HELLENIC REPUBLIC

National and Kapodistrian University of Athens

Department of Biology

Athens International Master's Programme in Neurosciences

Biomedical Research Foundation Academy of Athens (BRFAA)

RESEARCH THESIS PROJECT

EXTRACELLULAR VESICLES AS VEHICLES FOR IMMUNOTHERAPY IN NEURODEGENERATIVE DISEASES

2024

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Scientific Summary

Synucleinopathies, including Parkinson's Disease (PD), Dementia with Lewy Bodies (DLB) and Multiple System Atrophy (MSA) are characterised by the deposition of a misfolding-prone protein called alpha-synuclein (α-syn). Under these pathologic conditions, misfolded α-syn spreads to healthy neighbouring cells, inducing aggregation of the endogenous protein and thus impairing cellular homeostasis. Immunotherapies aiming to neutralise toxic species of α-syn have been studied for their disease modifying effects. In our study, we developed a passive immunization therapeutic scheme, combining conformation-specific antibodies and nanobodies, targeting oligomeric and fibrillar α-syn forms, and exosomes, a subtype of cup-shaped nanosized extracellular vesicles. Exosomes contain a wide range of micro-molecules and are secreted by all cell types, including brain cells, serving as mediators of inter-cellular communication. In the current study, we have developed a protocol for the generation of brain-derived exosome-antibody/nanobody complexes, as a means to favour the delivery of the conformation-specific agents to aberrant α-syn-bearing target cells. The immunotherapeutic agents were administered in *in vitro* and *in vivo* models of PD and their potential to reduce pathology was evaluated.

Highlights

- Nanobodies are internalized by SH-SY5Y cells after 48 hours of incubation.
- Intrastriatal injection of human recombinant α-syn preformed fibrils (PFFs) induce a PD-like pathology, one month post-injection.
- Nanobodies and antibodies targeting α-syn fibrils reduce α-syn burden in SH-SY5Y cells after 48 hours of incubation.
- Intranasal administration of nanobodies targeting α-syn fibrils, in PFF-injected mice can ameliorate α-syn pathology.

Keywords

Immunotherapy; Nanobodies; Extracellular Vesicles; Exosomes; α-synuclein preformed fibrils; protein misfolding; pathology transmission

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Lay Summary

Synucleinopathies, like Parkinson's Disease (PD), Dementia with Lewy Bodies (DLB), and Multiple System Atrophy (MSA), involve the buildup of a protein called alpha-synuclein (α-syn), which has the tendency to lose its native conformation and oligomerize into aberrant species. Under these conditions, misfolded α-syn spreads to nearby healthy cells, inducing further aggregation of the protein and disrupting normal cell function. Researchers are exploring immunotherapy-based (or immunotherapeutic) treatments to counteract these harmful α-syn species. Our study focused on a passive immunization approach, combining specialized antibodies and nanobodies, which are miniature antibodies, to target different forms of misfolded α-syn, with exosomes, cup-shaped nano-particles secreted by cells, including brain cells, facilitating cellular communication. We developed a method to generate antibody/nanobody-exosome complexes (Exo:Ab/Nb complexes) for effective delivery of therapeutic agents to target cells, aiming to reduce aberrant α-syn levels. The therapeutic potential of the produced Exo:Ab/Nb complexes OR The ability of the produced Exo:Ab/Nb complexes to reduce PD-related pathology, was assessed in *in vitro* and *in vivo* models of the disease.

Introduction

α-Syn, a small presynaptic protein encoded by the SNCA gene is one of the most abundant proteins in brain and its expression suggests a role in plasticity. (Lee and Trojanowski, 2006; Bendor et al., 2013). α-Syn is a natively unstructured presynaptic protein that performs many physiological functions in neurons including vesicular trafficking (Bendor et al., 2013). However, its physiological role is not fully described/deciphered yet. Understanding its typical function is crucial, as it likely affects its propensity to misfold and perturb cellular homeostasis. Misfolding and amyloid aggregation of α-syn play a critical part in the etiology of PD and other synucleinopathies (Spillantini et al, 2000; Stefanis et al. 2012; Kalia et al. 2015). Aggregated α-syn species are the major component of lewy bodies and lewy neurites which represent the classical neuropathological hallmark of PD (Spillantini et al., 1997; Spillantini et al., 1998). Nonetheless, the process by which native α-syn transitions into harmful misfolded amyloid aggregates is (not well) or poorly understood. This comprehension is essential, since uncovering the mechanisms responsible for the pathogenic processes leading to Parkinson's Diseases, can pave the way for disease modifying approaches able to delay or even halt the dilapidating symptoms of the disease (Chiti et al., 2017).

Oligomers are usually produced through a process called secondary nucleation. During this process α-syn monomers via their positively charged N-terminus interact with the negatively charged flexible C-terminus of the fibrils (Calabresi et al., 2023). α-Syn oligomers, which are highly lipophilic molecules, induce the permeabilization of cell membranes, allowing an increase in intracellular calcium, resulting in multiple Ca^{2+} homeostasis alterations (Virdi et al., 2022). This increase in Ca^{2+} levels results in higher levels of dopamine by upregulating the dopamine synthesis, however this newly synthesized dopamine cannot be properly incorporated into synaptic vesicles because of disrupted axonal transport, and cytosolic dopamine concentration rises (Wang and Hay, 2015). Apart from the cell membrane permeabilization, oligomers permeabilize also mitochondria, endoplasmic reticulum, and vesicles. Oligomers exert many other pathogenic effects, including cytoskeletal alterations,

increased reactive oxygen species (ROS) production, and synaptotoxicity in the form of decreased neuronal excitability and decreased synaptic firing (Danzer et al., 2007; Parihar et al., 2009; Colla et al., 2012; Choi et al., 2013). α-Syn oligomers also impair the cell's two major protein degradation systems, the autophagy-lysosomal and the ubiquitin-proteasomal system. α-Syn oligomers also cause alterations in the protein synthesis machinery in the brains of PD patients including alterations in expression of several mRNAs encoding ribosomal proteins and altered level of transcription factor eIF2 and eIF2 (Garcia-Esparcia et al., 2015). In a similar manner to oligomers, fibrillar α-syn species have been proposed to contribute to neurodegeneration by perturbing the cellular ion homeostasis, misbalancing cell proteostasis and/or by compromising the integrity or function of cytosolic organelles such as the endoplasmic reticulum, the Golgi, the mitochondria, and the lysosomes (Bousset et al., 2013; Morimoto, 2011; Brehme et al., 2013; Flavin et al., 2017). α-Syn fibrillar assemblies also trigger neurodegeneration through chronic inflammation and oxidative stress, by activating microglia and astrocytes. Neuroinflammation and oxidative stress, in turn, lead to further α-syn modification, misfolding, and aggregation, creating a vicious cycle (Gustot et al., 2015). However, a very important distinction is that, only the fibrillar assemblies have been shown to propagate, amplify by recruiting endogenous α-syn, and to trigger synucleinopathies when injected to animal models (Palaeski et al., 2014a).

To date, no cure is available for synucleinopathies, and therapy is limited to symptomatic treatment of motor and non-motor symptoms upon diagnosis. The successful use of various immunotherapeutic and immunomodulatory medications in treating multiple sclerosis (Baecher-Allan et al., 2018) has sparked hope for applying similar approaches to other neurodegenerative diseases/disorders. Immunotherapies targeting α-syn can be categorized into passive and active forms of immunization. Both types aim to counteract toxic forms of α-syn and have been investigated for their potential to modify disease progression. For these therapies to be effective, drugs must be capable of crossing the blood-brain barrier. Active immunization via vaccination involves exposure to short peptides that mimic the antigenic epitope of α-syn and generate antibodies against α-syn. On the other hand, passive immunization entails externally administering manufactured antibodies against α-syn to mitigate its neurotoxic effects (Vijayakumar and Jankovic, 2022). It has been demonstrated that these immunotherapeutics/antibodies may target toxic extra- and intra-cellular misfolded proteins involved in the pathogenesis of Alzheimer's disease (AD), PD, Frontotemporal dementia (FTD), Huntington's disease (HD), or transmissible spongiform encephalopathies (TSEs) (Frontzek and Aguzzi, 2020; Panza et al., 2020; Singh et al., 2023). While the clinical use of immunotherapy for PD holds promise, there exist several limitations and concerns, mainly concerning the inflammatory and autoimmunity-related adverse effects that need to be tackled to enhance the development of immunotherapeutic drugs and possibly avoid the challenges encountered in AD clinical trials (Chatterjee and Kordower, 2019).

The most significant challenge for α-syn immunotherapy drugs lies in the absence of reliable markers to assess α-syn proteopathic burden in patients and determine the stage of α-syn-mediated pathology. Unlike PET imaging of amyloid beta and tau, clinical imaging methods for synucleinopathies have not yielded clinically relevant results (Catafau and Bullich, 2017). Non-invasive imaging modalities, such as α-syn PET tracers, may prove crucial for effectively monitoring the α-syn disease state and evaluating target engagement for anti-α-syn clinical

modalities. Alternatively, the development of PET ligands targeting microglia and activated forms of CNS glial populations presents an intriguing imaging approach relevant to disease progression. Site-specific glial activation has been identified as a strong correlate of PD symptomatology and an indicator (or promoter) of Lewy body pathology. Additionally, although serum or cerebrospinal fluid (CSF) markers for α-syn show promise with advancements in α-syn antibody technology, their clinical validation is currently limited, posing a challenge in providing widespread relevance to all PD populations. This gap underscores the need to establish appropriate clinical outcome measures for evaluating efficient target engagement (Chatterjee and Kordower, 2019). Another primary concern for passive antibody therapies is the strain specificity for the conformers of pathological α-syn targeted. Although many passive antibody constructs exhibit significantly higher affinity towards aggregate species of α-syn, evidence of strain specificity is largely lacking and is typically only demonstrated within the context of the specific in vivo model used (Chaterjee and Kordower, 2019).

Currently, Prasinezumab (PRX002/RG7935) is a candidate for a clinically approved monoclonal antibody treatment for PD, with an estimated completion date of September 2026 (Jankovic et al., 2018). Prasinezumab, is a humanized IgG1 monoclonal antibody directed against the C terminus of aggregated forms of α-syn. When administered to healthy volunteers, it exhibited high toleration, as well as dose-dependent reduction of free α-syn in the serum (Jankovic et al., 2018), in keeping with preclinical assessments of PRX002 that demonstrated the ability to block cell-to-cell transmission and ameliorate memory and learning deficits in an α-syn over-expression model (mThy1-a-syn) (Spencer et al., 2017).

Besides from typical antibodies used so far in immunotherapy, there are different antibody formats currently studied in clinical trials for many diseases: antigen-binding fragments (Fab), single-chain fragment variable (scFv) consisting of the antigen-binding domains of Ig heavy (VH) and light (VL) chain regions, and single-domain antibody fragments (sdAbs), such as camelid heavy-chain variable domains (VHHs) and shark variable new antigen receptor (VNARs) (Pietersz et al., 2017; Bates et al., 2019).

Nanobodies (Nbs), which are heavy-chain-only antibodies naturally produced in camelids, can overcome many antibody limitations in terms of size, tissue penetration and possible immunogenicity. Thus, they offer new possibilities for the treatment of a variety of human diseases, including neurodegenerative diseases and PD (Jovcevska and Muyldermans, 2020). Nbs, have a molecular mass of 95 kDa, while their variable antigen-binding domains (VHH) have a prolate shape with dimensions of 4nm x 2.5nm x 3nm and are usually 12-14 kDa. The VHH domain is located at the N-terminal region and is structurally and functionally equivalent to the Fab fragment (antigen-binding fragment) of conventional antibodies. (Muyldermans, 2013). The sequence variability within V domains is localized in three hypervariable (HV) regions surrounded by four more conserved framework (FR) regions (Padlan, 1994). The particularly long complementarity-determining region, which recognizes the epitope, gives the paratope its convex shape, building protrusions that can reach cryptic epitopes often not accessible to cAbs. The nanobody scaffold typically includes two conserved cysteine residues that form a disulfide bond in oxidizing conditions (e.g., endoplasmic reticulum, Golgi, bacterial periplasm, extracellular environment, etc.) and are critical for the

stabilization of a nanobody's structural folding (Sundberg and Mariuzza, 2002; Muyldermans at al., 1994).

Nbs can also be produced easier, on a high scale and with low cost. Typically, Nbs are expressed in the periplasm of bacteria grown in simple shake flasks, and are characterized by a good expression yield and single-step purification protocols (Arbabi-Ghahroudi et al., 2005; Djender et al., 2014). Their final structure is generated/facilitated/shaped/assembled by chaperons which act in the oxidizing environment of the periplasm and assist in the proper folding of VHH domain, including the formation of disulfide bonds. Extraction of the periplasmic proteins via an osmotic shock yields several milligrams of Nbs per liter of culture (Djender et al., 2014; Zarschler et al., 2013). Nbs possess a high thermo-resistance (Van der Linden et al., 1999), their shelf life even when kept at 37°C is remarkably long and after 2 weeks at 37°C they still retain close to 100% antigen-binding activity (Arbari Ghahroudi et al., 1997). Therefore, it comes as no surprise that Nbs are protease resistant as well. Nanobodies, are being utilized as antibodies so target affinity and specificity are probably the most important parameters to be assessed. Nb target specificity is excellent, at least when selections during panning from immune libraries and screenings are performed properly. Nbs' small size and solubility in aqueous solutions reassure that they diffuse rapidly, even in dense tissues or viscous environments. (Debie et al., 2020)

In addition to conformer-specificity, target recognition efficacy and stability, required for immunotherapeutic reagents to be effective, blood-brain barrier (BBB) penetration consists another obstacle to be surmounted. Many studies have verified that antibody delivery to the central nervous system (CNS) can be extremely challenging due to the BBB (I. Zafir-Lavie *et al.*, *J. Control. Release*. **291**, 80–89,2018). Therefore, the development of delivery systems able to by-bass BBB is urgent. Exosomes, which are nanosized intraluminal extracellular vesicles, naturally secreted by all cell types, CNS cells included, are able due to cross BBB and thus have begun being the center of attention as a new delivery vehicle for higher tissue penetration and low immunogenicity in immunotherapeutic strategies. More specifically, exosomes are small vesicles (40–100 nm in diameter) released into the extracellular space by various cell types, including neurons, astrocytes, microglia and can be detected in body fluids such as blood, urine, and CSF (Thery et al., 2006). Exosomes are generated in a process that is initiated by the double invagination of the plasma membrane and the formation of intracellular multivesicular bodies (MVBs) containing intraluminal vesicles (ILVs) which are then secreted as exosomes through the MVB fusion to the plasma membrane and exocytosis. Exosomes are highly heterogeneous and contain membrane components, proteins, lipids, and microRNAs, which can be considered as a snapshot of the cell's components (Kowal et al., 2014; Hessvik and Lorente, 2018). In addition due to their ability to carry and horizontally transfer bioactive molecules between cells, exosomes are considered as intercellular communication mediators.

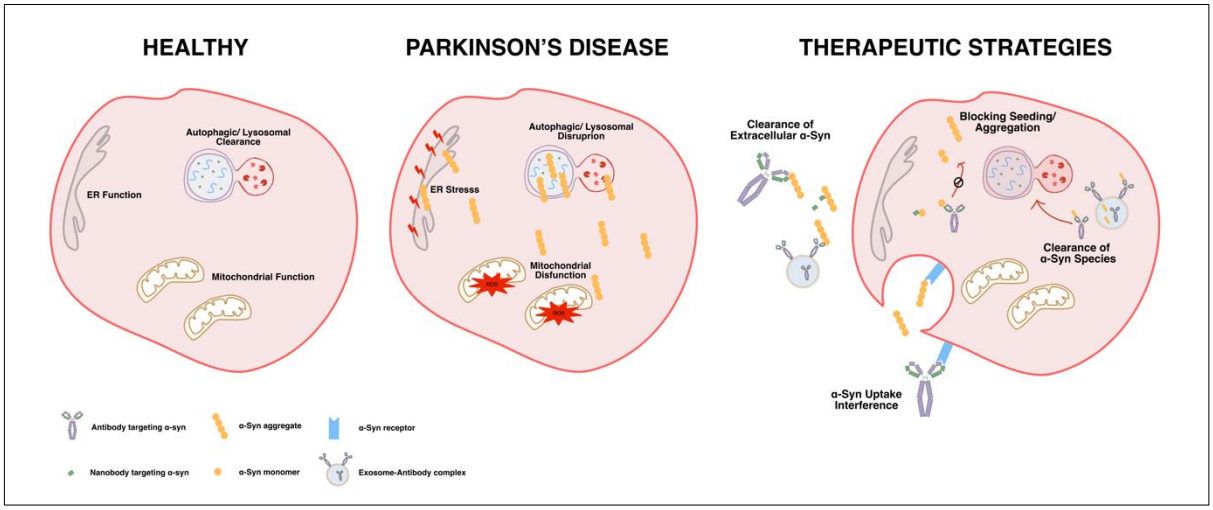

Figure 1. Implicated pathways in α-syn toxicity. Left: healthy cellular pathways are illustrated, middle: pathways altered in PD are shown, right: mechanistic targets of immunotherapy. In healthy conditions, the autophagosome lysosomal pathway degrades proteins in the cell, while in PD this pathway is blocked resulting in accumulation of misfolded α-syn and further aggregation of the protein. Additionally, ER stress causes calcium to leak into the cytoplasm, disrupting its function. Mitochondrial dysfunction in PD leads to increased production of reactive oxygen species, causing oxidative stress. Mechanistic targets of antibody/nanobody-based therapies for PD include the clearance of the α-syn in the extracellular milieu, the interference of the α-syn uptake by the cell by blocking the α-syn receptors, the blockage of the seeding process in the intracellular space and the transport of the aggregates to the lysosome for degradation.

Methods

Cell culture

Tet-Off SH-SY5Y human neuroblastoma cells inducibly over-expressing human wild type (WT) α-syn (Vekrellis et al., 2009), 10% fetal bovine serum (10,270; Gibco, Invitrogen, Carlsbad, CA, USA), and 1% penicillin-streptomycin (15140122; Thermo Fischer Scientific, Waltham, MA, USA). Inducible SH-SY5Y cells were cultured in the presence of 250μg/ml G418 (11811023; Thermo Fischer Scientific, Waltham, MA, USA) and 50 μg/ml hygromycin B (10843555001; Sigma-Aldrich) for clonal maintenance and 2 μg/ml doxycycline for suppression of the transgene. Removal of doxycycline from the medium results in activation of α-Syn expression, reaching maximum levels 6 days post. Neuronal differentiation was achieved with the addition of 10 μM all-trans retinoic acid (R2625; Sigma, St Louis, MO, USA) for 6 days and kept for another 4 days. The cells were kept in an incubator with optimal culture conditions of 37^{O}C and 5% CO_2, and the medium was replaced every other day. Cells were plated in 6-well plates ($3x10^5$cells/well) with 2 ml RPMI 1640 for protein extraction experiments or in 24-well plates ($12x10^3$ cells/well) with 0,5ml/well for immunocytochemistry. PFFs were added on the 6th day of differentiation at indicated concentrations and the therapeutic scheme was implemented from day 8 OR therapeutic agents were added on day 8. Two days post-PFF addition, cells were washed with phosphate-buffered saline (PBS) and lysed or fixed on day 10, for western immunoblotting of immunofluorescence, respectively. PFFs were stored at -80oC.

Preparation of exosome-depleted medium.

RPMI medium supplemented with 20% FBS and 1% penicillin/streptomycin was centrifuged at 100,000 x *g* for 16 h at 4°C. The supernatant was carefully removed, sterilized by filtering through a 0.2 µm filter and stored at 4°C.

Whole Brain Exosomes Isolation and Purification

α-Syn KO mice C57BL6/JOlaHsd mice were sacrificed, brains were excised and exosomes were isolated as previously described (Perez Gonzalez et al., 2012; Papadopoulos et al., 2018; Karampetsou et al., 2020) with slight modifications (Melachroinou et al, 2024). Brains were minced, homogenized in papain-containing serum free DMEM (20 units/ml) by 10 strokes in a glass Teflon and further dissociated at 37^{O}C for 30 min. Papain activity was halted by the addition of two volumes of ice cold serum-free DMEM solution. The suspension was centrifuged at 300 x *g* (10 min, 4^{O}C) and passed through a 40 um mesh cell strainer. The supernatant was sequentially filtered through a 0.45- µm and 0.2-µm PES filter and further centrifuged at 2,000 x *g* (10 min, 4^{O}C), 10,000 x *g* (30 min, 4^{O}C) and finally at 100,000 x *g* (70 min, 4^{O}C). The resulting crude exosome-enriched pellet was rinsed in 22–24 ml of cold PBS and spun down at 100,000 x *g* (70 min, 4^{O}C). The exosome-enriched pellet was reconstituted in 1.5 ml of 0.95 M sucrose/20 mM Hepes solution (pH7.4), and loaded on a sucrose gradient comprised of six fractions (2.0 M, 1.65 M, 1.3 M, 0.95 M, 0.60 M, 0.25 M, 1.5 ml each), and centrifuged at 200,000 x *g* (16 h, 4^{O}C). Seven fractions were separated according to the gradient (a–g from top to bottom). Fractions B, C and D were collected individually, diluted in PBS to 18-20 ml final volume and centrifuged at 100,000 x *g* (70 min, 4^{O}C). The resulting pellets were re-suspended in PBS and stored at -80^{O}C until used.

Electron Microscopy

Exosome-enriched fractions B, C, and D were fixed with 4% paraformaldehyde overnight at 4^{O}C, as previously described (Melachroinou et al 2024). Five microliters of the preparations were loaded on 300-mesh copper grids with carbon-coated formvar film and incubated for 20 min. Following a rinse in PBS, the grids were incubated with 1% glutaraldehyde for 5 min, stained with uranyl oxalate (pH 7, 5 min) and methyl cellulose–uranyl acetate (10 min on ice) and allowed to dry. For the assessment of the size and morphology of PFFs by EM, 5 µl of PFFs were loaded on Formvar-coated 400 mesh copper grids and following brief fixation with 0.5% glutaraldehyde (5µl), they were negatively stained with 2% uranyl acetate (Sigma-Aldrich, USA) (Melachroinou et al 2024). Samples were examined with a Philips 420 Transmission Electron Microscope at an acceleration voltage of 60 kV and photographed with a Megaview G2CCD camera (Olympus SIS, Münster, Germany) and iTEM image capture software. Size distribution of exosomes was evaluated using image Fiji v2.0.0 software.

Acetylcholinesterase activity assay

Acetylcholinesterase (AChE) activity was measured in the exosome-enriched fractions (B, C, and D), as previously described (Savina et al., 2002; Emmanouilidou 2010; Melachroinou 2024). Briefly, 10 µl of each fraction or known concentrations of recombinant AChE were added to individual wells on a 96-well flat-bottomed microplate; 1.25 mM of acetylthiocholine and 0.1 mM of 5,50-dithiobis (2-nitrobenzoic acid) in PBS were added to a final volume of 250 µl. Following 30-min incubation at RT, under agitation, in the dark, the

absorbance at 405 nm was measured. The ratio of the ng AChE/µl of each fraction normalized to the respective protein load (in µg/µl), as measured by Bradford protein assay, is indicative of the enrichment in exosomes.

Nanoparticle Tracking Analysis of exosomal preparations

NanoSight NS300 instrument (Malvern Instruments, Amesbury, UK), equipped with a 532 nm laser (green), a high sensitivity sCMOS camera and a syringe pump, was used for Nanoparticle tracking analysis (NTA) (Melachroinou et al 2024). The exosome preparations were diluted in particle-free PBS (0.22 µm filtered) to obtain a concentration within the recommended measurement range (1-10 × 10^8 particles/ mL), corresponding to approximately 0.8-1.0 µg of total protein. Each of the diluted samples was loaded on a 1 ml syringe, which was then placed to the pump. Autofocus was adjusted so that blurry particles were avoided. For each measurement, 5 videos with a duration of 30 seconds each, were captured under the following stable conditions: cell temperature: 25°C; Syringe speed: 100 µl/s. NanoSight NTA 3.4 build 3.4.4 software (Copyright 2020, Malvern) was used for the analysis of the acquired videos, following capture in script control mode. A total of 1500 frames were examined per sample.

Generation of exosome-antibody/nanobody complexes

For the generation of exosome-antibody or exosome-nanobody complexes (Exo:Abs) different conformation-specific antibodies and nanobodies were used, kindly provided by Dr. Omar El Agnaf. The moniclinal mouse antibody used was the SynO2 targeting α-syn fibrils, whereas IgG isotype was used as respective control. The nanobody used was Nb40 targeting human and rodent α-syn fibrils and Nb81 isotype was used as an irrelevant control nanobody. Both nanobodies were -HA tagged. As a source of exosomes α-Syn KO brain-derived exosomes were used. The *in vitro* binding assay was performed by incubation at RT for 45 min. The reaction mix contained 40 µg exosomes and 80 µg of therapeutic agent (monoclonal antibodies or nanobodies) in PBS to a final volume of 260µl. The binding was stopped by the addition of 1ml ice-cold PBS and the exosome-antibody/nanobody complexes were isolated by ultracentrifugation at 100,0000 xg for 1h at 4^{O}C. The pellet was reconstituted in 40ul sterile PBS and further analyzed by EM for validation of the exosome integrity and by WB for quantification of the bound antibodies/nanobodies to a given amount of exosomes.

Treatment of vesicles with Na_2CO_3

To remove non-integral membrane proteins, exosomes/Exo:Abs were treated with Na_2CO_3 1,0 N, pH 11, for 30 min at 4°C (Fujiki et al., 1982; Emmanouilidou 2024). After centrifugation at 50,000g for 2h at 4°C, integral exosomal membrane proteins were recovered in the pellet fraction, whereas the exosomal lumen proteins remained in the soluble fraction.

Stereotaxic Injections

Adult male C57/BL6 mice (3 months old), were injected under general isoflurane anesthesia by an apparatus adjusted to the stereotaxic frame (Kopf Instruments, United States), as previously described (Karampetsou 2020; Melachroinou 2024). The right dorsal striatum was targeted using the following coordinates from bregma: anteroposterior +0.5 mm, mediolateral −2 mm, and dorsoventral in two depths, −3.2 and −3.4 mm according to the mouse stereotaxic atlas (“The Mouse Brain in Stereotaxic Coordinates”, G. Paxinos & K.B.J Franklin) (Franklin, 2001). The injection material was administered at a constant flow rate of

0,27µl/min, whereas a 5-min interval was followed between target depths and the needle was slowly retracted 5min after the injection procedure was completed. Mice were injected with 5 µg of human PFFs diluted to a final volume of 2µl or with an equal volume of PBS, as a control. Animals were sacrificed at 1 month post-injection.

Intranasal administration of nanobodies

Intranasal administration was performed on anesthetized mice. PFF-injected adult male C57Bl6 mice were anesthetized by inhalation (isoflurane); each mouse was introduced into an induction chamber with isoflurane (3%-4%) mixed with oxygen (1%) until anesthetic takes effect. Following anesthesia, mice were laid on their back, with the head immobilized at upright position, as previously described (De Rosa et al., 2005). Ten µg of nb40 or nb80 diluted in water for injections (1 µg/ µl) or PBS alone were administered intranasally, 5µl per nostril at a time (10 µl in total). The dose was decided taking into account that the nanobodies reach directly the brain through the nasal route (El-Agnaf et al., 2018; Ekmark-Lewen et al., 2023). At the end of the procedure, the mice were left at the described supine position, until regaining consciousness. During this time, the breathing was monitored closely. The treatment protocol involved bi-weekly intranasal injections (10 µg/dose) over the span of a month. Following this treatment period, mice were trans-cardially perfused, their brains were excised and processed for subsequent analysis.

Immunohistochemistry

Mice were anesthetized with isoflurane anesthesia and intracardially perfused with ice-cold PBS (30 ml/mouse), followed by 4% paraformaldehyde (30 ml/mouse) in PBS, under a constant flow rate using a peristaltic pump. Brains were post-fixed overnight at 4°C and then gradually dehydrated by sequential incubation in 15% sucrose in PBS for over-day at 4°C and then in 30% sucrose in PBS for 24-48h. For snap freezing, brains were immersed into frozen iso-pentane (-45°C) for 45 sec and stored at -80°C. Free-floating cryo-sections of 30 µm were collected, using a Leica CM3050S cryostat at -25^{O}C. For immunohistochemistry, following treatment with antigen retrieval solution (citrate buffer, pH = 6) at 80°C for 20 min and 20 min on ice, 3x10 min PBS washes and a 60min blocking in 2% normal goat serum (NGS) in PBS containing 0.2% Triton X (blocking buffer), sections were incubated with the primary antibodies in blocking buffer for 16 h at 4 ^{O}C. Following 3 x 10 min PBS washes, secondary antibodies (Invitrogen) diluted in blocking buffer were added for 2 h at RT. The sections were rinsed again as above and mounted on Poly-D-lysine slides (VWR) using Dako fluorescence mounting medium. For phospho-S129 α-Syn immunostaining, no antigen retrieval was performed, whilst the blocking buffer used was 5% NSG, 0.1 Triton-X 100 in PBS. The following primary antibodies were used: tyrosine hydroxylase (TH) (mouse monoclonal, 1:2,000, Merck-Millipore), phospho-S129 α-Syn (rabbit monoclonal, 1:2,000, Abcam), GFAP (rabbit polyclonal, DAKO, 1:750), Iba-1 (mouse monoclonal, Merck-Millipore, 1:500). DAPI dihydrochloride (0.5 µg/ml) was used for nuclear staining.

Immunocytochemistry

For immunocytochemistry, cells grown on poly-d-lysine-coated glass coverslips and fixed by incubation in 3.7% formaldehyde for 30 min at RT. Fixed cells were washed three times with PBS and then were incubated in a blocking buffer containing 10% NGS and 0.4% Triton X-100 in PBS for 1 h at RT. Then the primary antibodies diluted in 2% NGS and 0.1% Triton X-100 in PBS were incubated for 16 h at 4°C. Following three washes in PBS, secondary antibodies

diluted in 2% NGS and 0.1% Triton X-100 in PBS were added for 2 h at RT and washed again as before. After final PBS washes, the coverslips were mounted on a slide using Dako fluorescence mounting medium. Cell nuclei were stained with DAPI dye, which was added together with the secondary antibodies.

Images were obtained with both Leica SP5 upright and inverted confocal microscopes and 3D reconstruction of confocal images was performed by Imaris 9.1.3 software.. Confocal stacks were imported into the Imaris Software and surfaces were built for the corresponding confocal channels. α-Syn pathology was measured after performing 3D reconstruction, creating a surface for the cell soma and a surface for the α-syn that is included under the cell soma surface. Pathology was counted as α-syn number of voxels normalized with the number of nuclei.

Cell Fractionation

For extraction of cellular proteins, cells were harvested, washed once with ice-cold PBS, and lysed with 1% Triton-X STET lysis buffer (containing 50mM Tris, pH 7.6,150mM NaCl, 1% Triton-X, 1% NP-40, 2mM EDTA, and protease- phosphatase inhibitors). The lysate was sonicated (three times for 5 sec, at 33% amplitude, with 1 min interval) at 4°C, incubated for 30 min at 4°C, and centrifuged at 15,000 xg for 30min at 4°C. The supernatant represents the triton-soluble fraction and the protein concentration was estimated by Bradford assay. The remaining pellets were re-suspended in 10% Sarcosyl STET lysis buffer and further dissolved via sonication (three times for 5 sec, at 33% amplitude, with 1 min interval) at 4°C. Following a 30 min-incubation at 4°C, triton-insoluble fraction was isolated by centrifugation at 15,000 x g for 30min at 4°C.

Western immunoblotting

Denaturing gel electrophoresis was performed on 10-13% SDS-PAGE Tris-glycine gels. The proteins were transferred onto nitrocellulose membranes (Whatman) and analyzed by immunoblotting, stained with antibodies specific against the target proteins. Membranes were blocked in blocking buffer (5 % non-fat milk, 0.05% Tween-20 in TBS), for 1 h at RT and probed overnight at 4ºC with the following primary antibodies: anti-α-Syn C20 (Santa Cruz, 1:1000), anti-α-syn Syn1 (BD biosciences, 1:1,000), anti-pS129 α-Syn (Abcam, 1:1,000), anti-Flotillin-1 (Santa Cruz, 1:1,000), anti-α-syn 4B12 (GeneTex, 1:1000), anti-alix (Cell Siganling, 1:1000), anti-TSG101 (abcam, 1:1000), anti-CD81 (abcam, 1:1000), anti-CD9 (abcam, 1:1000). Following 10 min washes with 0.05% Tween-20 in TBS (TBS-T) thrice, secondary antibodies (Merck-Millipore) diluted in blocking buffer (1:5000) were added for 2 h at RT. Membranes were rinsed again as above and Clarity Western ECL Substrate (Bio-Rad) was used for the detection of the proteins bound to nitrocellulose membranes. Differences in protein expression levels (immunoreactive band) were estimated using both Gel Analyser v1.0 and Fiji v 2.0.0 software, after normalization of all values with the appropriate loading controls (Alix and Flotillin-1 for exosomal protein levels and β-actin, γ-tubulin for total protein levels).

Fluorescence activated cell sorting (FACS)

SH-SY5Y human neuroblastoma cells were differentiated in the presence of 10 μM Trans-retinoic acid for 6 days. As mentioned earlier, from previous publications, 75 μg of Antibody were administered to 10^6 cells (Tran *et al*., 2014, Volpicelli-Daley *et al*., 2011). So, per $40x10^3$ cells/well, we treated with 3 μg exosomes associated-antibodies, or equivalent amount of

exosomes or free antibody alone, for 4 and 8 hours. Following treatment, recipient cells were collected, stained for the nuclei and fixed for FACS analysis. FACS acquisition was performed with the cytometer Cytomics FC500 (Beckman Coulter) and the data were analyzed using the Flowjo software (Tree Star, Inc., Ashland, OR).

Statistical Analysis

Data are expressed as the mean ± standard error of the mean (SEM). The statistical analysis was performed with the GraphPad Prism 8 software (Version 8.2.0) (San Diego, CA), using Unpaired Student's *t*-test or one-way ANOVA followed by Tukey's post-hoc test, for comparison between two or multiple groups, respectively.

Animals and ethical approval

For this study 5-6-month-old wild-type (WT) C57BL6/C3H (Jackson Laboratory, Bar Harbor, Main) and α-Syn knockout mice (α-Syn KO) (C57BL6/JOlaHsd mice, Harlan Laboratories) were used. Animals were housed in individually ventilated cages with free access to food and water, under a controlled light-dark cycle (12 h light-12 h dark) and temperature (21+1 ^{o}C), at the animal facility of the Biomedical Research Foundation of the Academy of Athens. All experimental protocols were performed in accordance with the EU directive guidelines for the care and use of experimental animals (86/609/EEC; 27/01/1992, No. 116) and were approved by the Institutional Ethics Committee for Use of Laboratory Animals, and an authorized veterinarian committee in accordance to Greek legislation (Presidential Decree 56/2013, in compliance with the European Directive 2010/63).

Table 1. List of materials, manufacturers and catalog numbers.

Materials	Manufacturer/supplier/developer, city state and country	Catalog number
RPMI 1640	Sigma-Aldrich	R8758
Fetal Bovine Serum	Gibco, Invitrogen, Carlsbad, CA, USA	10,270
penicillin-streptomycin	Thermo Fischer Scientific, Waltham, MA, USA	15140122
G418	Thermo Fischer Scientific, Waltham, MA, USA	11811023
hygromycin B	Sigma-Aldrich	10843555001
retinoic acid	Sigma, St Louis, MO, USA	R2625
papain	Worthington	LS003124
nitrocellulose membrane	Amersham	10600001
α-Synuclein C20 , (human, mouse, rat) (polyclonal)	Santa Cruz	sc-7011 1:800

α-Synuclein Syn1 (human, mouse, rat, monoclonal)	BD Transductions	610787
α-Synuclein (phospho S129) (monoclonal)	ABCAM	51253
α-Synuclein 4B12 (human, mouse, rat)	Thermo Fischer Scientific, Waltham, MA, USA	MA1-90346
anti-HA (mouse, monoclonal)	Cell Signaling	23675
β-Actin (mouse, monoclonal)	Cell Signaling	4970
γ-tubulin (mouse, monoclonal)	Sigma	T5326
β-tubulin III (Tuj- 1) (mouse, monoclonal)	Sigma	T8578
Tyrosine hydroxylase (TH) (mouse monoclonal)	Merck- Millipore	MAB318
GFAP (rabbit, polyclonal)	DAKO	Z0334
Iba-1 (mouse, monoclonal)	Merck- Millipore	MABN92
Flotillin-1 (rabbit, polyclonal)	Abcam	ab133497
Alix (mouse)	Cell Signalling	A2171
TSG101 (rabbit)	Abcam	Ab125011
CD81 (rabbit)	Abacam	Ab109201
CD9 (rabbit)	Abcam	Ab307085
Goat Anti-Mouse IgG-HRP conjugated	Biotium	20010
Goat Anti-Rabbit IgG-HRP conjugated	Biotium	20012
Donkey Anti- Mouse Alexa 568	Invitrogen	182787568
Donkey Anti- Mouse Alexa 647	Invitrogen	1839633
Goat Anti-Mouse Alexa 488	Biotium	20010
Donkey Anti- Rabbit Alexa 568	Invitrogen	1826664
Donkey Anti- Rabbit Alexa 647	Invitrogen	1826679
Goat Anti-Rabbit Alexa 488	Invitrogen	1910795C
α-synuclein (SynO2) (monoclonal)	Gift from Omar el Agnaf	
Nanobody 40	Gift from Omar el Agnaf	
wild-type (WT) C57BL6/C3H mice	Jackson Laboratory, Bar Harbor, Main	
α-Syn KO C57BL6/JOlaHsd mice	Harlan Laboratories	
Tet-Off SH-SY5Y human neuroblastoma cells	(Vekrellis et al., 2009)	

Results

Characterization of the *in vitro* PD model for the evaluation of the nanobody-based immunotherapy

To confirm the effective trasnport of anti-α-syn nanobodies into neuronal cells and assess their impact on α-syn pathology, we utilized the well characterized Tet–Off human neuroblastoma SH-SY5Y cells (Vekrellis et al. 2009). These cells were molecularly engineered to overexpress express wild-type human α-syn under the control of the Tet-Off promoter in an inducible manner (Vekrellis et al., 2009). Prior research from our laboratory has demonstrated that these cells can generate harmful oligomeric forms of α-syn (Vekrellis et al., 2009). In addition, in order to induce a neuronal-like phenotype to these cells, we cultured them in the presence of all-trans retinoic acid (Sahin et al., 2021). Finally, in order to trigger a more robust α-syn pathology, we established a 10-day protocol, in which we cultured the cells with retinoic acid and in the absence of dox for 6 days, and on the 6^{th} day we added α-syn PFFs for 48h. To evaluate the inducibility of the described *in vitro* system, 10 days post-differentiation, we performed biochemical and immunocytochemical characterization with anti-α-syn antibodies **(Figure 2)**. Immunocytochemistry staining for TUJ-1 and DAPI allowed visualization of neuronal morphology and nuclei, respectively, allowing the quantification of intracellular α-syn levels within neuronal populations. Our findings confirm that removal of doxycycline leads to upregulation of the total intracellular α-syn levels (designated as WT-dox), in comparison to the baseline expression observed in non-induced control WT+dox cells. This increase was also evident for the higher molecular weight α-syn species.

Additionally, the incubation of cells with α-syn PFFs significantly altered the cell's α-syn species composition. At the time point of 48H α-syn PFFs do not promote seeding of the endogenous protein but they enter the cells. Specifically, differentiated cells cultured without dox and treated with α-syn PFFs exhibited a marked increase in oligomeric α-syn species, as demonstrated by the enhanced staining with the conformation-specific α-syn aggregate antibody (mjfr14) and total α-syn antibodies (C-20, Syn1).

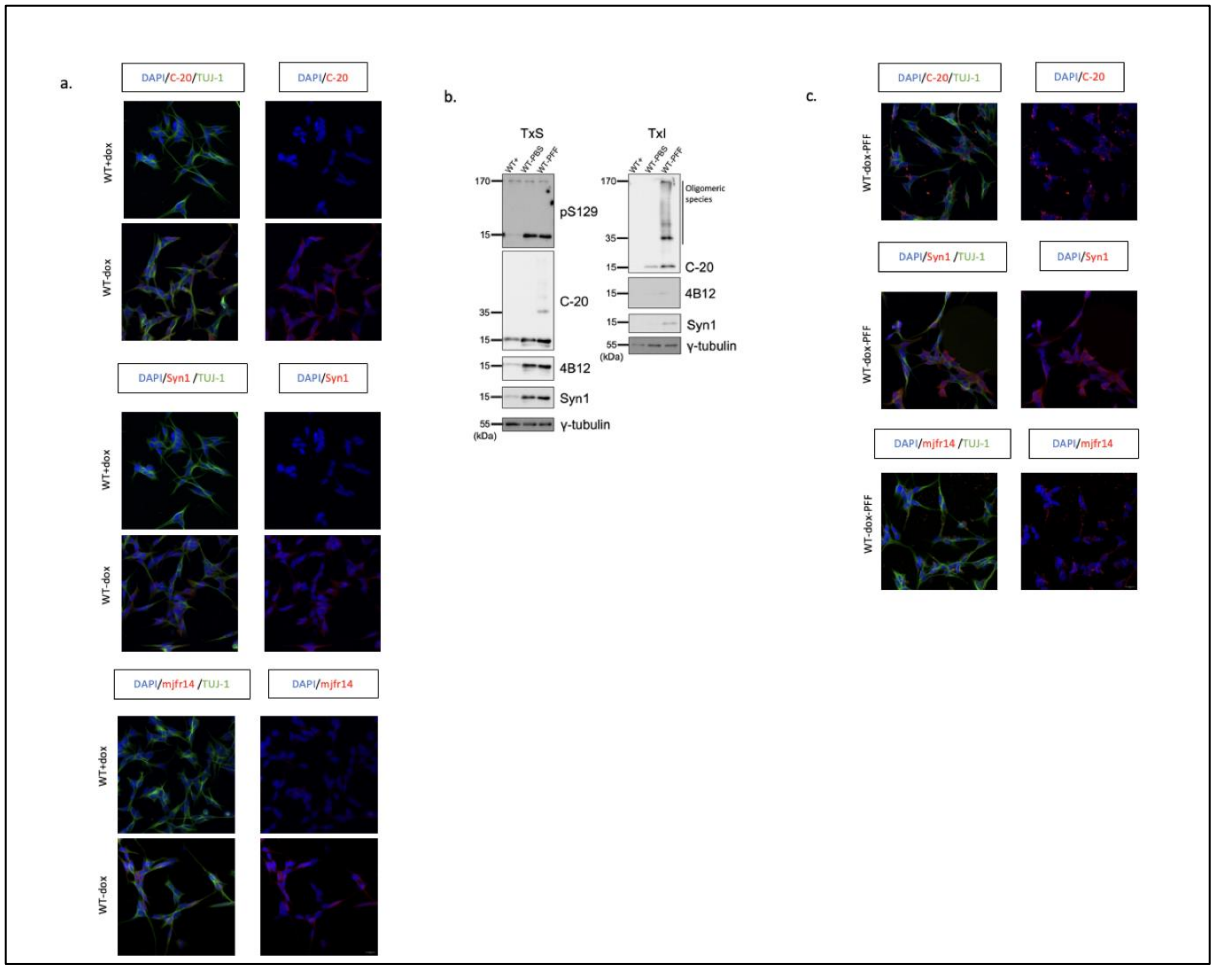

Figure 2. Characterization of the *in vitro* PD model. SH-SY5Y human neuroblastoma cells inducibly overexpressing human α-syn (WT-dox) or control cells expressing the basal levels of the protein (WT+dox) were characterized both with and without the addition of α-syn PFFs for the levels of α-syn by immunocytochemistry and western immunoblotting of their protein extracts. a) Differentiated cells with and without dox were stained for the conformation-specific α-syn aggregate antibody (mjfr14) and total α-syn antibodies (C-20, Syn1), red and TUJ-1, neuron-specific class III beta-tubulin antibody, green b) western blot analysis of the human α-syn PFFs for total α-syn (C-20, Syn1,4B12). c) Differentiated cells, cultured without dox and treated with α-syn PFFs were stained for C-20, total α-syn (green) and TUJ-1, pan-neuronal marker (red).

Nanobody40 that targets α-syn fibrils decreases the pathology in differentiated SH-SY5Y neuroblastoma cells

Here, we investigated the therapeutic potential of Nanobody40 (nb40), which has a high affinity to α-syn fibrils, in mitigating α-syn pathology in the previously described *in vitro* system of PD. As already mentioned, to induce α-syn pathology, SH-SY5Y cells were cultured in the absence of dox and treated with human α-syn preformed fibrils (PFFs) (0,5μg PFFs/300.000 cells), a well-established *in vitro* model for studying α-syn aggregation (**Figure 3a**). Forty eight hours after PFF treatment, cells were exposed to 0,25μM either nb40 or the control nanobody, nb81, of the same isotype. The effects of these treatments on intracellular α-syn levels were assessed using both immunocytochemistry and immunoblotting approaches.
Immunocytochemistry analysis, utilizing the anti-total α-syn antibody C-20, showed that upon PFF treatment, the diffuse α-syn staining changes to a punctated pattern, corresponding to the internalized fibrils and which have possibly recruited the endogenous protein as well, explaining the reduction of the diffuse signal (Figure 3b, WT-dox vs WT-dox/PFFs). Imporantly, Nb40 treatment results in a significant reduction of α-syn immunoreactive puncta compared

to the Nb81 control group (Figure 3b, WT-dox/PFF/Nb40 vs WT-dox/PFF/Nb81). 3D reconstruction of immunocytochemistry images provided not only a comprehensive visualization of α-syn distribution within cells, but also a more acurate/precise quantification of intracellular α-syn aggreagtes, further highlighting the efficacy of nb40 in reducing α-syn aggregation (**Figure 3c**). Immunoblotting analysis complemented these findings by demonstrating a decrease in both Triton-X soluble and insoluble α-syn levels in cells treated with nb40 compared to controls (**Figure 3d**). This reduction in α-syn levels suggests that nb40 may interfere with α-syn aggregation and promote its clearance from cells.
Importantly, Western blot analysis confirmed the intracellular presence of HA-tagged nb40, indicating its ability to enter SH-SY5Y cells and target α-syn aggregates directly (**Figure 3e**).
Overall, our results demonstrate the therapeutic potential of nb40 in ameliorating the PD-realted phentotype of intracellular α-syn accumulation. By reducing intracellular α-syn levels and mitigating α-syn aggregation, nb40 represents a promising immunotherapeutic approach for PD treatment. Further studies will validate the efficacy and safety of nb40 *in vivo* and to explore its potential as a disease-modifying therapy for PD.

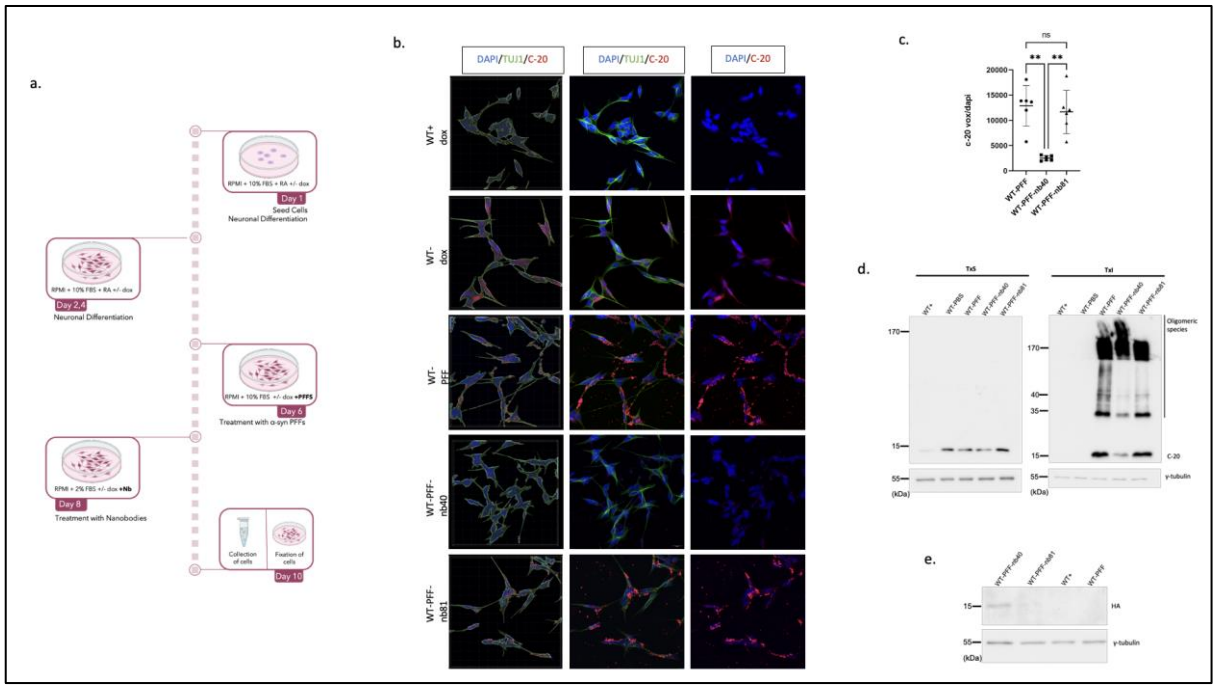

Figure 3. Nanobody40 enters SH-SY5Y cells and ameliorates α-syn pathology. SH-SY5Y cells genetically modified to over-express human WT α-syn in the absence of doxycycline (dox), were grown for 6 days in the presence of 10μM RA and in the absence of dox. On day 6 *in vitro* the cells were treated with α-syn preformed fibrils (PFFs) (0.25 nM) and on day 8, culture medium was replaced and supplemented with 0.25 μM of either nb40 or the respective control, nb81 of the same isotype. The effects of each treatment on the intracellular α-syn levels were assessed via immunocytochemistry or immunoblotting a) Schematic representation of the *in vitro* nanobody immunotherapy. b) Differentiated cells, treated with PFFs on day 6 and with nanobodies on day 8, were fixed on day 10 and stained with C-20 (total α-syn, red) and TUJ-1 (pan-neuronal marker, green). On the left column, 3D reconstruction using the Imaris software was conducted on the images obtained with the microscope (scale bar 20mμ). c) α-syn pathology was measured after performing 3D reconstruction, creating a surface for the cell soma and a surface for the α-syn that is included under the cell soma surface. Pathology was counted as α-syn number of voxels normalized with the number of nuclei. d) Western blot analysis of triton-X soluble and insoluble proteins from differentiated cells, treated with PFFs on day 6 and with nanobodies on day 8 for the presence of total α-syn (C-20). e) Western blot analysis of proteins from differentiated cells, treated with PFFs on day 6 and with nanobodies on day 8 for the presence HA-tagged nanobodies.

Characterization of the PFF mouse model of PD and evaluation of the nanobody-based immunotherapy

After refining our nanobody-based immunotherapy to effectively target α-syn in SHSY5Y cells, our focus shifted towards validating these promising results in an *in vivo* setting. To accomplish this, we used the well-characterized PFF-mouse model, which closely mimics the pathological hallmarks of Parkinson's disease (PD), particularly the formation of proteinaceous aggregates composed of α-syn (Karampetsou et al 2017; Melachroinou et al 2024). Unilateral inoculation of the dorsal *striatum* of wild-type C57BL/6 mice was conducted with either recombinant human PFFs or PBS as a control. One month post-injection, mice were sacrificed, and their brains were subjected to detailed immunohistochemical analysis. (Luk et al., 2012; Karampetsou et al., 2017; Melachroinou et al., 2024). As depicted in **Figure 4**, the presence of Ser129 phosphorylated aggregated α-syn was evident throughout the substantia nigra pars compacta (*SNpc*) and the respective striatal dopaminergic terminals of the ipsilateral side, at the 1-month post-injection time point. Conversely, minimal to no immunoreactivity against phospho-α-syn was observed in either the contralateral side of the PFF-injected mice or in the PBS control group. These findings highlight the ability of PFFs to induce robust α-syn pathology, replicating key features of PD pathology in the mouse model. Additionally, the selective distribution of phosphorylated α-syn aggregates emphasizes the regional specificity of α-synucleinopathy within the nigrostriatal pathway, further validating the relevance of our *in vivo* model.

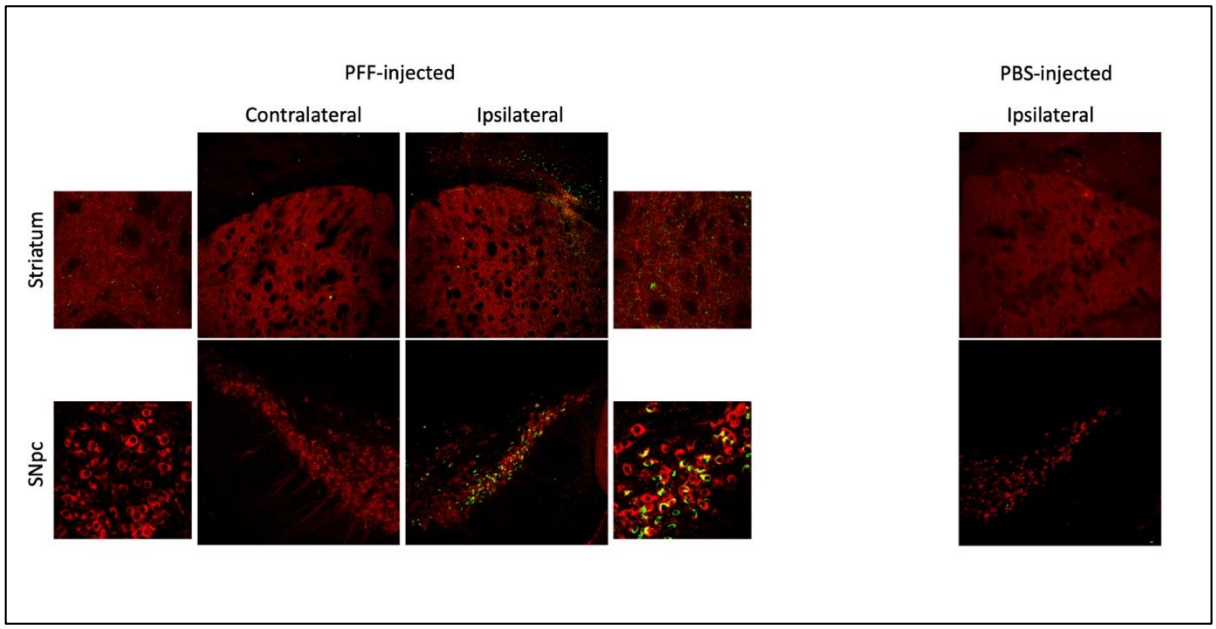

Figure 4. Human α-syn PFF-mouse model. Adult WTC57Bl6 mice received unilateral intrastriatal injections of 5 μg of human PFFs (hPFFs). Representative confocal images of the contralateral and ipsilateral striatum and SNpc of PFF- injected WT mice, one-month post-injection, showing double immunostaining of phosphorylated-α-Syn (green) and tyrosine hydroxylase (TH, red). Images were obtained with 10x lens (scale bar 20 μm) and high magnification insets with 63x lens (scale bar 20 μm). The respective images of the ipsilateral side of the PBS-injected control groups are included (right panel)

In our thorough investigation of the efficacy of nb-based immunotherapy targeting α-syn pathology in PD, we embarked on a comprehensive exploration of the therapeutic potential of nb40 treatment in the described PFF mouse model of PD. Our research paradigm was rooted in the imperative to bridge the translational gap between promising *in vitro* results and their real-world applicability in complex biological systems. Central to our investigation was the precise validation of nb40's therapeutic efficacy, a nanobody meticulously engineered to specifically target α-syn aggregates implicated in PD pathogenesis. While our primary focus

rested on assessing the direct impact of nb40 on α-syn pathology, we also investigated the potential influence on the brain's inflammatory state.

In order to assess the efficacy of nb40 *in vivo*, we precisely designed an experimental protocol that harnessed the unique advantages of intranasal administration (Gomes et al., 2018). This non-invasive delivery method offers several key benefits, including enhanced drug delivery to the brain via direct nose-to-brain transport, circumventing the BBB and minimizing systemic exposure. The intranasal route also facilitates rapid and efficient distribution of therapeutic agents throughout the CNS, optimizing treatment efficacy while mitigating potential systemic side effects, in a needle-free way.

Our experimental protocol commenced with the bi-weekly intranasal administration of nb40, nb81 (control nanobody), or phosphate-buffered saline (PBS) over the course of a month in PFF-induced PD mouse models. Specifically, 3-month old WT C57BL/6 mice were injected in the right dorsal *striatum* with 5μg of human PFFs and then were intranasally administered 10μg of nb40/81 or equal amount of PBS twice per week for a month, starting from the day of the surgery (**Figure 5**) (El-Agnaf et al., 2018, Games et al., 2014). This strategic approach ensured sustained and targeted delivery of nanobody therapeutics to the brain, maximizing their potential to mitigate α-syn pathology and modulate neuroinflammatory responses within the *striatum*, a critical brain region implicated in PD pathogenesis.

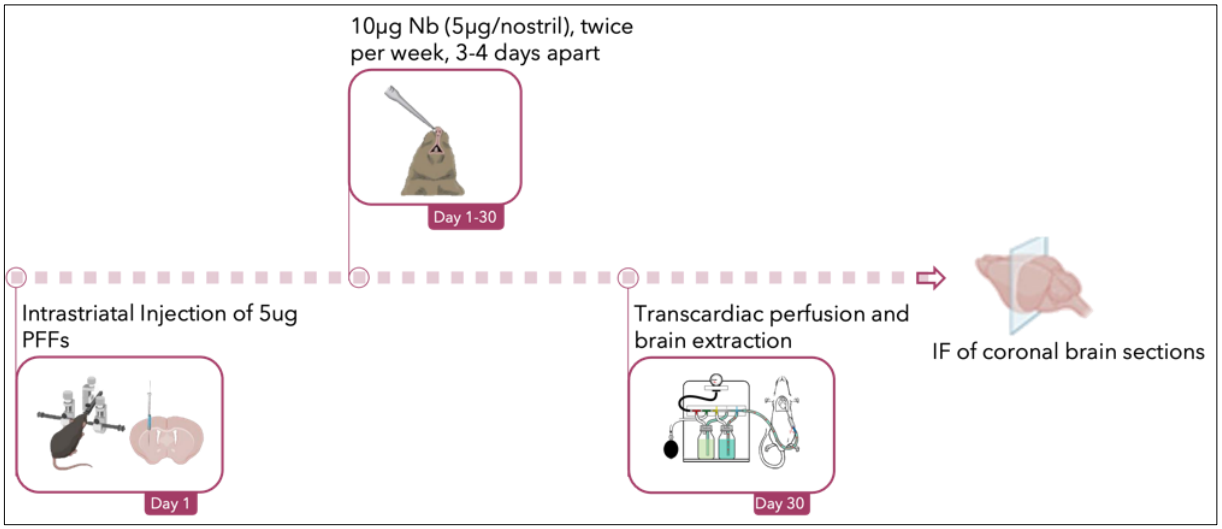

Figure 5. *In vivo* immunotherapy protocol. PFF-injected mice received nb40 or nb81 as a control, through the nasal route. The treatment protocol involved bi-weekly intranasal injections (10 μg/dose, 5 μg/nostril) over the span of a month. Following this treatment period, mice were trans-cardially perfused, their brains were excised and processed for subsequent analysis. Immunofluorescence analysis is conducted on 30 μm free-floating coronal brain sections to assess the effects of the nb40 on phospho-α-syn pathology along the ipsilateral nigrostriatal axis.

Following Nb treatment, trans-cardial perfusion and brain tissue processing were performed to prepare samples for subsequent immunofluorescence analysis. This comprehensive methodology encompassed the utilization of advanced imaging techniques to visualize and quantify α-syn pathology, astrocytic and microglia activation within key brain regions, including the *SNpc* and the *striatum*. By leveraging the advantages of intranasal administration and employing state-of-the-art imaging technologies, we aimed to provide a comprehensive assessment of the therapeutic efficacy of nb40 in PD, while unraveling a possible connection between α-syn pathology and neuroinflammatory processes.

In summary, our experimental protocol integrated innovative delivery methods with advanced imaging techniques to provide a holistic evaluation of nanobody-based immunotherapy in PD. By elucidating the therapeutic mechanisms underlying nb40 treatment and its impact on α-syn pathology and neuroinflammation, our study aims to contribute to the development of targeted therapeutic interventions for PD and other neurodegenerative disorders.

Our results showed that nb40 effectively lowers α-syn pathology, measured by the double positive phosphoS129 (pS129) and tyrosine hydroxylase (TH) neurons in the ipsilateral *SNpc* (**Figure 6a**). However, there was also an effect on the pathology by the control isotype nb81, which can be attributed to non-specific cross-reactivity of the nanobody. For this reason, we also plan to repeat the experiment, using lower concentration of the nanobodies, in order to diminish the non-specific effects. We also plan to include another control nb in our scheme.

Beyond the scope of α-syn pathology, our investigation delved into the complex landscape of neuroinflammation within the *striatum*, a pivotal brain region involved in PD pathophysiology. Using immunostaining techniques targeting astrocytic and microglial markers, we revealed a profound increase in both astrocytosis and microglial activation in nb40-treated mice, indicative of a potential immunomodulatory effect elicited by nanobody intervention (**Figure 6b,c**). Quantitative analysis of staining intensity using the Fiji imaging software provided quantitative metrics elucidating the correlation between diminished α-syn pathology and heightened neuroinflammation, particularly in the ipsilateral hemisphere. Intriguingly, a modest elevation in inflammatory markers was also observed in the nb81 group, further underscoring the complexity of immunotherapy outcomes and the need for meticulous dissection of underlying mechanisms.

This characterization not only sheds light on the multifaceted nature of immunotherapy in PD, but also underscores the intricate interplay between pathological and inflammatory processes underlying disease progression.

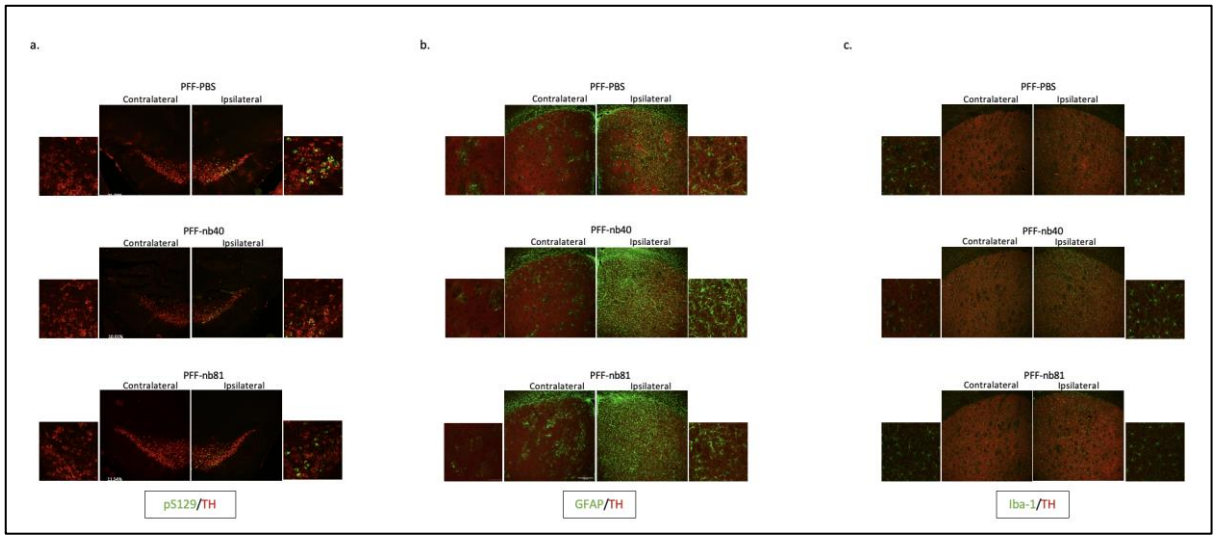

Figure 6. Nb40, Nb81 or PBS were administered through the nasal route, bi-weekly for a month in PFF-injected mice and α-syn pathology and neuro-inflammation were assesed. a) Representative confocal images of the contralateral and ipsilateral *SNpc* showing double immunostaining against the phosphorylated-α-Syn (green) and the dopaminergic-specific marker tyrosine hydroxylase (TH, red). The average pathology of each condition is presented on the left bottom. b) Representative confocal images of the contralateral and ipsilateral *striatum* showing double immunostaining of astrocytes (GFAP, green) and tyrosine hydroxylase (TH, red). c) Representative confocal images of the contralateral and ipsilateral *striatum* showing double immunostaining of microglia (Iba-1, green) and tyrosine hydroxylase (TH, red). Images were obtained with 10x lens (scale bar 20 μm) and high magnification insets with 63x lens (scale bar 20 μm).

Generation and characterization of exosome antibody α-syn complexes and optimization of exosome-mediated α-syn antibody/nanobody immunotherapy

Our next goal for this project will be to use exosomes in order to optimize the efficiency and delivery efficacy of nanobodies and antibodies that target α-syn.

The characterization of mouse brain-derived exosomes and their binding with antibodies was conducted through a series of well-established experimental procedures, as previously described by Melachroinou et al., 2024. Initially, exosomes were isolated from adult male α-syn null (KO) mouse brains. Following mild tissue homogenization with papain, brain membrane vesicles were isolated on a sucrose gradient ultracentrifugation protocol. The purified exosomal fractions obtained from the sucrose gradient were subjected to electron microscopy (EM) and western blot (WB) analysis to confirm their identity and assess the presence of exosome-specific markers, namely CD9 and CD81 (Figure 7a). Additionally, the enrichment of exosomes was quantified by measuring acetyl-cholinesterase (AChE) activity and normalizing it to the total protein load (Savina et al., 2002;emmanouilidou et al. 2010; Melachroinou et al. 2024) (Figure 7a). Nanoparticle tracking analysis was also employed to determine the size distribution and number of the extracellular vesicles, providing further insights into the effectiveness of our purification (**Figure 7a**).

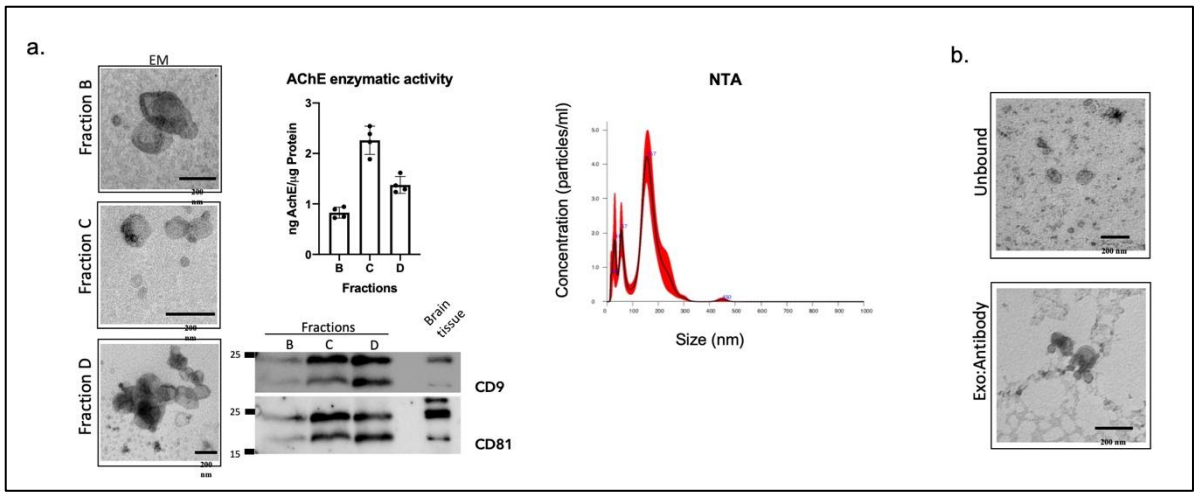

Figure 7. Characterization of brain-derived exosomes prior and after binding with antibodies. a) Exosomes have been isolated from whole mouse brains of α-syn knock-out adult mice (KO) using a sucrose-gradient ultracentrifugation protocol. The sucrose gradient purified fractions were characterised by EM and WB analysis against the exosome-specific markers CD9 and CD81. In addition, their enrichment in exosomes was further validated by measurement of the acetyl-cholinesterase (AChE) activity, expressed as ng AChE normalized to the total protein load, as measured by Bradford assay. Finally, nanoparticle tracking analysis (NTA) was performed to assess the size distribution and particle number of the isolated exosomes b) The exosome-antibody binding was achieved upon incubation in room temperature. The integrity of the exosomes before and after the binding was validated by EM.

In previous experiments from our lab, we have developed an *in vitro* binding assay to generate complexes of brain-derived exosomes and the monoclonal conformation-specific antibody SynO2. To this end, we mixed brain exosomes with SynO2 antibody at a ratio 1:2 in PBS and incubated at room temperature for 45 minutes. The generated exosome-SynO2 complexes (Exo:SynO2) were isolated by ultracentrifugation.

To validate the integrity of exosomes before and after antibody binding, electron microscopy was employed to visualize the structural integrity of the vesicles (**Figure 7b**). Quantification of the exosome-antibody complexes was performed via densitometric western blot analysis, utilizing known concentrations of the unbound antibody as a reference (**Figure 8a,b**). For precise localization of the antibodies within the complex, Na_2CO_3 treatment was employed to create pores in the membranes, allowing the isolation of both luminal and non-integral membrane proteins. The resulting membranous and luminal fractions were analyzed via western immunoblotting, utilizing specific antibodies against exosome markers (such as Alix and Flotillin) and the anti-mouse antibody that binds to $SynO_2$ (**Figure 8c**).

Through this comprehensive characterization, we gained valuable insights into the integrity and binding efficiency of brain-derived exosomes with antibodies. These findings not only do they provide a deeper understanding of exosome biology but also hold significant implications for the development of exosome-based therapeutic strategies for neurodegenerative diseases such as PD.

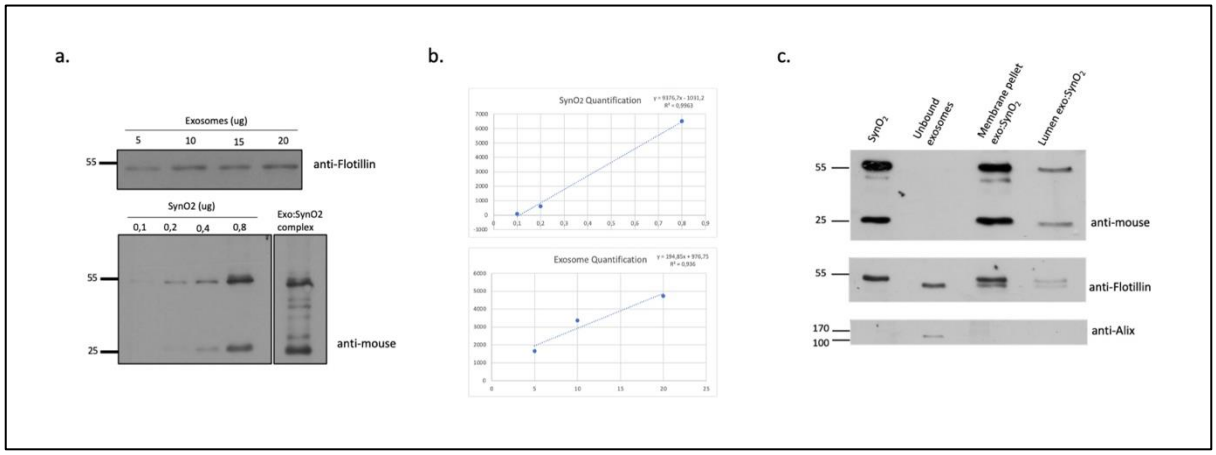

Figure 8. Quantification of the exosome:antibody complexes and localization of the antibody. a) Quantification of the exosome:antibody complex was performed via densitometric western blot analysis, using known concentrations of free antibody and unbound exosomes, detected either with the anti-mouse secondary HRP conjugated antibody or anti-flotillin antibody, respectively. b) The exact localization of the antibodies within the complex was assessed with Na_2CO_3 treatment. Na_2CO_3 creates pores on the membranes, thus allowing the isolation of both luminal and non-integral membrane proteins, following ultracentrifugation. The produced membranous and luminal fractions were analyzed by western immunoblotting. The anti-mouse secondary HRP conjugated antibody and antibodies against Alix and Flotillin, were used for the detection of SynO2 and the characterization of the exosomal fractions, respectively.

Optimization of exosome-mediated α-syn antibody/nanobody immunotherapy

After characterizing the exosome-antibody complexes we checked whether binding with exosomes facilitates the delivery and the effectiveness of the $SynO_2$ antibody *in vitro*. We have demonstrated that binding of antibodies with exosomes enhances their uptake by cells and results in a more robust/effective reduction of α-syn pathology. Specifically, we found that treatment of SH-SY5Y cells with exosome-bound SynO2 antibody led to a more efficient uptake compared to the free antibody alone, as evidenced by fluorescence-activated cell sorting (FACS) analysis (**Figure 9a**). Furthermore, when SH-SY5Y cells were treated with $SynO_2$ antibody bound to exosomes, a significant decrease in intracellular α-syn levels was observed and the effect was more robust compared to treatment with either free $SynO_2$ antibody or

exosomes alone, as assessed via immunocytochemistry using the total anti-α-syn antibody C-20 (**Figure 9b**). This reduction in α-syn pathology highlights the potential therapeutic efficacy of exosome-bound antibodies in ameliorating neurodegenerative disease pathology.

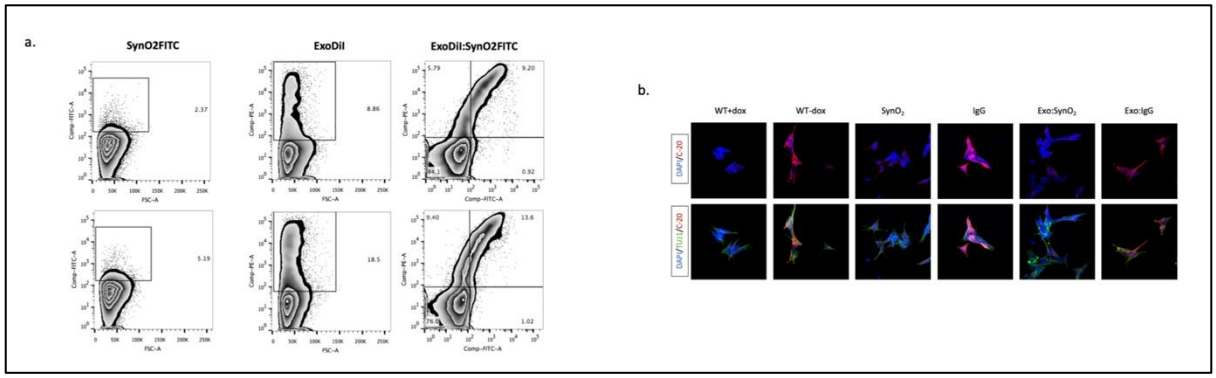

Figure 9. $SynO_2$ binding with exosomes favors its uptake by SH-SY5Y cells and ameliorates the α-syn pathology. a) SHSY5Y cells cultured in the presence of 10uM retinoic acid, were treated with DiI-labelled exosome:SynO2FITC complex, or equal amounts of DiI-labelled exosomes or SynO2FITC antibody alone for 4 and 8 hours. The free or exosome-bound SynO2 uptake was measured by FACS. The results show that the exosome:antibody complex is more efficiently uptaken by the cells compared to the free antibody. b) SH-SY5Y cells were grown for 6 days in the presence of 10uM RA and in the absence of dox. On days 6 and 8 the cells were treated with either Exo:SynO2 or the respective exo:IgG isotype or SynO2 or IgG alone. The effect of each treatment on the intracellular a-syn levels were assessed via immunocytochemistry, using the total anti-async antibody, C-20 (red). The neuronal marker TUJ-1 (green) and DAPI (blue) were used to stain the cytoskeleton and the nuclei, respectively.

Building upon these findings, we extended our investigation and examined the localization and quantification of nanobodies targeting fibrilar α-syn, bound to exosomes. Through densitometric western blot analysis, we quantified the exosome:nanobody complex and found that the nanobody is efficiently encapsulated within the exosomes (**Figure 10a**). Additionally, we employed Na_2CO_3 treatment to isolate membranous and luminal fractions, revealing the specific localization of the nanobody within the exosome complex (**Figure 10b**). Nanobodies were localized both in the membrane and the lumen with a substantial portion of the nanobody within the exosomal lumen. In contrast to the antibody localization, which predominantly resides on the exosomal membrane, the nanobody's hydrophilic nature likely contributes to its preferential localization within the exosome lumen. These results underscore the potential utility of exosome-bound nanobodies as targeted therapeutic agents for neurodegenerative diseases, which enhance nbs' ability to be functional within the harsh cytosolic environment, due to their structure.

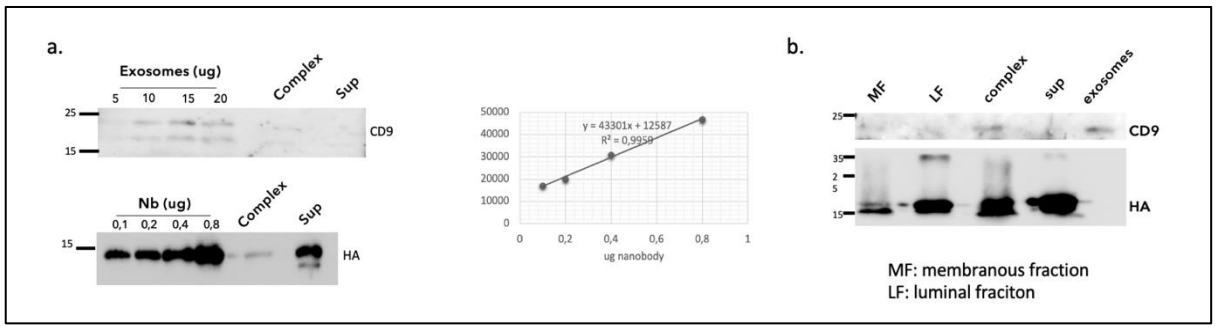

Figure 10. Quantification of the exosome:nanobody complexes and localization of the nanobody. a) Quantification of exosome:nanobody complexes was performed via densitometric western blot analysis, using known concentrations of the unbound nanobody, with the use of anti-HA antibody and the exosomes with the use of anti-flotillin antibody. b) The exact localization of the antibodies within the complex was assessed via Na2CO3 treatment. Na2CO3 creates pores on the membranes and allows the isolation of both luminal and non-integral membrane proteins, following ultracentrifugation. The produced membranous and luminal fractions were analyzed by western immunoblotting, with antibodies against Alix and Flotillin. The presence of nanobodies within these fractions was evaluated with the anti-HA antibody.

Moving forward, our study aims to further investigate the delivery and effectiveness of nanobodies when administered in association with exosomes, both *in vitro* and *in vivo*. By comparing the delivery efficiency and therapeutic efficacy of exosome-bound nanobodies to free nanobodies, we seek to elucidate the potential benefits of utilizing exosomes as delivery vehicles for targeted therapeutic interventions in neurodegenerative diseases. Through this comprehensive approach, we aim to advance our understanding of exosome-mediated drug delivery and pave the way for the development of novel and effective treatments for neurodegenerative disorders such as PD.

Discussion

Immunotherapies against α-syn currently appear to be a very promising therapeutic approach for the modification of disease progression in the treatment of patients with PD in early stages. (Games et al., 2014; Mandler et al., 2015). Several studies have been performed in animal models of PD, attempting to remove α-syn from the extracellular space and thereby to reduce the progressive deposition of α-syn aggregates throughout the brain. Active immunotherapy involves the production of self-generated antibodies against administered α-syn (or fragments), whereas passive immunotherapy is achieved by administration of antibodies against various domains of α-syn (Sardi et al., 2018). A successful approach to active immunization against PD necessitates ensuring effective transfer of antibodies across the BBB. Affiris, an Austrian company, conducted phase I clinical trials (registered as NCT01568099 and NCT02267434) for two vaccines targeting α-syn, PD01A and PD03A (known as AFFITOPE), which recognize the oligomeric forms of α-syn. These vaccines utilize short peptide fragments mimicking the C-terminal domain of α-syn (110–130 amino acids) coupled with carrier proteins and adjuvanted with aluminum hydroxide. Efficacy assessments in transgenic mouse models revealed high antibody titers against aggregated α-syn, detectable in plasma, CSF and brain parenchyma. Immunization with AFFITOPE resulted in reduced α-syn deposition, preservation of dopaminergic neurons, and amelioration of motor and spatial memory deficits (Mandler et al., 2015). Active immunization against α-syn ameliorates the degenerative pathology and prevents demyelination in a model of MSA. Notably, no adverse effects were reported, and repeated dosing of PD01A in randomized PD patients elicited a significant humoral immune response (NCT02758730). The molecule, now named ACI-7104, will undergo further investigation, in a two-pronged approach of biomarker-based early PD symptom monitoring (Volc et al., 2020). The first passive immunotherapy construct introduced, Prasinezumab (PRX002/RG7935), demonstrated efficacy in promoting α-syn clearance via autophagy in PDGFb-α-syn transgenic mice, resulting in improved movement and spatial cognitive perception (Masliah et al., 2011). Additional antibodies, 5C1 and 1H7, targetting the C-terminus of α-syn, exhibited high plasma titres, preventing cell-to-cell transmission of α-syn aggregates (Games et al., 2015). Phase I trials with PRX002 demonstrated safety and reduction in free serum α-syn levels in healthy participants and patients with mild-to-moderate PD,

although no improvement was observed in the Movement Disorder Society-Unified Parkinson's Disease Rating Scale (MDS-UPDRS) (Schenk et al., 2017). A phase II study (PASADENA) with PRX002 in early stage PD patients did not show significant differences in dopamine transporter levels or clinically defined endpoints, emphasizing the importance of patient stratification in clinical trials (Jankovic et al., 2018). Another antibody, BIIB054, targeting the N-terminal domain of α-syn, exhibited high affinity against aggregated α-syn in preclinical studies, but a phase II study (SPARK) failed to meet its primary and secondary endpoints, leading to the suspension of further development (Weihofen et al., 2019; Lang et al., 2022). Finally, AstraZeneca and Takeda Pharmaceutical Company developed MEDI-1341 (TAK-341), an antibody targeting both monomeric and aggregated α-syn, which showed promising results in preclinical studies and acceptable safety profiles in phase I and II trials. Ongoing phase II trials with MEDI-1341 in patients with MSA aim to further assess its therapeutic potential in synucleinopathies (Schofield et al., 2019). The most significant challenges to α-syn immunotherapy drugs are target specificity, lack of reliable biomarkers, and the limited BBB penetration of antibodies. Developing strategies to enhance the delivery of therapeutic antibodies across the BBB is a significant challenge in the field.

In our study we aimed to utilize the intrinsic properties of exosomes and conformation-specific nanobodies targeting oligomeric and fibrilar forms of α-syn in order to confront some of the major challenges in immunotherapy so far.

In 1993, Hamers-Casterman et al. discovered in camels a naturally existing heavy-chain only antibody (HCAb) that lack the first domain of its constant region (CH1) (Hamers-Casterman et al., 2018). The antigen-binding region of this chain is called the variable domain of heavy chain of heavy-chain only antibody (VHH) has a molecular weight of 15kDa and a size in the nanometer scale. Therefore, VHH is widely mentioned as nanobody (Fan et al., 2017). Due to their special structure, nbs have many advantages comparing to the conventional antibodies, including high solubility (Muyldermans et al., 1994), high stability (Perez et al., 2001), high antigen-binding ability (Stijlemans et al., 2004), low immunogenicity (Cortez-Retamozo et al., 2004), and strong tissue penetration (Ruiz-Lopez et al., 2021). Thanks to these advantages, nbs can be an excellent tool both for basic and translational research. For the treatment of PD, the strategies for reducing the pathological aggregation of α-syn can be divided into two categories: the first is to inhibit the aggregation of α-syn into fibrils, and the second is to enhance α-syn degradation. For our immunotherapy protocol we used a conformation-specific nanobody targeting α-syn with a preference to α-syn fibrils, named nb40, which was generated and kindly provided by Omar M. A. El-Agnaf. To assess the efficacy of the nb40 to be delivered into recipient cells and subsequently to reduce the α-syn pathology we designed an *in vitro* and an *in vivo* immunotherapy protocol. For the *in vitro* protocol, we used a tet-off system, that was previously generated and described by our lab (Vekrellis et al., 2009), in which SH-SY5Y human neuroblastoma cell line was genetically modified to express human wild type (WT) α-syn, under the control of a tet-off promoter. In the presence of doxyxycline the transgene is switched-off and thus only basal levels of the protein are expressed, whereas in the absence of doxyxycline the overexpression of α-syn is induced (Vekrellis et al., 2009; Emmanouilidou et al., 2010) (Figure 2a). To further enhance α-syn pathology in these cells, after culturing them for 6 days in the absence of doxyxycline and in the presence of RA, for induction of neuronal-like phenotype, we treated them with human PFFs for 48H (Figure 2b,c,d). α-Syn overexpressing cells treated with PFFs (WT-dox+PFFs) produced both low and high oligomeric species of α-syn, α-syn overexpressing cells (WT-dox) produce high levels of the monomeric form of α-syn, while the control WT+dox cells only produce the basal levels of

the protein (Fig 2b). The higher species of α-syn can be detected by western immunoblotting in both the triton-soluble and the triton-insoluble fraction with a higher enrichment in the triton-insoluble fraction. To study the effectiveness of nb40 in the *in vitro* model described above, SH-SY5Y were grown for 6 days in the presence of 10μM RA and in the absence of dox. On day 6 the cells were treated with PFFs and on day 8, culture medium was replaced and supplemented with 0.25 μM of either nb40 or the respective control, nb81 of the same isotype. The effects of each treatment on the intracellular α-syn levels were assessed via immunocytochemistry or immunoblotting (Figure 3a). More specifically, we performed immunofluorescence staining against the total α-syn antibody C-20, conducted a 3D reconstruction using the Imaris software, creating a surface for the cell soma and a surface for the α-syn that was included within the cell soma surface. Pathology was estimated as α-syn number of voxels normalized to the number of nuclei (Figure 3b,c) and as the levels of total α-syn in the triton-X soluble and insoluble fraction from differentiated cells, treated with PFFs on day 6 and with nanobodies on day 8. Our results from both immunofluorescence and immunoblotting analysis show a significant reduction of α-syn pathology after treatment with the nb40 (Figure 3d). Finally, in order to validate that nanobodies can enter the cells and act in the intracellular space we performed immunoblotting in cell extracts from differentiated cells, treated with PFFs on day 6 and with nanobodies on day 8 for the presence HA-tagged nanobodies (Figure 3e). By this experiment, we showed that, indeed, nanobodies have the ability to penetrate the cell membrane and reduce α-syn pathology. Having these encouraging results from our therapeutic *in vitro* protocol we decided to test the efficacy of nb40 in reducing α-syn pathology *in vivo*. For this purpose, we used a PD model established in our lab, which is based on the striatal inoculation of wild-type mice, with human PFFs. In this model, at one month post-injection, phospho-positive α-syn rings are developed within the TH positive neurons, along the ipsilateral *SNpc* (Figure 4). To test the efficacy of nb40 in this animal model of PD we performed a semi-preventive immunotherapy protocol in which the treatment with nanobodies started on the same day to/as PFF inoculation. Importantly, in order to enhance the drug delivery and mitigate potential systemic side-effects we utilized the intranasal route for administering the nanobodies to the mice. Our protocol included bi-weekly administration of the nanobodies for 1 month starting on the day of PFF inoculation. Specifically, the mice received 10μg (5μg/nostril) of nb40 or control nb81 or PBS, twice a week for a month and after this period they were euthanatized, perfused and their brain were extracted, dehydrated and frozen in order to be processed for subsequent analysis (Figure 5). For the assessment of pathology, immunohistochemistry was performed in 30 μm brain slices of the *striatum* and *SNpc*, via staining against phosphorylated α-syn (pS129) and tyrosine hydroxylase. The pathology was counted as described before, by measuring phospho-positive α-syn rings developed in TH neurons of the ipsilateral *SNpc*. Our results showed that nb40 is able to lower α-syn pathology, when administered intranasally in PFF-inoculated mice. Surprisingly, the control isotype nb81 also influenced the pathology although at a lower scale compared to nb40. This can be attributed to a non-specific effect of the nanobody, therefore, in future experiments we plan to use lower concentrations of the nanobodies in order to achieve a more specific effect and also use different control nbs. Finally, considering the major role of inflammation in the development of PD and the implications of microglia and astrocytes in the propagation of α-syn pathology, we wanted to extract information about the inflammatory state of the mice's brain and how it is affected by nanobody administration. For this purpose, we performed immunohistochemistry in striatal brain slices, via staining against the astrocytic, microglial and dopaminergic neuron markers, GFAP, Iba-1 and TH, respectively.

Using Fiji imaging software, the quantitative analysis of staining intensity provided metrics that showed a connection between decreased α-syn pathology and increased neuroinflammation, particularly on the ipsilateral side. Comparing the contralateral sides between the Nb40, Nb81, and PBS groups, we observed no significant difference in terms of glial cell activation, indicating that the induction of inflammation we observed is related specifically to α-syn pathology rather than the treatment itself. Interestingly, a slight increase in inflammatory markers was also observed in the nb81 group. Activation of microglia and astrocytes facilitates the clearance of α-syn aggregates through phagocytosis, contributing to the resolution of protein accumulation in PD. Upon activation, microglia undergo morphological changes and upregulate phagocytic receptors to engulf extracellular α-syn aggregates, while astrocytes enhance α-syn clearance through increased expression of phagocytic receptors and protein degradation enzymes. This coordinated effort between microglia and astrocytes highlights the importance of glial-mediated phagocytosis in maintaining protein homeostasis and neuronal integrity in neurodegenerative diseases (Basurco et al., 2022).

Our ultimate goal is to generate a delivery system for a more effective and targeted delivery of therapeutic agents in the CNS. To this end, we optimized the conditions for the formation of complexes between naturally produced brain exosomes and conformation-specific antibodies and nanobodies and to tested how this combination can boost the therapeutic efficacy of these compounds, as well as enhance their delivery. Exosomes are cup-shaped, nanosized, lipid bilayer-enclosed vesicles, naturally secreted from cells after fusion of intracellular multivesicular bodies with the plasma membrane. In our lab, we have established a protocol for isolation and purification of high yield intact interstitial exosomes from mouse brain (Figure 7). Previous experiments from our lab have shown that binding of the conformation-specific $SynO_2$ antibody, that targets α-syn fibrils, to exosomes amelioratedits uptake by the cells (Figure 9a). Also, treatment of cells with $SynO_2$ resulted in significant decrease in intracellular α-syn levels and this effect was higher when the antibody was administered in complex with exosomes (Figure 9b). Moving on to this direction we performed binding of nb40 to exosomes, quantified the exosome:nanobody complexes and assessed the nanobody localization within the complex (Figure 10). Our next step, will be to use our established *in vivo* and *in vitro* protocols to assess the effectiveness of the nb:exosome complexes.

To sum up, we firmly believe that nanobodies can be used as a therapeutic tool in brain diseases and particularly in PD pathology as they offer many advantages comparing to conventional antibodies and their association to exosomes, that have the innate ability for horizontal transfer of bioactive molecules, secures their effective penetration to the CNS.

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Title: Extracellular Vesicles as Vehicles for Immunotherapy in Neurodegenerative Diseases
Student Name: Bourtouli Nikoleta

Research proposal

"Developing Exosome-Nanobody Complexes for Enhanced Immunotherapy in Parkinson's Disease"

Project Summary

Current immunotherapies against α-synuclein (α-syn) present a promising avenue for modifying disease progression in early-stage Parkinson's Disease (PD). Despite their potential, these therapies face significant hurdles, including limited blood-brain barrier (BBB) penetration, off-target effects, and lack of reliable biomarkers. This project aims to address these challenges by leveraging the unique properties of exosomes and nanobodies (nbs). Exosomes, naturally occurring nanosized vesicles, can effectively cross the BBB and deliver therapeutic agents directly to target cells. Nbs, due to their small size and high stability, offer several advantages over conventional antibodies, including better tissue penetration and reduced immunogenicity. By conjugating nbs with exosomes, we propose to create a more efficient and targeted delivery system for α-syn immunotherapy. This project will explore the binding efficiency, stability, and therapeutic efficacy of exosome-nb complexes in both *in vitro* and *in vivo* models of PD. We aim to demonstrate that this novel approach can significantly enhance the delivery and effectiveness of immunotherapy, potentially leading to improved clinical outcomes for PD patients.

Specifics Aims

In this project, we will focus on the development and evaluation of exosome-nb complexes as a potential treatment for PD. By leveraging the unique properties of nbs and exosomes, we aim to create a system that can effectively target and reduce α-syn pathology, while also exploring the therapeutic implications of targeting different α-syn species. Our specific objectives include the following:

Objective 1: Evaluate the binding efficiency and stability of nbs to exosomes. We will quantify the binding efficiency and stability of the nb-exosome complexes using advanced immunoassays and electron microscopy techniques.

Objective 2: Assess the therapeutic efficacy of exosome-nb complexes in *in vitro* models of α-syn pathology. Using a well-established tet-off SH-SY5Y cell system expressing human α-syn as well as primary neuronal and mixed neuronal/glial cultures we will investigate the impact of these complexes on reducing α-syn aggregation and toxicity.

Objective 3: Study the pharmacokinetics of nb and exosome-nb complexes. The distribution and concentration in the brain of nb or nb-exosome complexes will be assessed after intranasal administration.

Objective 4: Investigate the impact of exosome-nb complexes on α-syn pathology and neuroinflammation in *in vivo* PD models. In a mouse model of PD, we will evaluate the ability

of these complexes to mitigate α-syn pathology and associated neuroinflammation when administered intranasally.
Objective 5: Investigate the role of different α-syn species. Nbs targeting α-syn monomers, oligomers and fibrils specifically will be used in order to evaluate which of these species are more toxic and would be more efficient to target.

Introduction and Significance

PD is the second-most common neurodegenerative disease, affecting at least 0,3% of the worldwide population and over 3% of those over 80 years old (Pringsheim et al., 2014). It is characterized by the accumulation of α-syn aggregates in the brain, leading to the degeneration of dopaminergic neurons in the Substancia Nigra pars compacta (SNpc) (Rodrigues e Silva et al., 2010). Current therapeutic strategies, including both active and passive immunotherapies targeting α-syn, show considerable promise but are limited by several significant challenges. One of the primary obstacles is the efficient delivery of therapeutic agents across the BBB, a selective permeability barrier that restricts the entry of most large molecules into the brain. Additionally, off-target effects and a lack of reliable biomarkers for disease progression further complicate the development of effective treatments (Vijayakumar et al., 2022).

IgG antibodies, known as heavy-chain antibodies (HCAbs), are devoid of the L chain polypeptide and are unique because they lack the first constant domain (CH1). Heavy chain only antibodies have a molecular mass of 95 kDa, while their variable antigen-binding domains (VHH) have a prolate shape and are usually 12-14kDa. Due to their small size, nbs exhibit excellent tissue penetration, high stability, and low immunogenicity. These properties make them particularly suited for targeting intracellular and extracellular protein aggregates in neurodegenerative diseases such as PD (Muyldermans et al., 2013).

Exosomes, on the other hand, are nanosized, lipid bilayer-enclosed vesicles naturally secreted by cells intact, following fusion of the plasma membrane with the multivesicular bodies (Vekrellis et al., 2011). They play a crucial role in intercellular communication but they have also been associated with the pathogenesis of many neurodegenerative disorders. They have a remarkable ability to cross the BBB, and are characterized by a wide range of surface adhesion proteins and vector ligands making them ideal candidates for drug delivery vehicles as they can interact and target the cellular membrane (Vella et al., 2016).

The convergence of these two promising technologies—nbs and exosomes—offers a novel approach to overcoming the limitations of current α-syn immunotherapies. By conjugating nbs with exosomes, we aim to enhance the targeted delivery and therapeutic efficacy of nbs in PD. Our preliminary studies have demonstrated the potential of nbs to reduce α-syn pathology in vitro, and we have developed protocols for isolating and purifying high-yield exosomes from mouse brain tissue.

In order to achieve the optimal delivery of our complexes in the CNS of mice, we will use the intranasal nose to brain delivery route. In general, CNS drug delivery via the olfactory, trigeminal or optic nerves bypasses the BBB and could thereby dramatically advance the CNS drug delivery. (Pardeshi et al., 2013). In particular, intranasal nose to brain delivery has

emerged as an exciting and attractive minimally invasive delivery option and so far intranasal delivery of small proteins, such as insulin, scFvs and nbs has been successfully demonstrated. (Gomes et al., 2018).

This research project will build on these preliminary findings by systematically evaluating the binding efficiency and stability of nb-exosome complexes, assessing their therapeutic efficacy in vitro, and investigating their impact on α-syn pathology and neuroinflammation in a mouse model of PD. We hypothesize that the exosome-bound nbs will demonstrate superior BBB penetration, enhanced targeting of α-syn aggregates, and reduced neuroinflammation compared to free nbs.

Finally, a very important point of this project will be to evaluate which α-syn species is more efficient to target. While purified α-syn oligomers are more toxic toward SHSY5Y cells than fibrils on a weight basis, fibrils win out on a particle number basis as determined by a combination of analytical ultracentrifugation and quantitative length distribution measurements (Pieri et al., 2012) (Lorenzen et al., 2014b). Besides the higher per-particle activity, fibrillar species possess one additional advantage over oligomers in terms of toxicity: their ability to propagate and amplify by seeding the aggregation of endogenous α-syn. So far, studies in animal models have shown contradictory results into which species are more toxic. Utilizing nbs that target specifically α -syn monomers, oligomers or fibrils will help us better understand the mechanism of α-syn pathology propagation.

If successful, this approach could pave the way for more effective immunotherapies for PD and potentially other neurodegenerative diseases characterized by protein aggregation.

Research Strategy

Work Package 1: Evaluate the binding efficiency and stability of conformation-specific nanobodies to exosomes.
Exosomes from adult mouse brains which are knock out for the wild type α-syn gene will be purified by homogenization, ultracentrifugation with sucrose gradient. Size and shape of exosomes will be observed by Transmission Electron Microscopy (TEM) and Nanoparticle Tracking Analysis (NTA). Quantification of exosomes will be estimated by Micro-Bradford and Acetylcholinesterase (AChE) assay reaction methods. The nanobody will be conjugated to the exosomes using established biochemical methods. The conjugation efficiency will be quantified using enzyme-linked immunosorbent assays. The stability of the exosome-nanobody complexes will be assessed under various conditions, including different pH levels, temperatures, and in the presence of serum proteins. Stability will be evaluated by monitoring the integrity and binding capability of the complexes over time using Dynamic Light Scattering (DLS) and immunoassays. We will analyze the data to determine the optimal conditions for maintaining the stability and binding efficiency of the exosome-nb complexes. These findings will guide the preparation of complexes for subsequent *in vitro* and *in vivo* studies.

Work Package 2: Assess the therapeutic potential of exosome-nanobody complexes in *in vitro* models of α-syn pathology. We will use both a tet-off SH-SY5Y cell system, genetically

modified to express human wild-type α-syn under the control of a tetracycline-responsive promoter and neuronal and glial primary cultures. SH-SY5Y cells will be cultured and induced to overexpress α-syn by removing doxycycline from the medium. Primary cortical neurons, and neuronal/glial mixed cultures will be prepared by allowing the neurons to grow in the presence of glial cells. These cultures will provide a more physiologically relevant environment to study the effects of the nanobody-exosome complexes. To promote α-syn pathology, cells will be treated with α-syn PFFs for 48 hours. This treatment mimics the aggregation process observed in PD and creates a relevant model for testing the efficacy of the exosome-nanobody complexes. We will use nbs targeting α-syn monomers, oligomers and fibrils as well as control nbs. The cells will be treated with exosome-bound nb, free nb, and PBS. Treatments will be administered at specific concentrations optimized from preliminary studies. We will evaluate α-syn aggregation and toxicity through various techniques, including immunocytochemistry (ICC) using α-syn-specific antibodies, western blot analysis to quantify different α-syn species, and confocal microscopy for detailed imaging. We will use 3D reconstruction software to quantify intracellular α-syn levels and analyze the effectiveness of the treatments. Quantitative analysis will be performed to compare the impact of different treatments on α-syn pathology. Statistical methods will be used to assess the significance of observed differences.

Work Package 3: Investigate the impact of exosome-nanobody complexes on α-syn pathology and neuroinflammation in a mouse model of PD. First, in order to study the pharmacokinetics of nbs and exosome-nb complexes we will administer intranasally in WT mice different concentrations of the nanobodies and exosome-nbs complexes. The nbs will then be quantified in brain extracts by binding ELISA. Afterwards we will check the efficiency of the nb and nb-exosome complexes in alleviating the pathology in a PD mouse model. We will employ a well- established PD model involving the nigrostriatal inoculation of wild-type mice with human α-syn PFFs. This model reliably reproduces key features of PD, including α-syn aggregation and dopaminergic neuron loss. Mice will be divided into groups receiving different treatments: exosome-bound nb, free nb, and PBS. Treatments will be administered intranasally bi-weekly for one month, starting on the day of PFF inoculation. The intranasal route is chosen to enhance BBB penetration and minimize systemic side effects. Throughout the treatment period, we will conduct a series of behavioral assessments to monitor motor and cognitive functions. These tests will include the rotarod test for motor coordination, the open field test for general activity, and the Barnes maze test for spatial memory. Brain sections will be stained for phosphorylated α-syn (pS129) and tyrosine hydroxylase (TH). We will quantify α-syn pathology by measuring pS129-positive inclusions within TH-positive neurons. The intensity of neuroinflammation markers (GFAP for astrocytes and Iba-1 for microglia) will also be quantified using imaging software. Additionally, we will perform RT-PCR on brain tissue samples to measure the expression levels of cytokines and other inflammatory factors, providing a comprehensive view of the neuroinflammatory response. Data from behavioral tests, immunohistochemistry, and biochemical analyses will be

compiled and statistically analyzed to determine the efficacy of the treatments. Comparisons will be made between the different treatment groups to identify significant differences in α-syn pathology and neuroinflammation.

Budget

Table 1: Project budget and Justification

BUDGET		
Category		**Total in €**
Direct Costs Personnel		
Post-Doc Researcher(s)		**50.000,00**
PhD Candidate(s)		**30.000,00**
	Total Direct costs for Personnel	**80.000,00**
Other Direct Costs	**Justification**	
6.1.2 Consumables	General Lab Expences, Experimental Model Expenses, Antibodies, Cell Culture Exprenses, Molecular Biology Methods Expences, etc)	**80.000,00**
6.1.3 Travel	Conferences	**3.000,00**
6.1.4 Dissemination	Conferences	**1.500,00**
6.1.5 Other Costs	Publication	**2.000,00**
6.1.6 Purchase of animals	CD57BL6/JOIaHsd mice, Harlan Laboratories	**5.000,00**
	Total "other direct costs"	**91.500,00**
	Total Direct Costs	**80.000,00**
	Indirect Costs (Institution overhead, 10%)	**9.150,00**
	Total Budget	**180.650,00**

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Vijayakumar, D., Jankovic, J. (2022) Slowing Parkinson's Disease Progression with Vaccination and Other Immunotherapies. CNS Drugs 36, 327-343

Title: Extracellular Vesicles as Vehicles for Immunotherapy in Neurodegenerative Diseases
Student Name: Bourtouli Nikoleta

Curriculum Vitae

Bourtouli Nikoleta, Chemist

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EDUCATION

10.2021-07.2024- Master's degree in Athens International Master's Programme in Neurosciences, NKUA
10.2017-09.2021- Bachelor's degree in Chemistry, NKUA (Grade:8,38/10,00)

RESEARCH EXPERIENCE

02.2024-07.2024 Master's Internship in Institute of Mediterranean Neurobiology, INSERM, Marseille, under the supervision of Dr. Thomas Marissal and Dr. Nathalie Bernard-Marissal. My project focused on the interplay between oligodendrocytes and the energy voracious parvalbumin interneurons at the hippocampal CA1 region. Laboratory techniques: hippocampal organotypic slice culture, drug and AAV viral vector-based cell specific knock-down, calcium imaging using two-photon microscopes, immunohistochemistry and confocal imaging, data analysis.
07.2022-07.2024 Master's Research Project in Biomedical Research Foundation of the Academy of Athens (BRFAA), under the supervision of Dr. Konstantinos Vekrellis, Researcher A', Deivision of Basic Research. My project was about the role of exosomes both as vehicles for immunotherapy and as source of biomarkers in synucleinopathies. Laboratory techniques: mouse brain extraction and microdissection of brain regions, interstitial brain exosome isolation, immunofluorescence of free-floating brain slices and confocal imaging, cell line culture, immunocytochemistry, immunoblot analysis, fluorescence-activated cell sorting.
03.2022-05.2022 Master's Lab Rotation, Biomedical Research Foundation of thr Academy of Athens (BRFAA), under the supervision of Dr. Popi Syntichaki, Researcher C', Division of Basic Research. My project was related to the understanding of the role of the mitochondrial LON proteases in autophagy, in the C. elegans model. Laboratory techniques: maintenance of C. elegans, forward and reverse mutagenesis in C. elegans, DNA, RNA and protein extraction from C. elegans, PCR and RT-PCR, immunoblot analysis, confocal imaging. Grade: 10/10.
12.2021-02.2022 Master's Lab Rotation, Biomedical Research Foundation of thr Academy of Athens (BRFAA), under the supervision of Dr. Kostas Vekrellis, Researcher A', Division of Basic Research. My project focused on the investigation of the mechanisms governing astrocytic responses under physiological and pathological conditions mimicking Parkinson's Disease. Laboratory techniques: isolation and culture of primary neurons and glial cells, immunocytochemistry and confocal imaging, immunoblot analysis. Grade: 10/10.

LANGUAGES

Greek: Fluent (Native).
English: Fluent, C2, ECPE, University of Michigan
French: Fluent, B2, DELF