has been demonstrated previously that αSyn, released from two neuroblastoma cell lines (B103 and SH-SY5Y) into the medium, can spread to co-cultured cells in an insert-based model (Games et al. 2014; Schofield et al. 2019). In both cell lines, the cell-to-cell transmission of αSyn was reduced by treatment with αSyn-specific antibodies. In these insert-based models the αSyn-releasing cells and the recipient cells share the same medium but are not in proximity hence cannot interact via synapses (Games et al. 2014; Schofield et al. 2019). Findings from other groups indicated that αSyn can also propagate between primary mouse neurons via axonal trafficking in micro-fluid-chamber-models, which can also be reduced by passive treatment with antibodies (Tran et al. 2014). Especially *in vivo,* axonal propagation plays an important role. Spencer et al. (2017) demonstrated axonal propagation of αSyn in wildtype mice and a transgenic mouse model, where αSyn is overexpressed under the mThy1 promoter (line 61) (Chesselet et al. 2012). If an αSyn-related pathology is induced by lentiviral αSyn injection in one hemisphere in these mice, αSyn also accumulates in the contra-lateral hemisphere (Spencer et al. 2017). Since αSyn was detected in commissural fibers and axons in the corpus callosum, both known to be connecting the hemispheres, the authors concluded that the propagation to the contralateral hemisphere happens via axons in these mice (Spencer et al. 2017). Furthermore, the accumulation of αSyn in the contralateral hemisphere was reduced by antibody-treatment (Spencer et al. 2017). LUHMES neurons form neuronal networks and express axonal and synaptic markers (Smirnova et al. 2016). Therefore, αSyn most likely also propagates via axons between LUHMES neurons. Previously used models only address αSyn propagation either via medium or via axonal trafficking. In our new cell model, αSyn possibly spreads through the medium and via axonal trafficking. Therefore, this model delivers a unique opportunity to study therapeutics targeting αSyn spreading. The underlying mechanisms in our model are likely to be comparable to the *in vivo* spreading of the αSyn pathology.

The fact that αSyn-specific antibodies protected GFP^+ cells from toxicity by scavenging significant amounts of αSyn from the medium, as shown by immunoprecipitation and ELISA, strongly suggests that αSyn is the main pathogenic agent in our model. Different mechanisms of αSyn release from cells into the medium have been described (Grozdanov et al. 2018). αSyn was shown to be released actively by conventional and Golgi- or endoplasmic-reticulum-independent exocytosis (Lee et al. 2005) or by exosomes (Fussi et al. 2018). Furthermore, αSyn can be released passively by diffusion over an intact cell membrane or through a compromised cell membrane, due to cell death (Grozdanov et al. 2018). PD patients are characterized by progressive neuron loss in the substantia nigra and other parts of the brain (Obeso et al. 2017). We found αSyn in the medium prior to cell death, which indicates that αSyn is released independent of cell death and membrane permeability to some extent in our model. Whether extracellular αSyn originates from apoptotic or living cells might be pathophysiological irrelevant since the tested antibodies reduce the extracellular amount of αSyn independent of the

release mechanism. In PD patients, extracellular αSyn might influence cells in proximity in the same way as it does in our model. Over the course of the co-culture experiment a small fraction of all LUHMES cells in the culture dies and also releases other cytosolic components like electrolytes, proteases, and cytokines into the medium (Elmore 2007). To what extent these released components affect the GFP^+ population in our model is unknown. The fact that by scavenging αSyn the toxicity can be reduced almost completely in our model suggests that the impact of these cytosolic components is only marginal.

10.2 Tool antibodies´ properties and their protective abilities

We investigated the protective efficacy of three anti-αSyn antibodies against toxicity associated with αSyn in our new neuron-based model. Two of the tested antibodies demonstrated protective properties against the αSyn-mediated toxicity: antibody 23E8 and 8A5 reduced cell death in the GFP^+ population significantly. Neither the control antibody EG27/1 nor the anti-αSyn tool antibody 5D12 were able to reduce cell death. As expected, the control antibody EG27/1 showed no intrinsic affinity to αSyn in the performed sensitivity tests or the epitope mapping and also did not have an effect on cell viability in our cell model. Tool antibody 5D12 presented similar sensitivity to recombinant αSyn monomers in comparison to the protective antibodies 23E8 and 8A5. Therefore, we concluded that the sensitivity to recombinant αSyn monomers is not sufficient to explain the disparities in the protective properties of the tested antibodies. However, 5D12 did not bind to cell-derived αSyn to the same extent as 23E8 and 8A5 as shown using Western blots and immunoprecipitation. This discrepancy between the sensitivity to recombinant and cell-derived αSyn demonstrates the importance of testing potential therapeutics preferably under conditions as physiological as possible. Different post-translational modifications of αSyn including truncation, acetylation, phosphorylation, ubiquitylation, and nitration have been described (Hiroyasu et al. 2013). These modifications could be one possible explanation for the differences in the antibodies' binding to recombinant and cell-derived αSyn. Post-translational modifications influence antigen-antibody-interactions substantially and require complex models to mimic physiological conditions (Bergström et al. 2016). However, we did not investigate in what way αSyn undergoes post-translational modifications in our model and therefore cannot make final conclusions.

The role of the anti-αSyn antibodies' epitope in passive immunotherapy has been investigated, but inconclusive data has been collected by various groups. On the one hand, Games et al. (2014) proposed that a C-terminal binding to αSyn might be essential, since pathologically relevant truncation via

calpain-1 takes place in the C-terminus. They delivered evidence that a C-terminal antibody (9E4), that blocked the binding site of calpain-1 at AA 120, reduced αSyn-mediated pathology in a mouse model. On the other hand, they also found that the anti-αSyn antibody 5D12 was not protective in a mThy1-αSyn transgenic mouse model despite the fact that it also bound αSyn at the calpain-1 cleavage site. This is in line with our observations that 5D12 binds in the C-terminus. Furthermore, 5D12 also showed no protective efficacy in our model. Games et al. (2014) proposed that these differences in the protective efficacies between the antibodies binding at the calpain-1 binding site could be due to conformational differences in the antibodies' preferences for αSyn. Additionally, we investigated another C-terminal antibody, 8A5, that has previously been reported to bind to AA 125-140 (Masliah et al. 2011) and therefore not at the calpain-1 binding site. Nevertheless, we also found 8A5 to be protective in our model. Furthermore, we also found that the N-terminal antibody 23E8 protected from aSyn mediated toxicity in our model, showing that both C- and N-terminal antibodies can protect against aSyn mediated toxicity. Therefore, our findings suggest that C-terminal binding, especially at the calpain-1 binding site, is not essential for a potentially therapeutic antibody. This is also supported by data from Weihofen et al. (2019), who demonstrated that a N-terminal antibody, BIIB054, could be a promising candidate for clinical testing since it displayed protective efficacy in a A53T transgenic mouse model. These mice display PD-like motor impairments and overexpress αSyn, which carries a mutation in AA 53 and is more prone to form oligomeric species (Lee et al. 2002; Conway et al. 1998). After treatment with BIIB054, these mice suffered from less motor impairments and displayed a higher dopamine transporter density in the striatum (Weihofen et al. 2019). In another study, Fisher 344 rats, a commonly used rat model for cancer and aging research, were injected with an adeno-associated viral vector encoding WT human aSyn and consecutively treated with a N-terminal anti-αSyn antibody. Rats treated with the N-terminal antibody suffered from less dopaminergic cell loss and less behavioral deficits (Shahaduzzaman et al. 2015). Additionally, a study conducted in Sprague–Dawley rats, also a broadly used rat model, delivered further evidence for the effectiveness of an antibody-based approach for different epitopes. In this study, Chatterjee et al. (2018) induced pathology by adeno-associated viral vector encoding for human WT αSyn and treated the rats with so-called nanobodies directed against aSyn. Nanobodies are antibody fragments with comparable binding specificity that can be introduced into cells via adeno-associated viral vectors and are then expressed intracellularly. In this study, a C-terminal nanobody as well as a nanobody directed against the NAC domain reduced behavioral deficits significantly. Taken together, our data, supplemented by data from other groups, suggests that the epitope specificity is not the most important property of potentially therapeutic αSyn antibodies.

10.3 Implications on the principles of immunotherapy against α-synuclein

We tested our antibodies against αSyn in the therapeutic molar range that is currently investigated for protective efficacy in patients. In clinical trials, patients receive up to 6 g of anti-αSyn antibodies, which equals 1 mg/ml at an average blood volume of six liters (Jankovic et al. 2018). Antibodies, peripherally administered, have been reported to cross the blood-brain-barrier in small amounts of approximately 0.4% (Schofield et al. 2019; Bard et al. 2000; Yu et al. 2013), which corresponds to an intracerebral concentration of 4 µg/ml in the patient. We tested the protective efficacy of our antibodies at a concentration of 25 nM. Assuming a molecular weight of IgG of 140 kDa, this corresponds to 3.75 µg/ml in the cell culture medium, showing that we tested our antibodies in a concentration that can very well be achieved in the patient, supporting the relevance of our model.

Different αSyn target species for passive immunotherapy have been proposed over the past decade. Antibodies directed against the αSyn monomers (Schofield et al. 2019; Games et al. 2014), oligomeric αSyn (Gustafsson, Lindström, et al. 2017; Gustafsson, Eriksson, et al. 2017; El-agnaf et al. 2017; Schofield et al. 2019), and αSyn fibrils (Weihofen et al. 2019; El-agnaf et al. 2017) have been tested and found to be protective against αSyn-mediated toxicity in cell culture and mouse models. In our model, we did not identify a distinct preference of our antibodies for any oligomeric or fibrillar species. However, we deliver further evidence that monoclonal antibodies can protect neurons from the toxic effect of extracellular αSyn. Our findings indicate that spreading of αSyn-induced pathology can be reduced by scavenging αSyn in the extracellular space independent of a preference for a distinct αSyn species.

In physiological conditions, various other proteins might compete with the antibody to bind to the harmful αSyn species. Thus, quick binding kinetics and stable antibody-antigen-complexes are important. Therefore, we investigated the stability of the antibody-antigen-complexes by challenging the binding with a chaotropic agent (Svobodova 2013). We found the complexes of αSyn and 23E8 and 8A5 to display a higher stability compared to complexes of the unprotective antibody 5D12 and αSyn. This suggests that antibodies that form more stable antibodies-antigen-complexes are more effective in scavenging αSyn from the extracellular space.

Antibody-antigen-interactions follow complex biochemical rules. Therefore, we conducted a series of experiments to investigate named interactions. We started in a rather unphysiological setting using recombinant αSyn and proceeded to physiological conditions to the greatest possible extent *in vitro*. In the biochemistry of antibody-antigen-interactions a profound nomenclature is used. In general, the term affinity describes the interaction of an individual epitope to an individual binding site of the

antibody, while the avidity is the sum of the functional binding of all antibody-antigen interactions from different binding sites (Rudnick et al. 2009). Physiologically, avidity might play a greater role than the monovalent affinity. Affinity, respectively avidity, cannot be measured directly (Goldberg and Djavadi-Ohaniance 1993). However, to estimate differences in the binding properties between the antibodies, we measured the amount of antibody that binds to a distinct amount of antigen (αSyn) and referred to it as sensitivity. At first, we tested the sensitivity to recombinant, nitrocellulose membrane-bound αSyn in a dot blot experiment. In this setting, we found no significant difference between protective and not protective antibodies. Since we used recombinant αSyn in this experiment, conformational differences between physiological αSyn and recombinant αSyn must be considered. Conformational changes in the protein might make an epitope not accessible for the antibody. Therefore, a more physiological approach to the binding behavior of the antibodies might be needed to explain the differences in the protective efficacies. In a next step, we investigated the antibodies' sensitivity against cell-derived αSyn in Western blots of the cell medium. In this condition, we expected the conformation of αSyn to be more physiological compared to recombinant αSyn. Since both protective antibodies detected αSyn in the medium on day four after transduction, while the not protective antibody did not, this indicates that our protective antibodies have a higher sensitivity to cell-derived, physiological αSyn compared to the not protective antibody. However, during a Western blot the antigen is also fixed to a membrane and the physiological binding capacity in solution might be even higher. In physiological conditions in patients, the antigen and the antibody are in solution, which offers more degrees of freedom for them to interact. Less degrees of freedom, as presented in the dot blot or Western blot, might influence the binding behavior of the antibody to the antigen, which partly might influence our findings. To address antigen-antibody binding in solution we conducted an immunoprecipitation. We found that the protective antibodies 23E8 and 8A5 scavenged higher amounts of αSyn from the medium compared to the not protective antibody 5D12. The protective antibodies reduced the amount of αSyn in the medium by 70%. Scavenging significant amounts of αSyn from the extracellular space is known to be one possible mechanism of action of passive immunotherapy (Bergström et al. 2016). We delivered evidence that antibodies that scavenge αSyn from the extracellular space in a significant amount, reduce the amount of αSyn in the medium and therefore protect against neuronal cell death. Antibodies for clinical testing cannot be selected by their sensitivity to recombinant αSyn but should be selected by their ability to scavenge and reduce extracellular, cell-derived αSyn.

Even though our model is well suited to investigate measures to reduce extracellular aSyn derived from a cellular context including principles of passive immunization, there are some limitations. One downside of the presented co-culture model is the approximate 4 fold increase of αSyn by adenoviral overexpression, that might not occur in PD patients (Höllerhage et al. 2014). Furthermore, the role of

other cell types of the central nervous system like astrocytes and oligodendrocytes in the pathophysiology of α-synucleinopathies and their role in passive immunotherapy cannot be investigated in this model. In PD patients, neurodegeneration occurs over a period of years, in animal models for PD it also takes weeks to months for the pathology to occur (Obeso et al. 2017; Lee et al. 2002). However, in our model an experiment can be conducted in a time course of 8 days from seeding of the cells to the readout measurement. This quick approach enables researchers to screen a greater number of potential antibodies in this model.

In summary, the observed differences in the sensitivity to recombinant αSyn between the antibodies did not correlate with the antibodies' protective efficacies and therefore the sensitivity to recombinant αSyn alone is not suitable to select antibodies for clinical testing. In conditions in which we used cell-derived αSyn and therefore are closer to physiology, as addressed by immunoprecipitation and Western blots, the protective antibodies bound to αSyn monomers from the medium to a greater extent compared to the not protective one. They also reduced the amount of αSyn present in the medium significantly. Therefore, we not only demonstrated one possible mechanism of action for immunotherapy, but we also concluded that future investigations of potential therapeutic antibodies against αSyn should be conducted in models that are as close to the physiology as possible. In the future, the reactivity of anti-αSyn antibodies to human αSyn from PD patients could shed light on the antigen-antibody interactions in the patient. Samples of the cerebrospinal fluid (Hansson et al. 2014) or of the interstitial fluid collected by brain microdialysis during brain surgery (Chefer et al. 2009; Yamada et al. 2011) could be used for this purpose. Our data suggest that the choice of the right analytic methods for the investigation of the properties of potentially protective αSyn antibodies is very important.

11 Conclusion

Our model delivers an easily accessible, neuron-based, convenient system to test potentially therapeutic approaches to prevent αSyn spreading. The effectiveness of different drugs can be measured quantitively.

Two out of three of the tested tool antibodies against αSyn were able to ameliorate toxicity mediated by extracellular αSyn. Neither the antibodies' epitope nor the sensitivity towards the recombinant αSyn monomer was sufficient to explain the observed differences in the protective efficacies. However, the two protective antibodies were able to scavenge αSyn and deplete αSyn from the medium to a significantly greater extent than the unprotective antibody. The protective antibodies 23E8 and 8A5 formed more stable complexes with αSyn, while the complexes of the unprotective antibody 5D12 dissociated easier.

Two conclusions regarding a promising immunotherapeutic approach for PD and other α-synucleinopathies can be drawn. Firstly, our findings indicate that the formation of stable antibody-antigen-complexes is necessary for an antibody to be protective, which enables the antibody to scavenge cell-derived αSyn species sufficiently. Furthermore, we demonstrated a discrepancy between sensitivity towards recombinant αSyn monomer and the antibodies' protective properties. Therefore, we secondly conclude that it is of highest importance to test potentially therapeutic antibodies in models as physiological as possible.

By automated digital readout of the cell culture images various anti-αSyn antibodies could be screened for their protective efficacy in this model. Additionally, the binding of the protective antibodies to human αSyn, possibly extracted from PD patients' serum or brain, should be investigated.

12 Literature

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