Pathogenic α-synuclein (α-syn) is a prion-like protein that drives the pathogenesis of Lewy Body Dementia (LBD) and Parkinson’s Disease (PD). To target pathogenic α-syn preformed fibrils (PFF), here we designed extracellular disulfide bond-free synthetic nanobody libraries in yeast. Following selection, we identified a nanobody, PFFNB2, that can specifically recognize α-syn PFF over α-syn monomers. PFFNB2 cannot inhibit the aggregation of α-syn monomer, but can significantly dissociate α-syn fibrils. Furthermore, adeno-associated virus (AAV)-encoding EGFP fused to PFFNB2 (AAV-EGFP-PFFNB2) can inhibit PFF-induced α-syn serine 129 phosphorylation (pS129) in mouse primary cortical neurons, and prevent α-syn pathology spreading to the cortex in the transgenic mice expressing human wild type (WT) α-syn by intrastriatal-PFF injection. The pS129 immunoreactivity is negatively correlated with the expression of AAV-EGFP-PFFNB2. In conclusion, PFFNB2 holds a promise for mechanistic exploration and therapeutic development in α-syn-related pathogenesis.
Lewy body dementia (LBD) is one of the most common dementias, including Dementia with Lewy Body (DLB) and Parkinson’s Disease (PD) with Dementia (PDD)\(^1\)–\(^3\). Moreover, Alzheimer’s Disease (AD) patients with Lewy body pathology exhibit a more rapid and severe cognitive decline than AD alone\(^4\). There is an urgent need for effective therapies for LBD. LBD is characterized by the accumulation of aggregated α-synuclein (α-syn) in the cortex. Substantial post-mortem studies by Braak et al.\(^5\)–\(^8\) and others\(^9\)–\(^12\) showed that misfolded α-syn is a prion-like protein, and its pathology spreads stereotypically. A single administration of recombinant α-syn preformed fibrils (PFF) can induce endogenous α-syn monomers to aggregate and subsequent cell-to-cell transmission\(^13\)–\(^15\). Both clinical and experimental observations support the prion-like hypothesis of aggregated α-syn fibrils\(^16\). Thus, targeting α-syn fibrils could provide an alternative approach to study the pathogenesis and to treat LBD and related α-synucleinopathies.

Of note, α-syn pathology and propagation are observed mainly inside neurons and α-syn is more abundant in neurons than in glial cells, indicating that the intracellular α-syn significantly drives the pathogenesis\(^17\)–\(^19\). There have been tremendous efforts on the development of antibodies against α-syn (reviewed in Vaikath et al.\(^20\)); however, due to the large size and structural complexity, antibodies have limited penetrability through the plasma membrane\(^21\)–\(^22\). Consequently, α-syn antibodies typically do not enter cells but only target the extracellular α-syn fibrils\(^23\)–\(^27\), not accessible to the essential target, the intracellular pathologic α-syn species. Furthermore, antibodies are usually not functional in the reducing environment of the cytosol due to the reduction of the disulfide bonds, which are critical for the correct folding of antibodies\(^28\). In addition, antibody treatment could be costly and inconvenient in long-term disease progression\(^29\). All these gaps indicate an urgent need to generate a reagent targeting the intracellular pathogenic α-syn.

Nanobodies are single-domain antibodies with several advantages: (1) high stability\(^30\)–\(^31\), (2) small size (15 kDa)\(^31\),\(^32\) and improved brain permeability\(^33\), and (3) suitability for intracellular expression\(^34\)–\(^35\). Recent advances in adeno-associated virus (AAV)-based gene delivery have provided an attractive approach to continuously express recombinant proteins binding to pathogenic targets in long-term disease treatment\(^22\),\(^25\)–\(^36\). Some nanobodies,\(^37\)–\(^38\) including NbSyn2 and NbSyn87, were designed to bind to total α-syn (both monomers and aggregates). In particular, NbSyn87 targeted to the proteasome was found to reduce α-syn pathology both in vitro and in vivo\(^39\)–\(^40\). However, the total α-syn-targeting nanobodies may perturb the physiological function of α-syn monomers, and show reduced efficacy against α-syn pathology due to the competitive binding with α-syn monomers. Therefore, it is necessary to develop nanobodies that specifically bind to α-syn aggregates.

Nanobodies are normally screened under extracellular oxidizing conditions\(^41\)–\(^42\) and then applied for intracellular application under reducing conditions (e.g., intracellular cytosol). In the traditional nanobody scaffold, there are two conserved cysteine residues that form a disulfide bond in oxidizing conditions (e.g., endoplasmic reticulum, Golgi, bacterial periplasm, extracellular environment, etc.), which is critical for the stabilization of a nanobody’s structural folding\(^43\). However, the disulfide bond can be disrupted in reducing conditions, resulting in changes in the stability, folding, and function of the nanobody in some circumstances\(^43\). Thus, it is desired to develop an extracellular disulfide bond-free nanobody selection platform to ensure consistent nanobody folding.

In this study, we designed extracellular disulfide bond-free synthetic nanobody libraries by mutagenesis of the two conserved cysteine residues. We generated nanobodies that preferentially bind to α-syn fibrils, but not α-syn monomers. We determined the specificity and efficacy of one nanobody in vitro and in vivo. The α-syn fibril-specific nanobody will provide a tool for pathogenesis exploration and hold promise for therapeutic application.

**Results**

**Preparation of human α-syn monomers and PFF.** To obtain nanobodies that preferentially bind to α-syn fibrils but not α-syn monomers, we prepared both human recombinant α-syn monomers and PFF following the established protocol\(^44\). Seven days after agitation, mature α-syn fibrils were generated from α-syn monomers, and then sonicated for α-syn PFF. Transmission electron microscopy (TEM) showed the short fibrillar morphology (average length 53.8 nm) of α-syn PFF, and irregular morphology of α-syn monomers (Supplementary Fig. 1a, b). α-Syn PFF and monomers were validated with a thioflavin T (ThT) fluorescence assay (Supplementary Fig. 1c)\(^45\),\(^46\).

**Design of the disulfide bond-free nanobody libraries by mutagenesis of the conserved cysteine residues and randomization of the binding loops.** Nanobodies have a framework region consisting of β-sheets (Fig. 1a), and three variable loops that correspond to the complementary determining regions (CDR1, 2, and 3) constituting the antigen-binding site (Fig. 1a). Under oxidizing conditions, two conserved cysteine residues inside the β-sheets form a disulfide bond (orange line, Fig. 1a), stabilizing the nanobody scaffold\(^31\). Because the folding and function of traditional nanobodies with the disulfide bond may change in reducing conditions from oxidizing conditions, we generated nanobody libraries with the disulfide bond removed, by mutagenesis of the two conserved cysteine residues, to select nanobodies for intracellular applications.

To evaluate whether nanobodies can fold correctly and function without the conserved disulfide bond, we introduced C22L and C96A mutations into a published nanobody against GFP (GFPNB, PDB, 3K1K)\(^47\). Supplementary Fig. 2 shows that the disulfide bond-free GFPNB can still bind to EGF to a substantial degree on the yeast cell surface. This is consistent with a previous study that GFPNB with these two cysteine residues mutated maintains the nanobody’s binding function\(^48\). Therefore, we incorporated C22L and C95A (corresponding to C96 in GFPNB) mutations to the synthetic nanobody scaffold to generate the disulfide bond-free nanobody libraries (Fig. 1a, Supplementary Fig. 3a). Without immunization, we constructed the synthetic nanobody libraries using degenerated primers to randomize CDR1, 2, and 3 following a recent protocol\(^42\) (detailed PCR construction in Supplementary S3 and Supplementary Method). The CDR3 was constructed with three different lengths with 7, 11, or 15 amino acids totally randomized with NNK degenerated codons to generate three libraries: DNA library 7, 11, and 15. The nanobody library size obtained for each library was ~1 × 10^7, covering only part of all the possible amino acid combinations. These nanobody libraries were displayed on the yeast surface via fusion to the binding subunit of α-agglutinin protein Aga2p, a yeast surface protein (Fig. 1b) following an established protocol\(^49\).

**Selection of nanobodies against α-syn PFF.** Next, we performed the selection of disulfide bond-free nanobodies against α-syn PFF. As illustrated in Fig. 1b, nanobodies were expressed on the yeast surface by fusion to the C-terminus of Aga2p, followed by the FLAG tag. α-Syn PFF was incubated with the yeast cell libraries to allow binding to nanobody clones. Then, mouse anti-α-syn monoclonal antibody (mAb) (BD Biosciences) was added to label
α-syn PFF bound on the yeast cell surface, followed by the incubation with anti-mouse IgG conjugated with, either magnetic beads for magnetic-activated cell sorting (MACS) or fluorophores for fluorescence-activated cell sorting (FACS). By using MACS, nanobody clones binding with α-syn PFF were selected in the first round of sorting from a large population of yeast cells (~5 × 10⁸ cells). From the MACS enriched clones, we performed 5 more rounds of FACS and MACS (Fig. 1b) to enrich nanobody clones binding with α-syn PFF (Fig. 1c). Details of the selection process are described in Supplementary Fig. 3b, c.

From the enriched yeast cells, we extracted the plasmid DNA and re-transformed the DNA into bacterial cells for individual clone sequencing. From the 40 nanobody clones that were sequenced, 28 unique clones were identified (Supplementary Table 1). We re-transformed these nanobody clones into yeast cells for further characterization of the individual clones. Supplementary Fig. 4 shows that all these 28 clones showed preferential binding to α-syn PFF over α-syn monomers. Henceforward, we refer to these nanobodies as PFF-nanobodies (PFFNBs).

Because CDR3 is the antigen-binding loop with the most variations, we performed an amino acid analysis of loop3 for the selected PFFNBs. The majority of the selected clones consist of a CDR3 with 7 amino acids randomized (Supplementary Fig. 5a). The selected PFFNBs tend to be rich in hydrophobic and positively-charged residues in the CDR3 (Supplementary Fig. 5b). These PFFNBs may exhibit preferential binding to α-syn PFF through hydrophobic and ionic interactions.

In vitro characterization and validation of the selected PFFNB2 binding to α-syn PFF. To validate the PFFNBs’ binding with α-syn PFF, we made PFFNB constructs fused with maltose binding protein (MBP) at its N-terminus for protein expression in E. coli (BL21). However, all of these nanobody proteins were retained in the cell pellet when expressed in E. coli (BL21) except for the positive control, GFPNB (C22L, C95A). This indicated that these PFFNBs are less stable than GFPNB (C22L, C95A) (Supplementary Fig. 6a). Successful expression of soluble PFFNBs was achieved by supplementing chaperon protein (plasmid pGro7) to BL21(C14) E. coli cells. Recombinant MBP-PFFNBs with a prominent band at the correct molecular weight were then observed in both the crude cell lysate and the semi-purified protein extract (Supplementary Fig. 6b) with polyacrylamide gel electrophoresis (PAGE) analysis. Supplementary Fig. 6c showed PAGE analysis of recombinant proteins which were later used in this study.

We picked seven out of the 28 PFFNB clones for the initial testing. These nanobody clones were expressed, purified, and immunoblotted against α-syn PFF and monomers with native-PAGE. As published, anti-α-syn mAb (BD Biosciences) can detect total α-syn (monomers and aggregates) (Fig. 2a)³⁰. Among the seven nanobody clones tested, MBP-PFFNB2 was identified to specifically bind α-syn aggregates, but not to α-syn monomers (Fig. 2a). Next, we performed ELISA to evaluate PFFNB2’s selective binding for α-syn PFF. MBP-PFFNB2 showed preferential binding to α-syn PFF (EC50, 163.0 nM) over α-syn monomers (EC50, undetermined) (Fig. 2b). This ELISA result
Fig. 2 In vitro characterization of PFFNB2 binding to α-syn PFF and aggregates. a Native-PAGE immunoblot of human α-syn monomers and PFF with PFFNB2 and anti-α-syn monoclonal antibody (mAb). PFFNB2 binds selectively to the high molecular weight (MW) α-syn but not to the low MW α-syn. Anti-α-syn mAb binds to both the high and low MW α-syn forms. M, α-syn monomers. P, α-syn PFF. mAb, anti-α-syn monoclonal antibody. The experiment was replicated three times with similar results. b ELISA result of PFFNB2 binding to α-syn PFF, monomers, and control (blank). Wells were coated with 3 ng/μl of α-syn PFF or monomers, and then titrated with 3.3, 33.3, 66.7, 133.3, 266.7, 666.7, and, 1333.3 nM of PFFNB2. Three data points were collected for each concentration and shown as mean ± SEM. The experiment was replicated once with similar result. c AAV-transduced EGFP-PFFNB2 (green) signal co-localized with the immunostaining of anti-pS129 in HEK293T cells stably expressing α-syn(A53T) induced by α-syn PFF. Green, EGFP-PFFNB2 signal. Red, anti-pS129 immunofluorescence signal. White arrows indicated the co-localization between EGFP-PFFNB2 and pS129 α-syn. Scale bar, 40 μm. d Quantification of co-localization between pS129 α-syn signal to PFFNB2 using Pearson correlation. Data were analyzed from 103 puncta. The box ranges from the first to the third quartile of the distribution with median indicated as line across the box. The whiskers are the minimum and maxima of the data. e ELISA analysis of PFFNB2 binding to mouse brain lysate. KO, Snca knock-out mouse; PBS, transgenic mouse expressing human α-syn with striatal-PBS injection; PFF, transgenic mouse expressing human α-syn with striatal-PFF injection. Wells were coated with 3 ng/μl of each brain lysate, and then detected with 2, 15, 50, 100 nM of PFFNB2. Two data points were collected for each concentration. The experiment was replicated once with similar result. Source data are provided as a Source Data file.
is consistent with the result of native-PAGE immunoblot. To exclude any role of MBP in the binding, we further determined that MBP does not bind to α-syn PFF with ELISA (Supplementary Fig. 7a). Anti-α-syn mAb50 and MBP-NbSyn8738 exhibited comparable (non-selective) binding affinities toward α-syn PFF and monomers (Supplementary Fig. 7b, c). Further characterization with ELISA showed a similar binding affinity of MBP-PFFNB2 to human α-syn (A53T) PFF (EC50, 176.4 nM), a familial PD mutant51, compared to human wild type (WT) α-syn PFF (Supplementary Fig. 7d).

To evaluate the functional difference of PFFNB2 with or without disulfide bond, we added back cysteines to residues 22 and 95. Re-introduction of the disulfide bond significantly reduced the binding affinity of PFFNB2 with α-syn PFF (Supplementary Fig. 7e). This suggests that disulfide bond may cause changes in the nanobody scaffold resulting in loss of functionality. MBP-PFFNB2 fusion protein (green) co-localized with the immunostaining of PFFNB2 in fixed and immunostained samples. This result indicated that intracellularly-expressed PFFNB2 has the proper folding and function. More importantly, PFFNB2 not only binds to recombinant α-syn PFF, but also binds to the cellular inclusion of pS129-positive α-syn aggregates (Fig. 2c, d).

AAV-EGFP-PFFNB2 decreased α-syn pathology in primary cortical neurons. Encouraged by the dissociation result, we further evaluated whether PFFNB2 can reduce α-syn pathology in WT mouse primary cortical neurons. AAV encoding EGFP-PFFNB2 (AAV-EGFP-PFFNB2) was added to cortical neurons at 5 days in vitro (DIV), followed by the administration of α-syn PFF at 7 DIV. AAV encoding EGFP (AAV-EGFP) was used as the control. Both substantial expression of EGFP in the AAV-EGFP and AAV-EGFP-PFFNB2 groups appeared at 11 DIV (Supplementary Figs. 9a, 8b). There was no difference in neurotoxicity 10 days after AAV transduction (neurons 15 DIV) between these two groups (Supplementary Fig. 9c, d). Substantial immunoreactivity of anti-pS129 in the AAV-EGFP group was observed in cortical neurons 7 days after α-syn PFF administration as published10. In contrast, AAV-EGFP-PFFNB2 induced a significant reduction of the immunoreactivity of anti-pS129 (Fig. 3f, g). Of note, in the experimental timeline, α-syn PFF was administered at 7 DIV, and EGFP-PFFNB2 expression was prominent at 11 DIV cortical neurons, 4 days after the PFF administration. This indicated that AAV-EGFP-PFFNB2 exhibited efficacy in inhibiting α-syn PFF-induced pathology in cortical neurons.
Fig. 3 PFFNB2 dissociates α-syn fibrils and inhibits α-syn pathology induced by PFF in vitro. a Disaggregation of α-syn fibrils monitored by ThT fluorescence assay. α-syn PFF was incubated alone (black), with MBP-PFFNB2 (red), or with MBP (blue). Quantification data are the means ± SEM, n = 3 independent experimental replicates, two-way ANOVA with Tukey correction. (α-syn fibrils vs. α-syn fibrils + MBP-PFFNB2, P values are Day5 = 0.0388, Day8 = 0.0199, Day12 = 0.0005, Day15 = 0.0001) *P < 0.05, **P < 0.01, ***P < 0.001, ns, not significant. b TEM images of α-syn fibrils with MBP-PFFNB2 or MBP. Scale bar, 100 nm. c Quantification of fibril length from (b). Quantification data are the means ± SEM, n = 50 datapoints per group, one-way ANOVA with Tukey correction. (α-syn fibrils vs. α-syn fibrils + MBP-PFFNB2, P = 0.0001) ***P < 0.001, ns, not significant. d Circular dichroism (CD) spectra for α-syn PFF with MBP-PFFNB2 or MBP. e Dynamic light scattering (DLS) analysis for α-syn fibrils with MBP-PFFNB2 or MBP. Quantification data are the means ± SEM, n = 3 independent experiments, P values were determined by one-way ANOVA with Tukey correction. (α-syn fibrils vs. α-syn fibrils + MBP-PFFNB2, P = 0.0001). ****P < 0.0001, ns, not significant. f WT mouse primary cortical neurons were transduced with AAV encoding EGFP (control group) or EGFP-PFFNB2 (PFFNB2 group) at day 5 in vitro and α-syn PFF at day 7 in vitro. The α-syn pathology level was assessed with anti-phosphorylated serine129 (pS129) immunostaining 7 days after α-syn PFF treatment. Scale bar, 50 μm. g Quantification of the pS129 immunoreactivity normalized by Hoechst. Quantification data are the means ± SEM, n = 6 independent experiments, P values were determined by two-sided Student’s t test. (AAV-EGFP vs. AAV-EGFP-PFFNB2 P = 0.0001). ****P < 0.0001. Source data are provided as a Source Data file.

Two months after AAV injection, intrastriatal injection of α-syn PFF was performed on these mice when in the adult age, to induce α-syn pathology spreading to the cortex that was examined one month later (Fig. 4a).

One month post-striatal-PFF injection, we assessed the EGFP expression in the cortex, and the immunoreactivity of anti-pS129 in the cortex of these mice. The intraventricular injection of the AAV with synapsin promoter resulted in the expression of EGFP in the neurons of the cerebral cortex (Figs. 4a, c, 5a–c), consistent with the published study. The EGFP was mainly expressed in the motor and somatosensory cortical regions (~12–18%) (Fig. 5b, c). Two cortex sub-regions with high intensity expression of EGFP were chosen for analysis. Substantial immunoreactivity of anti-pS129 was observed in the AAV-EGFP group (Figs. 4b–e, 5a–c), indicating that α-syn pathology spread from the striatum to the cortex. In contrast, a significant decrease of pS129 level was detected in the AAV-EGFP-PFFNB2 group (Figs. 4b–e, 5a–c). Furthermore, we performed the correlation analysis between the intensity of EGFP expression and pS129 immunoreactivity. The results showed that pS129 immunoreactivity is negatively correlated with the intensity of EGFP in the AAV-EGFP-PFFNB2 group, and there is no relevant correlation in the AAV-EGFP...
group (Fig. 5d). In another, there is no significant reduction of pS129 level in the striatum of the AAV-EGFP-PFFNB2 group, which could be attributed to the absence of EGFP-PFFNB2 expression in the striatum (Supplementary Fig. 10a, b). Taken together, AAV-EGFP-PFFNB2 can effectively prevent pathogenic α-syn spreading to the cortex in the striatal-PFF mice model.

Discussion

Fibrillar α-syn aggregates are prion-like seeds propagating throughout the brain, which is a major driver in the pathogenesis of LBD, PD, and related α-synucleinopathies7–9. It is important and necessary to generate reagents that specifically target α-syn fibrils. To address this, we establish an extracellular disulfide bond-free nanobody selection platform that allows the generation of nanobodies with consistent folding and function in both intracellular and extracellular environments. We generated and identified a nanobody (PFFNB2) that specifically binds to α-syn PFF, but not to α-syn monomers. PFFNB2 dissociated α-syn fibrils in the solution, inhibited α-syn pathology in vitro, and prevented α-syn pathology from spreading to the cortex by AAV-transduction. We expect that these PFFNB-related agents hold great promise as a potential therapeutic strategy against α-syn-related pathogenesis.

Native α-syn monomers play an important role in vesicle trafficking and refilling at synapses60–62. Thus, targeting α-syn monomers may result in the imbalance between the reserved and releasable vesicles, and impair neurotransmitter uptake by the
vesicles. Non-conformation specific reagents (e.g., antibody, nanobody) might also disrupt the balance of α-syn monomers and tetramers, and α-syn tetramer has been reported to inhibit α-syn aggregation. Of note, α-syn fibrils are more toxic than other species (e.g., oligomer, ribbon). Thus, targeting α-syn fibrils is critical for therapeutic development against LBD, PD, and related α-synucleinopathies.

Considering the observation of cellular α-syn pathological inclusions and abundant expression of cytosolic α-syn monomers in neurons, it is critical to block the intracellular propagation of exogenous α-syn induced by the internalized α-syn seeds. The efficacy of α-syn fibril-specific antibodies in reducing α-syn pathology has been reported. However, antibodies have poor membrane penetration ability and are usually not functional in the reducing cytosolic environment, and therefore, can only target extracellular α-syn fibril, limiting their impact on the pre-existing intracellular α-syn aggregates. A study has reported that single-chain antibody fragment (scFv) D5 binding to α-syn oligomer can inhibit α-syn fibrillation and toxicity. However, extracellular expression of scFV tends to aggregate, which could compromise its therapeutic effects. Overall, nanobodies are preferred over conventional antibodies or scFVs for intracellular applications, as they are small, monomeric, stable, and commonly expressed inside cells. We believe this work provides a proof-of-concept that targets intracellular α-syn aggregates using AAV transduced α-syn-fibril-specific nanobody can effectively reduce pathology propagation.

In this study, we focus on using AAV-PFFNB2 to target intracellular α-syn aggregates against pathogenic α-syn spreading. Of note, emerging evidence also suggests the importance of exogenous α-syn aggregates in α-syn cell-to-cell transmission, by entering neurons and seeding endogenous α-syn aggregation. Encouragingly, two α-syn antibodies, PRX002 and MEDI134174 that bind to total α-syn, have exhibited efficacy in ameliorating disease phenotype of PD models when administered extracellularly, and they are currently in Phase II (ID: NCT03100149) and Phase I (ID: NCT04449484) clinical trial, respectively. More study is needed to evaluate the efficacy of exogenously applied PFFNB2 in preventing the neuronal uptake of α-syn PFF and subsequent α-syn propagation. If recombinant PFFNB2 can inhibit the effects of exogenous α-syn PFF when administered extracellularly, we will then modify the AAV-PFFNB2 system to secret PFFNB2 to the extracellular space. PFFNB2 could also be expressed in a bacteria-based expression system, which is higher-yielding and more cost-effective than the eukaryotic cell-based system used for conventional antibody production. Further bioengineering efforts on PFFNB2 will also be necessary to improve brain penetration.

Because the conserved cysteine residues will not form a disulfide bond in the reducing environment of the bacterial cytoplasm during the selection process, an intracellular bacterial selection platform could be advantageous for selecting nanobodies without forming a disulfide bond. However, an extracellular nanobody selection platform, the conserved cysteine residues would form a disulfide bond during the selection process. Therefore, in our library design, we introduced cysteine mutations to remove the disulfide bond even in the extracellular oxidizing condition. Although a previous study showed that removing the conserved disulfide bond in nanobodies does not allow them to fold correctly, we found that the conserved cysteine residues were still able to form a disulfide bond in the extracellular oxidizing condition. We hypothesized that this might be due to the presence of reducing agents such as glutathione in the extracellular environment.
have a significant effect on their binding affinity in the reducing environment, our study suggests that the disulfide bond can change the binding property of a nanobody. Thus, it is important to generate a nanobody framework exhibiting consistent binding property from the selection stage to final applications.

In summary, we have demonstrated the feasibility of selecting disulfide bond-free nanobodies that can be stably expressed in the bacterial host E. coli. The methodology described here could facilitate the production of a large number of nanobodies with high binding affinity for proteins of interest. The success of this approach hinges on the selection of a library that is well-suited for the target protein of interest. Further investigation and development of this technology will enable the generation of nanobodies with improved properties for a wide range of applications.

Methods

This research complies with all relevant ethical regulations. The animal studies were approved by the Johns Hopkins Animal Care and Use Committee (ACUC). All animal studies were performed according to the NIH Guide for the Care and Use of Experimental Animals and the guidelines of the Institutional Animal Care Committee of the Johns Hopkins University.

α-Syn PFF generation

Recombinant human WT and A53T α-syn monomers were purified following the established protocol28. Briefly, full length human WT or A53T α-syn containing prk1/72 vector were expressed in BL21(DE3) E. coli. α-syn monomers were subsequently purified on Ni-NTA resin (Thermo Fisher Scientific) followed by incubation with 50 μM Thiolutin V (ThT). The ThT fluorescence was measured using a plate reader (Varioskan LUX plate reader, Thermo Fisher Scientific) with excitation at 450 nm and emission at 485 nm. To perform TEM analysis, a drop of α-syn monomer solution was loaded onto carbon-coated electron microscopy grids and negatively stained with phosphotungstic acid. The images were acquired with Ultraview FEI Tecnai G2 F20 Transmission Electron Microscope.

Nanobody library generation

Nanobodies libraries were constructed by overlap-lapping polymerase chain reaction (PCR), combining a series of degenerated primers to generate the DNA encoding full-length nanobody. Degenerated bases were used to randomize CDRI, 2, and 3. Three different lengths were constructed for CD2R to generate Library 7, 11, and 15 (the number corresponds to the number of amino acids randomized on CD3R). To ensure homologous recombination of these randomized nanobody DNA constructs into the yeast vector backbone, these DNA constructs are flanked with 42 base pairs that are overlapping with the pCTCON2 vector backbone. The pCTCON2 vector was previously engineered to contain Aga2p and FLASH-tag with Nhel and BamH1 cut sites in between for nanobody gene insertion. After homologous recombination, nanobodies were expressed on yeast surface with FLAG-tag at its C-terminus to monitor nanobody expression. Details of construction with primer sequences and the sequence of nanobody libraries could be found in Supplementary Table 3 and 4. A detailed description of the nanobody library construction using overlap-lapping PCR is included in the Supplementary Methodology.

After preparation of the linearized vector and randomized nanobody PCR fragments, we followed the established protocol for yeast competent cell preparation and electroporation29. Briefly, EBY100 yeast competent cells (Thermo Fisher Scientific) were pre-conditioned with 100 mM lithium acetate and 1 mM dithiothreitol and then electroporated with a mix of the nanobody PCR fragments and linearized vector. Electroporated yeast cells were grown in SDCAA media (synthetic dextrose plus casein amino acid, 2% dextrose, 0.67% yeast nitrogen base without amino acids (Bio Basic), 0.5% Bacto casein amino acid (Difco), 0.54% disodium phosphate, 0.856% monosodium phosphate) lacking thymophan as a selection marker at 30 °C and 200 r.p.m. for 24 h. The library size of the nanobody libraries generated was ~1 x 10^6 for each library.

PFFNB selection and DNA sequence analysis

Roughly 1.5 x 10^8 yeast cells from each library (Library 7, 11, and 15) were cultured and protein expression was induced with 1:10 dilution of SDCAA in SGCAA media (synthetic galactose plus casein amino acid, 2% galactose, 0.67% yeast nitrogen base without amino acids (Bio Basic), 0.5% Bacto casein amino acid (Difco), 0.54% disodium phosphate, 0.856% monosodium phosphate) overnight at 30 °C. The next day, the yeast cells grew up to 10^8 cells/ml. About 5 x 10^8 cells were pelleted to be incubated with α-syn PFF for 1 h at room temperature (RT). Following that, the yeast cells were immunostained with mouse anti-α-syn mAb (1:200 dilution, BD Biosciences, Cat no. 610787) and rabbit anti-FLAG antibody (1:200 dilution, Sigma, Cat no. F7425) and then secondary antibodies goat anti-mouse IgG AlexaFluor 647 antibody (1:400 dilution, Thermo Fisher Scientific, Cat no. A11004) and goat anti-rabbit IgG AlexaFluor 488 antibody (1:400 dilution, Thermo Fisher Scientific, A21245). FACS sorting was performed using BD FACSaria III cell sorter and BD FACSdiva software (BD Biosciences). Analysis of the FACS plot was performed using FlowJo software. After several rounds of alternating MACS and FACS, the nanobody DNA plasmids were isolated and re-transformed to XL-Blue E. coli competent cells. Forty PFFNB clones were sequenced and analyzed with Sanger sequencing.

Yeast re-transformation

From the 40 PFFNB clones sequenced, 28 unique clones were identified and re-transformed into yeast chemical competent cells individually using EZ yeast Transformation kit (Zymo Research). Each of these nanobody clones was labelled with α-syn PFF or monomers and then immunostained and analyzed using BD FACSaria III (BD Biosciences) as described above in PFFNB selection.

PFFNB protein expression and purification

The PFFNBs were cloned into the pPF161 plasmid for protein expression and purification (listed in Supplementary Table 4). For Native-PAGE gel immunoblotting experiment, ELISA and dissociation analysis, HisTag-MBP-FLAG, MBP-PFFNB2-FLAG, MBP-PFFNB2(L22C, A95C)-FLAG, and MBP-Nbsyn87-FLAG were used (see Supplementary Table 3 for details). The PFFNB clones were transformed into BL21 E. Coli (C14) (gift from Dr. Haoming Zhang, University of Michigan) expressing a set of chaperones from plasmid pGro7 (Takara Bio). The transformed BL21 cells were grown in 5 ml Luria Broth (LB) media overnight at 37 °C and 220 r.p.m. The next morning, the 5 ml bacterial cultures were transferred to 500 ml LB and grown until OD600 = 3. Then, 1 ml IPTG was added to cells to induce protein expression. Cells were shaken at 220 r.p.m. and 16 °C for 16 h.

The cells were pelleted and suspended using B-PER Bacterial Protein Extraction Reagent (Thermo Fisher Scientific). The soluble proteins in the cell lysates were extracted first with Ni-NTA resin (New England Biolabs) and then size exclusion gel chromatography (AKTA Go, Cytiva). The purified proteins were analyzed with SDS-PAGE.

Mouse brain lysis and fractionation

Soluble and insoluble fractions of brain lysates were prepared30. Briefly, male C57BL/6 mice were intracardially perfused with 100 mM phosphate buffered saline and brains were harvested and homogenized in soluble lysis buffer (10 mM Tris-HCL, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 0.5% Sodium deoxycholate). The homogenate were centrifuged at 20,000 x g for 30 min and the supernatant (soluble fraction) was collected and stored. The pellet was washed with soluble lysis buffer and the resulting pellet was resuspended into soluble lysis buffer (10 mM Tris-HCL, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 0.5% Sodium deoxycholate) containing 1% SDS and 0.5% sodium deoxycholate. The homogenate was sonicated for 10 s (on 2 min, off 3 min) and centrifuged at 20,000 g for 30 min to collect the insoluble fraction.

Immunoblot analysis

Samples were separated on Native-PAGE Bis-Tris Gels (Thermo Fisher Scientific) and transferred to PVDF membranes (Bio-Rad). The membranes were blocked with 5% BSA in TBST (Tris-buffered saline-Tween 20) overnight at 4 °C. Then the membranes were incubated with purified MBP-PFPNB2-FLAG (~4 μg/ml) or mouse anti-α-syn mAb (1:2000 dilution, BD Biosciences, Cat no. 610787) in TBST with 3% Bovine Serum Albumin overnight at 4 °C. Mouse anti-FLAG-HP antibody (1:5000 dilution, Sigma-Aldrich, Cat no. A8592) or anti-mouse IgG-HP (1:5000 dilution, GE Healthcare, Cat no. NA931) were used as secondary antibody followed by incubation with SuperSignal West Pico Plus Chemiluminescent substrate (Thermo Fisher Scientific). The images were acquired and processed with ImageQuant LAS 4000mini scanner (GE Healthcare Life Sciences) and Adobe Photoshop (GE Healthcare Life Sciences). The soluble and insoluble fractions of brain lysates were resolved on 15% Tris-glycine gel and transferred to PVDF membranes for analysis with rabbit anti-α-syn
ELISA. To determine the EC50 of PFFNB2 (in Fig. 2b), 300 ng of α-syn PFF or monomers were diluted in 100 µl of coating buffer (0.2 M sodium carbonate buffer pH 9.4). α-Syn PFF or monomers were plated on 96-well plates (Greiner Bio-One) (100 µl/well) followed by shaking at 200 r.p.m. at 4 °C overnight. Wells incubated with coating buffer served as the blank. The next day, all treatments were done at RT, with 200 r.p.m. shaking on a microplate shaker. First, each well was decanted and washed with 200 µl washing buffer (25 mM Tris, 150 mM NaCl; Tween 0.05%, pH 7.2) for 3 min. The washing step was repeated three times. Then the wells were blocked with SuperBlock blocking buffer containing 0.05% Tween-20 (Thermo Fisher Scientific) for 30 min, followed by incubation with purified recombinant MBP-PFFNB2-FLAG protein in SuperBlock at concentrations of 3.3, 33.3, 66.7, 133.3, 266.7, 666.7 µg/ml and 1333.3 µg/ml. The wells were then washed three times before incubation with mouse-anti-FLAG antibody (Sigma-Aldrich, 1:1000 dilution, Cat no. F3165) in SuperBlock for 1 h. Lastly, the wells were washed and incubated with Goat anti-mouse IgG-HRP antibody (Thermo Fisher Scientific, 1:5000 dilution, Cat no. 31430) in SuperBlock for 1 h. Lastly, the wells were washed and incubated with Goat anti-mouse IgG-HRP antibody (Thermo Fisher Scientific, 1:5000 dilution, Cat no. 31430) in SuperBlock for 1 h. 100 µl of TMB substrate (Thermo Fisher Scientific) was added to each well. The signal was detected using SuperSignal ELISA Pico chemiluminescent substrate (Thermo Fisher Scientific). The absorbance of processed TMB substrate at 450 nm was quantified with a microplate reader (BioTek Cytation 5) and Gen5 software. Similar procedures were applied to acquire data in Fig. 2e, Supplementary Fig. 7a with a microplate reader (BioTek Cytation 5) and Gen5 software. The absorbance of processed TMB substrate at 450 nm was quantified with a microplate reader (BioTek Cytation 5) and Gen5 software. Similar procedures were applied to acquire data in Fig. 2e, Supplementary Fig. 7a with a microplate reader (BioTek Cytation 5) and Gen5 software. The absorbance of processed TMB substrate at 450 nm was quantified with a microplate reader (BioTek Cytation 5) and Gen5 software. Similar procedures were applied to acquire data in Fig. 2e, Supplementary Fig. 7a with a microplate reader (BioTek Cytation 5) and Gen5 software. The absorbance of processed TMB substrate at 450 nm was quantified with a microplate reader (BioTek Cytation 5) and Gen5 software. Similar procedures were applied to acquire data in Fig. 2e, Supplementary Fig. 7a with a microplate reader (BioTek Cytation 5) and Gen5 software. The absorbance of processed TMB substrate at 450 nm was quantified with a microplate reader (BioTek Cytation 5) and Gen5 software. Similar procedures were applied to acquire data in Fig. 2e, Supplementary Fig. 7a with a microplate reader (BioTek Cytation 5) and Gen5 software. The absorbance of processed TMB substrate at 450 nm was quantified with a microplate reader (BioTek Cytation 5) and Gen5 software. Similar procedures were applied to acquire data in Fig. 2e, Supplementary Fig. 7a with a microplate reader (BioTek Cytation 5) and Gen5 software. The absorbance of processed TMB substrate at 450 nm was quantified with a microplate reader (BioTek Cytation 5) and Gen5 software. Similar procedures were applied to acquire data in Fig. 2e, Supplementary Fig. 7a with a microplate reader (BioTek Cytation 5) and Gen5 software.
Dynamic light scattering (DLS) analysis. DLS experiments were performed to study the changes in the mean diameter of α-syn fibrils in the presence and absence of PFFN2. 10 μl of fibrils were mixed with 990 μl of filtered PBS. Measurements were performed in Zetasizer Nano-ZS from Malvern Instruments with He-Ne laser. Each sample was measured in single-use polystyrene semi-commercial disposable cuvettes (Fisher Scientific, Loughborough, The Netherlands) with a path length of 1 cm. The cell holder was maintained at 25 °C for all measurements. For each sample, 10 runs were performed, with three repetitions. Data were processed using the Malvern Zetasizer Software. The error bars displayed on the DLS graphs were obtained by the SD of measurements in triplicates.

Circular dichroism (CD) spectroscopy. The far-UV CD spectra were recorded from 195–250 nm using an Aviv model 420 spectrometer (Aviv Biomedical, Inc, Lakewood, NJ, USA). CD spectra were collected in a 1 mm path length cuvette at 25 °C with the data pitch of 1 nm. For all spectra, an average of three scans was obtained. Smoothing of CD data was done by keeping points of the window 3° for removing noise from signals. The secondary structural changes in α-syn fibrils with PFFN2 were predicted based on its negative peak at 218 nm and positive peak at 195 nm emission wavelength using a Varioskan lux (Thermo scientific). α-Syn fibrils were first generated by incubation of 2 mg/ml α-syn in PBS, pH 7.4 at 37 °C for 4 days at 1000 r.p.m. For in vitro dissociation assay, 5 μM of α-syn fibrils were incubated with 250 nM MBP or MBP-PFFN2 for up to 15 days at 37 °C for 7 days at 1000 r.p.m. The reaction mixture was taken out from an incubation solution at a different time interval (0, 2, 4, 8, 12, 15 days). 20 μM final concentration of ThT was added into clear bottom 96 well microplate (Invitrogen, Cat no. M33089), and different time interval (0, 2, 4, 8, 12, 15 days). 20 μl of samples were diluted ten times and adsorbed on 400 mesh carbon-coated copper reader. All measurements were performed in triplicate. All ThT fluorescence results were normalized with the ThT signal at zero time. The morphological analysis of PFFN2 mediated α-syn fibril dissociation was analyzed with TEM. Briefly, 10 μl of samples were diluted ten times and adsorbed on 400 mesh carbon-coated copper grids (Ted Pella, Inc, USA). Excess liquid was adsorbed by lint-free tissue paper and incubated for 1 min, following negatively stained with 2% uranyl acetate for 1 min. After the film dried, images were captured via TEM (Hitachi) with accelerating voltage at 80 kV. The length of the fibrils was measured using the open-source image processing program ImageJ.

Data availability
PDB-4LDE and PDB-3KIK cited in this study are accessible from Protein Data Bank. All the data supporting the findings of this study are available within the paper and its supplementary information files. Source data are provided with this paper. All the DNA constructs used in this study are available upon request to the corresponding author.

Received: 31 May 2021; Accepted: 1 July 2022; Published online: 19 July 2022

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Competing interests
The authors declare the following competing interests: patent application filed by W.W., X.M., Y.B., Y.L., and R.K. with title 'Compositions and methods for treating alpha-synucleinopathies.' U.S. Provisional Patent Application No. 63/222,141, filed Jul 15, 2021. Applicants: The Regents of the University of Michigan and The Johns Hopkins University. Patent pending. Some aspects of this paper is included in the patent such as disulfide-bond free PFFNB, AAV delivery of PFFNB2 to treat Parkinson’s Disease. All other authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-022-31787-2.

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Peer review information Nature Communications thanks Serge Muyldermans, Daniel Otzen and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

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