A nanobody-based fluorescent reporter reveals human α-synuclein in the cell cytosol

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Aggregation and spreading of α-Synuclein (αSyn) are hallmarks of several neurodegenerative diseases, thus monitoring human αSyn (hαSyn) in animal models or cell cultures is vital for the field. However, the detection of native hαSyn in such systems is challenging. We show that the nanobody NbSyn87, previously described to bind hαSyn, also shows cross-reactivity for the proteasomal subunit Rpn10. As such, when the NbSyn87 is expressed in the absence of hαSyn, it is continuously degraded by the proteasome, while it is stabilized when it binds to hαSyn. Here, we exploit this feature to design a new Fluorescent Reporter for hαSyn (FluoReSyn) by fusing NbSyn87 to fluorescent proteins, which results in fluorescence signal fluctuations depending on the presence and amounts of intracellular hαSyn. We characterize this biosensor in cells and tissues to finally reveal the presence of transmittable αSyn in human cerebrospinal fluid, demonstrating the potential of FluoReSyn for clinical research and diagnostics.
α-synuclein (αSyn) aggregation disorders including Parkinson’s disease (PD), Lewy Body dementia, and multiple system atrophy, are a group of disorders characterized by the pathological occurrence of intracellular inclusions filled with insoluble aggregates of αSyn. These aggregations can be found in various cell types and regions of the central nervous system. αSyn is a 140 amino-acid long protein that is highly enriched in presynaptic nerve terminals. Despite extensive efforts, the central molecular and physiological role of αSyn remains to be determined. Emerging evidence, however, suggests αSyn to be involved in the regulation and possible maturation of synaptic vesicles, particularly via its role in the assembly of N-ethylmaleimide-sensitive factor attachment receptor complexes; key players in synaptic vesicle docking and fusion with the presynaptic membrane. In addition, other functions have been attributed to αSyn including the regulation of glucose levels, serving as an antioxidant or a chaperone, and suppressing apoptosis in dopaminergic neurons.

Under normal physiological conditions, αSyn is found as a monomeric protein existing in equilibrium between an intrinsically disordered form in the cytosol and a membrane bound, helical form. Although still subjected to debate, observation of helical αSyn tetramers has also been reported under certain native environments.

It is widely accepted that arrangements of αSyn monomers into small-to-intermediate oligomeric or larger insoluble assemblies is associated with pathogenesis of αSyn aggregation disorders. Importantly, there is growing evidence for prion-like cell-to-cell transmission properties of different αSyn conformations where “toxic” αSyn species with seeding properties are suggested to get internalized by a host cell and trigger the aggregation of endogenous αSyn, an ambiguous notion. These mainly concern pinpointing of the disease underlying αSyn species (e.g., oligomers vs fibrils, phosphorylated αSyn, etc.), mechanistic steps of the cell-to-cell transmission paradigm and the downstream effects of pathological αSyn accumulation that eventually lead to neuronal injury.

Development of tools that can be used for reliable and reproducible detection and tracking of αSyn in vivo is a very important goal for deciphering molecular mechanisms of disease pathogenesis. A common strategy employed in studies addressing the cellular uptake, seeding, and transmission phenomena involve manipulation of the αSyn protein itself. Accordingly, αSyn is either pre-labeled with fluorescent dyes, or reconstituted as expressed a fusion protein. Concerning the latter, several different approaches are described which include tagging αSyn with a small epitope tag (e.g., myc, V5, etc.) for subsequent antibody-mediated immunodetection, or reconstituted as an alpha fusion protein. Concerning the latter, several different approaches are described which include tagging αSyn with a small epitope tag (e.g., myc, V5, etc.) for subsequent antibody-mediated immunodetection, or reconstituted as an alpha fusion protein (e.g., YFP-αSyn, DsRed-αSyn, etc.) for direct visualization or employing the protein complementation assay principles whereby αSyn is tagged with either N-terminal or C-terminal portions of a split fluorescent or bio-luminescent reporter. Although they have proven to be very helpful in advancing the knowledge about synuclein-related pathologies, one common caveat in such approaches is their limited potential in recapitulating the natural behavior of untagged native αSyn. For example, it has been shown that fluorescent protein fusions might result in the wrong localization of the studied protein or some organic dyes have a propensity to bind to biological membranes. Considering that a critical role is attributed to the lipid interacting properties of αSyn in its pathological behavior, the employment of untagged or native forms of αSyn may be a more-appropriate strategy when attempting to investigate the molecular mechanisms of αSyn pathology. The detection of untagged, native, or endogenous αSyn in vitro or in vivo model systems requires the use of other tools. Certain dyes such as Thioflavin S, derived from the histological dye Congo red, have been commonly utilized for detecting mature protein aggregates in vivo models of αSyn propagation and PD pathology. However, these dyes have the disadvantage of binding any protein capable of taking an amyloid conformation and thus do not provide an exclusive labeling of αSyn. In contrast, antibody-mediated immunodetection is a conventional approach for specific detection of αSyn. Hereby, a large number of αSyn-targeting antibodies, including conformation specific-ones and engineered antibody fragments are commonly utilized in a variety of applications. In the recent years, camelid-originated single-domain antibodies, also termed nanobodies, emerged as a promising alternative as they confer several advantages including recombinant production, enhanced tissue penetration, small size (ideal for super-resolution microscopy), and the ability to be expressed as intrabodies in mammalian cells. The latter feature is particularly attractive as it confers the ability to track and manipulate specific target proteins in living cells. Nanobodies are increasingly being used for investigation of diseases associated with protein misfolding and aggregation. Recently, two nanobodies against αSyn have been identified, NbSyn2 (ref. 39) and NbSyn87 (ref. 40), each binding distinct epitopes at the C-terminal region of αSyn. These have been biochemically well characterized and assessed for their potential therapeutic use by several studies.

In this study, we make use of a previously unknown feature of NbSyn87, namely its weak affinity to the 26 S proteasomal subunit Rpn10, which is located at the entrance of the proteasome and functions as a receptor for poly-ubiquitinated proteins that will undergo proteolysis. We show that this interaction is sufficient to drive a continuous proteasome-mediated degradation of intracellularly expressed NbSyn87 unless it is bound to haSyn. The presence of haSyn, on the other hand, results in the avoidance of the degradation of NbSyn87 by formation of a stabilized NbSyn87:haSyn complex. Accordingly, we exploit this mechanism to create and characterize a nanobody-based Fluorescent Reporter for human αSyn (FluoReSyn), which is able to report the presence or absence of cytosolic haSyn. Our results demonstrate the unique ability of FluoReSyn to report small amounts of cytosolic haSyn in cell lines and transduced primary rat hippocampal neurons. Expression of FluoReSyn in olfactory system of Xenopus laevis also shows its ability to operate and report haSyn in vivo. Furthermore, cells stably expressing FluoReSyn (Reporter-cells) report the presence of haSyn in their cytoplasm after exposing them to human cerebrospinal fluid (CSF) samples. The results presented here indicate that this biosensor is a valuable instrument for studying the transmission of αSyn and has great potential to be further optimized and validated as a diagnostic tool for αSyn aggregation disorders.

**Results**

**Reporting the presence of untagged haSyn in the cytoplasm.** We had previously observed in cells transiently expressing the NbSyn87 (ref. 40) fused to EGFP that their fluorescent signal correlated with the presence or absence of haSyn (Fig. 1a). In an attempt to comprehend this observation, we used the Basic Local Alignment Search Tool (BLAST) to find out if the described haSyn epitope sequence (VDPDNEAYEMPS) was recognized by the NbSyn87 might be present in another endogenous protein. The BLAST result showed a high identity (Fig. 1b) to a subunit of the 26 S proteasome (the 26 S proteasome non-ATPase regulatory subunit four homolog, also known as Rpn10). This
protein resides at the entrance of the 26 S proteasome and has an important role in the recognition of poly-ubiquitinated proteins that will be processed in the ubiquitin proteasome-mediated proteolysis (UPP). Using a dot-blot assay with purified Rpn10 and hαSyn, we were able to verify that the NbSyn87 can bind weakly to human Rpn10 (Supplementary Fig. 1a, b). Therefore, taking our results together, we hypothesized that the degradation of NbSyn87 in the absence of hαSyn may be mediated by its weak but continuous recruitment to the proteasome upon binding the endogenous Rpn10 (Fig. 1c).

In order to test our hypothesis and further characterize NbSyn87 in terms of this special feature, we decided to generate a stable cell line expressing NbSyn87 fused to EGFP and have mCherry signal as an expression reporter using the self-cleavable domain T2A (Fig. 1d). In addition, we added a NLS sequence at the C-terminus of NbSyn87-EGFP to concentrate the EGFP signal in the nucleus and gain sensitivity during imaging. The expression of this protein-chimera was controlled under the tetracycline-inducible promoter system (TetOn). Optimal induction duration to maximize the NbSyn87-EGFP-NLS expression was determined by analyzing the mCherry reporter signal (Supplementary Fig. 1c). In line with our original observations, we detected a clear nuclear EGFP signal in doxycycline-induced NbSyn87-EGFP-NLS stable cell line when we transiently transfected them with wild type and untagged hαSyn (Fig. 1d).

The strength of this effect was dependent on the amount of hαSyn.

**Fig. 1** αSyn-dependent accumulation of FluoReSyn in HEK293 cells. 

- **a** Schematic representation of the initial observation of cells transiently expressing NbSyn87-EGFP alone (left) or together with hαSyn (right). Although the former group of cells showed minimal fluorescence, the latter presented with a strong EGFP signal. 
- **b** Alignment of the amino-acid sequence from hαSyn and Rpn10 across different species. Rpn10 residues similar to hαSyn in the putative epitope are displayed in red. 
- **c** Schematic representation of the proposed mechanism of degradation versus stabilization of SynNb87-EGFP in the presence or absence of hαSyn. The following Protein Data Bank (PDB) accession numbers were used and modified to assemble the schematic: 2Y0G (EGFP), 2 × 6 M (Nanobody), 1XQ8 (hαSyn), 6MSK (Proteasome). 
- **d** Schemes of constructs used to transfect HEK293 cells (left). For the stably transfected cells, a tetracycline-inducible promoter (TetON) was used, followed by a mCherry reporter sequence, a cleavable T2A sequence, and FluoReSyn made of NbSyn87, EGFP and a nuclear localization signal (NLS) sequence. Transient expression of untagged wild-type human hαSyn was driven by a plasmid containing a cytomegalovirus (CMV) promotor. Equally scaled, representative images of doxycycline-induced Reporter-cells (right). Cells were either mock transfected (control) or transiently transfected with the hαSyn expression constructs. Scale bar represents 10 µm.
- **e** Quantitative analysis of EGFP-positive cells transfected with variable quantities of hαSyn plasmid. Per replication and condition >1000 cells were analyzed. Error bars represent the SEM from three independent experiments (n = 3). 
- **f** Western blot analysis of lysates from Reporter-cells transfected with variable quantities of hαSyn. Immunoblotted (IB) anti-EGFP represents FluoReSyn. Loading control is IB Beta-Actin. Full length blots are displayed in Supplementary Fig. 1d. Source data is available as a Source data file.
Fig. 2 The FluoReSyn is rapidly degraded by the proteasome machinery. a Equally scaled, representative epifluorescence images of MG132 treated and untreated Reporter-cells which were fixed 2, 4, 8, or 16 h post treatment. The FluoReSyn accumulates in response to prolonged MG132 treatment of Reporter-cells as revealed by the increasing nuclear EGFP intensity. Reporter-cells untreated with MG132 yielded virtually no EGFP signal even after 16 h. Scale bar represents 20 µm. b Quantitative analyses of FluoReSyn signal (EGFP) in arbitrary units (a.u.) when Reporter-cells were treated or untreated with MG132. Per replication and condition more than 540 cells were analyzed. c FluoReSyn signal normalized by mCherry signal (EGFP to mCherry signal intensity). Error bars represent the SEM from three independent experiments (n = 3). Source data is available as a Source data file.

Proteasome-mediated degradation of FluoReSyn. In order to validate our proposed mechanism for the degradation of FluoReSyn, we treated doxycycline-induced Reporter-cells (without expressing haSyn) over different time periods with the MG132 proteasome inhibitor. MG132 concentration was optimized to provide a strong gain of EGFP and mCherry signal while minimizing its adverse toxic effects on the Reporter-cells (Supplementary Fig. 2). The results clearly showed that, following treatment with 1 µM of MG132, FluoReSyn started to accumulate in the nuclei of Reporter-cells already from the 4th h on without the presence of haSyn (Fig. 2a, b). Induced Reporter-cells untreated with MG132, showed in contrast virtually no FluoReSyn signal throughout the whole duration (Fig. 2a, b). These suggested that the reporter is regularly produced but also continuously degraded via the proteasome in the cell (fast turnover) under normal conditions. The mCherry and FluoReSyn are produced from a single mRNA, making a fusion protein that is efficiently cleaved at the T2A domain17. It is expected that this strategy results in stoichiometric amounts of FluoReSyn and mCherry, which allowed us to normalize the signal of FluoReSyn with the mCherry signal. In induced Reporter-cells untreated with MG132, the relation of FluoReSyn to mCherry signal was maintained during the 16 h experiments (Fig. 2c). However, when using MG132, already after 2 h, the ratio between FluoReSyn signal and mCherry doubled and kept growing over time, showing that the accumulation of FluoReSyn exceeds that of mCherry and that the former gets particularly enriched in the Reporter-cells when the proteasome machinery is inhibited (Fig. 2c). This observation suggests that the proteasomal degradation is particularly accelerated for FluoReSyn under normal conditions (uninhibited proteasome machinery), and thus substantiates our proposition that the nanobody NbSyn87 specifically targets the proteasome and is degraded by it.

FluoReSyn reports haSyn in vivo. As Rpn10 is a well-conserved protein across different species (Fig. 1b), we presumed that our proposed mechanism should also operate in a model organism that lacks endogenous haSyn and possesses the conserved Rpn10 epitope recognized by NbSyn87. Accordingly, we chose to validate the proposed mechanism in living Xenopus laevis tadpoles, a time- and cost-efficient model organism.48 Xenopus laevis expresses endogenously the same Rpn10 epitope needed for FluoReSyn to operate and offers a straightforward electroporation-mediated approach for gene delivery to the olfactory receptor neurons in the olfactory epithelium of living animals49 (Fig. 3a, b). Accordingly, the plasmid encoding for FluoReSyn was electroporated either alone or together with a plasmid encoding for haSyn fused to mCherry into the right or left nostrils of anesthetized tadpoles, respectively. By in vivo imaging of tadpoles with two-photon microscopy we observed many GFP-positive nuclei co-localizing with the mCherry signal in the left nostrils of the animals (Fig. 3c, e, g, high magnification example on 3j), which was clearly in contrast with the right nostrils presenting seldom any GFP-positive nucleus (Fig. 3d, f, h). Analysis of the distribution of GFP fluorescence intensity further confirmed the clear distinction between left and right nostrils (Fig. 3i). Altogether, these data further validated our previous conclusions by demonstrating that the same mechanism seems to be operational in vivo whereby FluoReSyn is stabilized upon haSyn binding and cleared from the cell by proteasome-mediated degradation in the absence of this interaction.
The specificity of the FluoReSyn for hαSyn. As a next step, we assessed whether FluoReSyn binds specifically to human αSyn or has an affinity towards other synuclein species. For this purpose, Reporter-cells were transiently transfected with plasmids encoding for hαSyn, human βSyn (hβSyn), or rat αSyn (rαSyn). We analyzed the proportion of cells with a positive EGFP signal by epifluorescence microscopy (Fig. 4). The expressions of the different synuclein species were controlled by immunostaining with an antibody that recognizes all transfected variants. Although no significant differences were observed among the different synuclein species in terms of their expression (Supplementary Fig. 3a), only in hαSyn-transfected Reporter-cells a substantial proportion of the cells were positive for the FluoReSyn signal (Fig. 4b). This result, therefore, proposes that FluoReSyn binds primarily to human αSyn. It is important to note that the epitope sequence of the hαSyn recognized by NbSny87 has a great identity with the rαSyn sequence with the epitopes differing from each other only by two residues (Supplementary Fig. 3b). It is noteworthy, that FluoReSyn seems to bind with higher affinity to Rpn10 than to the rαSyn.

Detection of hαSyn uptake from the culture medium. In relation to the pathological αSyn transmission phenomenon, we evaluated the ability of FluoReSyn to report the entry of foreign hαSyn into the cellular cytosol. For this purpose, purified recombinant hαSyn was administered to the culture mediums of induced Reporter-cells either on its own or in a mixed state with a...
cationic liposome reagent (i.e., RNAiMAX) to enhance the protein uptake as suggested previously. We observed that, if hαSyn was associated with RNAiMAX, it manages to get into the cytosol and stabilize FluoReSyn as evidenced by the accumulated EGFP signal in the nucleus (Fig. 5). On the other hand, administering hßSyn associated with RNAiMAX or just naked hαSyn generated only background levels (i.e., induced Reporter-cells not exposed to anything) of EGFP-positive cells, which suggests that hαSyn on its own failed to go across the cell plasma membrane efficiently.

Subsequently, we setup a co-culture of Reporter-cells and HEK293 cells stably expressing untagged and wild-type hαSyn under an inducible promotor (TetON-hαSyn cells; Supplementary Fig. 4) to investigate if mammalian produced hαSyn was able to leave the cells and then enter into the neighboring Reporter-cells. As a control condition, Reporter-cells were co-cultured with wild-type HEK293 cells, which lack endogenous hαSyn expression. The analysis of co-cultures maintained for 2–5 days revealed no significant differences between the test and control groups failing to confirm the occurrence of any hαSyn transmission events between the hαSyn producing cells and the Reporter-cells (Supplementary Fig. 5).

Uptake of hαSyn from the culture medium by primary neurons. As a next step, we assessed the functionality of FluoReSyn in neurons, by investigating whether it can report the cytosolic presence of hαSyn, after adding recombinant hαSyn to the medium of primary neuronal cultures. For this purpose, we prepared rat hippocampal neuron cultures and infected them at DIV ~14 with an adeno-associated virus (AAV) encoding for NbSyn87-mCherry-NLS (a red version of FluoReSyn). We administered both the monomeric and large fibrillar forms of hαSyn extracellularly to the culture medium of FluoReSyn-transduced hippocampal neurons. The analysis suggested that naked monomeric hαSyn can reach the cytosol of neurons and can produce a detectable FluoReSyn signal in their nuclei.

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**Fig. 4 The FluoReSyn is a specific sensor for human αSyn.** a Equally scaled representative epifluorescence images of Reporter-cells transfected with hαSyn, hßSyn, or rαSyn expression constructs. Scale bar represents 10 µm. b Quantification of the proportion of EGFP-positive cells. Error bars represent the SEM from the independent experiments (n = 3). Per replication and condition >6000 cells were analyzed. Ordinary one-way ANOVA resulted in ****p < 0.0001. Source data is available as a Source data file.

**Fig. 5 Transmission of recombinant hαSyn into Reporter-cells.** a Equally scaled representative epifluorescence images of Reporter-cells after incubation for 14 h with recombinant hαSyn monomers added to the culture medium. Monomers were either administered alone (hαSyn) or pre-mixed with the commercial cationic lipid mixture RNAiMAX (hαSyn + iMAX). hßSyn + iMAX was used as negative control. Scale bar represents 20 µm. b Quantification of the percentage of EGFP-positive Reporter-cells. Error bars represent the SEM from three independent experiments (n = 3). Per replication and condition >2300 cells were analyzed. One-way ANOVA with multiple comparison Tukey’s post hoc test **p < 0.01, ns: not significant. Source data is available as a Source data file.
(Fig. 6a, b). This was in contrast to the large haSyn fibrils, which failed to induce FluorReSyn signals above that of the control neurons (Fig. 6b).

Based on our previous observations with the Reporter-cell line, where we also administered the synuclein species pre-mixed with the cationic lipid RNAiMAX (Fig. 6a, c). The results revealed a higher proportion of neurons with FluorReSyn positive nuclei when haSyn monomers were pre-mixed with the cationic lipids from RNAiMAX and added to the culture medium (Fig. 6a, c). It was interesting to observe that both, the naked monomers (Fig. 6b) and those complexed with lipids (Fig. 6c) were able to go across the neuron plasma membranes. This is clearly distinctive to the HEK293-based Reporter-cells, which displayed internalization of haSyn only when complexed to cationic lipids (Fig. 5b).

The use of cationic lipids did not influence the outcome for fibrils. Similar to the previous observation with uncoated fibrils (Fig. 6b), no FluorReSyn signal was detected in neurons exposed to RNAiMAX-haSyn fibril complexes (Fig. 6c). Importantly, we showed that NbSyn87 can clearly bind to our in vitro generated fibrils (Fig. 6d), which also confirmed previous reports.40 Thereby, we can exclude that the negative observations were owing to the inability of FluorReSyn to detect our fibrils (Fig. 6d), but are most likely a proof that fibrils have not succeeded in entering the cytoplasm of neurons.

Detection of haSyn in CSF samples. Confident that FluorReSyn can reliably report cytosolic haSyn, we decided to evaluate the ability of the Reporter-cells to detect haSyn species in human-originated biological samples. The main rationale here was to explore the potential usability of this cellular system for future diagnostic purposes. Therefore, we exposed induced Reporter-cells cultured on a 96-well plate to CSF samples from 42 individuals diagnosed with variable neurological disorders that were unrelated to αSyn aggregation disorders. Reporter-cells not exposed to CSF, as well as wild-type HEK293 cells exposed and not exposed to CSF, were used as negative controls. Cells were fixed 24 h post treatment and dozens of images of randomized and non-overlapping locations were automatically acquired per well (Fig. 7a). Data analysis showed a small but clear trend of positive Reporter-cells that were incubated with human CSF (Fig. 7b). We also correlated the specific clinical diagnosis of each patient and the total αSyn concentrations in their CSF to the Reporter-cells activity, as displayed in Supplementary Fig. 6. As controls, Reporter-cells not exposed to CSF or wild-type HEK293 cells (not producing any FluorReSyn chimera) incubated with or without CSF, all displayed background levels of positive signal (Fig. 7b). This observation reassured that the small percentage of positive Reporter-cells observed upon CSF exposure is a specific and trustworthy response to some forms of haSyn present in human CSF that can reach the cytosol of our model Reporter-cells. Altogether, this result not only suggests that human CSF may contain a transmittable form of αSyn that is capable of entering into cells but also opens the possibility to optimize this system for generating a unique cell-based diagnostic tool for αSyn aggregation disorders.

Discussion
Here, we present for the first time a unique feature of the NbSyn87, namely, its natural tendency to bind to the proteasomal subunit Rpn10 that leads to its own degradation and eventual clearance from the cell cytoplasm in the absence of haSyn. This special feature allowed us to develop FluorReSyn, the nanobody-based fluorescent reporter for haSyn, which is capable of detecting the presence or absence of haSyn in the cellular cytoplasm. Furthermore, our Reporter-cells stably expressing FluorReSyn were able to detect a transmittable form of haSyn present in human CSF.
Uptake of toxic αSyn species by cells and their subsequent intracellular trafficking is a crucial part of the proposed αSyn transmission pathology. Accordingly, we first characterized and assessed the potential of FluoReSyn Reporter-cells to be used as a research tool for investigating the transmission phenomenon. Our results after introducing recombinant αSyn to the culture medium suggested that the Reporter-cells could reliably report the uptake of extracellular αSyn. Similar to other reports, we did not detect direct cytoplasmic internalization of naked monomeric αSyn by the Reporter-cells (derived from HEK293 cells). We rather observed that the cytosolic uptake required assistance with lipid-based elements as also had been shown in other studies. Different from the HEK-based Reporter-cells, we observed that entry of naked recombinant αSyn monomers into primary neurons expressing FluoReSyn is more likely to occur, and lipid-based facilitating agents like the RNAiMAX make the entry process even more efficient. These observations expose the influence of the cellular context on the translocation of αSyn from the extracellular to the intracellular space (e.g., requirement of distinct receptor interactions or membrane translocators for αSyn) and can be interpreted as a reflection of the neuronal nature of αSyn transmission pathology. In line with this interpretation, we did not observe a transmission event when HEK293-based cells stably transfected with αSyn were co-cultured with the Reporter-cells. On the other hand, these transmission events might be highly dependent on the concentration of αSyn and duration of exposure. With our setup we could not employ higher concentrations of αSyn in order to avoid toxic effects to the cells and furthermore, we were limited in terms of exposure durations as a longer maintenance of dividing cells in culture became difficult after several days.

In the current study, we did not observe a positive FluoReSyn signal in transduced neurons following their exposure to fibrillar αSyn. This was not an unexpected observation since fibrils are large structures (in the µm range) that should not enter the cellular cytosol easily, especially as they are known to not form pore-like structures on membranes. Accordingly, it is probable that our observations merely reflected the inability of these bulky structures to penetrate into the neuronal cytosol.

It has been proposed that the uptake of larger arrangements such as oligomers, fibrils, or aggregated αSyn might be mediated by regular endocytosis. In this case, FluoReSyn would not detect compartmentalized αSyn in endocytosed vesicles unless αSyn finds a way to escape into the cytosol. It has recently been shown that αSyn pre-formed fibrils (pffs; <50 nm of length) that were internalized by cultured primary neurons remained confined to endo-lysosomal compartments up to 7 days, without a major escape from the endocytic pathway. Thus, even small fibrils have difficulties penetrating into the neuronal cytosol.

Finally, these results do not exclude the possibility that other types of large assemblies or fibrils do penetrate into the cell cytosol, and would therefore be detected by the sensor. This issue could be tested more thoroughly in the future. It is known that many types of fibril-like assemblies with different morphologies and structures can be obtained by changing the preparation and incubation conditions (e.g., pH, salinity, temperature, presence of modulators). Structural variabilities of recombinant fibrils were also shown to propagate to in vitro and in vivo functional properties of the formed assemblies. For example, it has been shown that upon reducing the incubation pH, the morphology of formed high-molecular weight αSyn assemblies shifted from fibrillar to more amorphous with the latter showing reduced in vitro seeding efficiency. Another study demonstrated that simply by changing from physiological salt concentrations to a lower salt condition, or by adding a chelating agent to the incubation buffer the appearance of the formed high-molecular weight assemblies of αSyn changed from cylindrical to flat. These assemblies, referred to as fibrils or ribbons, respectively, were shown not only to differ in other structural aspects but also in terms of their in vitro-, in vivo seeding, and propagation properties as well as the degrees of cellular toxicity they induced.

Our results demonstrated that the FluoReSyn works both in a cell line and primary neurons, and can report the uptake of recombinant αSyn monomers. Introducing recombinant or tissue derived αSyn extracellularly to in vitro cell cultures is a common approach for investigating the internalization and subsequent seeding activities of αSyn in the context of transmission paradigm. However, the readily available cellular models and tools are generally limited in terms of offering an unambiguous and spatially resolved discernment of internalized αSyn from the extracellularly applied αSyn seeds. Therefore, there is a necessity to develop novel tools that would enable selective visualization of internalized αSyn and deliver a quantitative characterization of αSyn uptake phenomenon. In a recent study where neuronal cultures were exposed to GFP-tagged αSyn
pre-formed fibrils (pfi's), a membrane-impermeable fluorescence quencher dye was used to exclusively quench the fluorescence of extracellular fibrils, thus enabling the selective imaging of only internalized seeds\(^5\). Similarly, we propose that, FluorReSyn is an optimal tool for this purpose as it can exclusively detect cytosolic aSyn. A major advantage of our system is that it avoids the necessity of employing tagged or covalently conjugated forms of aSyn, which as discussed previously may not recapitulate the normal behavior of native, untagged aSyn. Accordingly, provided that the nanobody epitope is exposed, FluorReSyn can be used for tracking the behavior of any endogenous species of native aSyn obtained from human materials.

Our data suggest that FluorReSyn is a useful research tool not only for neuronal cultures, but also for in vivo set-ups like in our experiments with living \(X. Laevis\). Besides, the FluorReSyn's specificity to h\(\alpha\)Syn would be of advantage when studying h\(\alpha\)Syn transmission in animal models (e.g., mice, rat) as any interference from endogenous aSyn present in rodents can be disregarded.

As a next step in further characterizing the cellular reporter system, we evaluated its ability to detect human-originated aSyn with the future perspective of developing it further into a diagnostic tool\(^62\)-\(^65\). Here, we used CSF samples from a readily available cohort of individuals (42). The results showed that our Reporter-cells could indeed detect specific species of aSyn in human CSF, which are able to enter into their cytoplasm. Considering our observations with recombinant aSyn monomers that required a lipid coating agent for entering the Reporter-cells, it is plausible that the aSyn molecules we detected in CSF samples were in vesicular structures such as exosomes as already suggested by other\(^66\),\(^67\), or were species other than monomers, such as oligomers with different proposed means of entering the cell\(^68\)-\(^71\).

The presence of aSyn in CSF can be measured biochemically (e.g., ELISA)\(^72\), and many efforts have been directed at developing assays for detection of CSF-originated aSyn as a biomarker\(^73\),\(^74\). It is important to emphasize that our system provides extra information by detecting aSyn forms that are able to get into the cytosol of cells. Therefore, it is plausible that we are detecting the species that are more prone to transmission related pathology. This is particularly relevant in the light of emerging evidence, which suggests that particular species of aSyn (e.g., oligomers, aggregates) that are associated with toxicity can serve as far-better biomarkers than total aSyn in CSF\(^74\).

Although our initial results in Fig. 1 show that the FluorReSyn signal correlates to the amount of aSyn, our Reporter-cells are not yet quantitative enough to precisely determine the concentrations of transmittable aSyn in human CSF. Nevertheless, this is a fascinating first proof-of-concept. To the best of our knowledge, our cellular haSyn reporter system is so far the sole approach developed for the detection of transmittable haSyn species present in human body fluids. We believe that major optimizations can be performed to this system to increase its sensitivity and accuracy. These enhancements might include employment of a faster maturing and brighter EGFP variant, tuning of the FluorReSyn sensitivity biochemically, and destabilizing the antigen-unbound NbSyn87 (ref.\(^75\)). Once these optimizations are performed and validated, a logical next step would be the generation of a knock-in (KI) mice, to thus generate an animal model for studying the mechanical and molecular aspects of aSyn transmission. Using primary cells from these KI mice, it will then be possible to establish a neuron-based reporter system, which would presumably hold more promise in a diagnostic context. We hope such efforts might help to achieve more sensitive and quantitative read-outs, which would in turn pave the way for the development of an accurate and reliable diagnostic or prognostic tool for aSyn associated disorders.

### Methods

#### Plasmon transmission and virus infections

Transfections with pcDNA 3.1 (+) vectors (THERMO FISHER SCIENTIFIC) encoding for haSyn (accession number NM_000345.4), human β-actin (h\(\beta\)-actin; accession number NM_001001502.3) or rat aSyn (raSyn; accession number NM_019169.2) sequences were performed with Lipofectamine 2000 (Invitrogen, THERMO FISHER SCIENTIFIC) and Opti-MEM I Reduced Serum Medium (Opti-MEM from Gibco, THERMO FISHER SCIENTIFIC, Waltham, MA, USA) according to manufacturer’s instructions. Transfected cells were typically used after 48 h. The AAV coding for NbSyn87 fused to mCherry and nuclear localization signal (NLS) sequences (NbSyn87-mCherry-NLS) was kindly provided by Dr. Sebastian Kügler, Department of Neurology, Viral Vectors Laboratory, University Medical Center Göttingen, Germany.

#### Protein purification

Rpn10, haSyn, h\(\alpha\)Syn were produced using NEB Express Competent Escherichia coli (New England Biolabs Inc., Ipswich, MA, USA). Bacteria were grown overnight with the plasmid of interest in Lysogeny Broth (Sigma-Aldrich) and the respective antibiotics. Next day, cells were further cultured in Terrific Broth (Sigma-Aldrich) and antibiotics until OD was ~2–3 at 37 °C and induced with 0.8 mM IPTG for 4 h. After adding 5 mM EDTA, cells were harvested by centrifugation at ~3,000 × g for 30 min at 10 °C and frozen at ~20 °C until further processing. Pellets were resuspended on ice with 1 mM DTT, 25 mM Imidazole, and 1 mM PMSF in a His-binding buffer (50 mM HEPES pH 8.0, 500 mM NaCl, 5 mM MgCl\(_2\), and 10% glycerol) and bacteria were lysed by sonication on ice. Cell debris was separated by centrifugation at ~13,000 × g for 1 h at 1 °C. The supernatant was incubated with 2 ml of pre-equilibrated Ni-beads (Complete His-Tag Purification Resin, Roche, Switzerland) for 1 h and then transferred to a column to be washed consecutively with His-binding buffer (50 mM HEPES pH 8.0, 1000 mM NaCl, 10 mM MgCl\(_2\), 25 mM Imidazole and 5% glycerol) and low concentration buffer (50 mM HEPES pH 7.5, 500 mM NaCl, 5 mM MgCl\(_2\), 25 mM Imidazole, 5% glycerol) with each of them containing 25 mM Imidazole. Finally, protein was eluted by 500 mM Imidazole and His-Tag was cleaved with SUMO protease and removed by reverse binding to nickel beads. Pure proteins were desalted into PBS, purity was confirmed by polyacrylamide gels (PAGE) and concentrations were determined using nanodrop spectrophotometer considering the protein molecular weight and extinction coefficient.

#### NbSyn87 coupling to Alexa647 fluorophore

NbSyn87 protein was obtained from a custom production service offered by NanoTag Biotechnologies GmbH (Göttingen, Germany), and the nanobody was equipped with one ectopic cysteine at its C-terminal. 1 mg of pure NbSyn87 was reduced by adding TCEP (Sigma-Aldrich) to a final concentration of 5 mM for 1 h. The reduced sample was then desalted using gravity columns Nap10 (GE Healthcare Life Sciences) in nitrogen bubbled PBS (pH 7.4) and immediately added to five molar excess of maleimide-functionalized Alexa647 (Thermo Scientific) for 1 h. Excess of free dye was separated from the conjugated nanobody with an Akta HPLC equipped with a Superdex 75 increase column (GE Healthcare Life Sciences).

#### Generation of haSyn fibrils

Monomeric haSyn was expressed recombinantly in E. coli BL21 (DE3) and purified by anion exchange and size-exclusion chromatography\(^58\). Cell lysis was conducted by French press (Avenin Emulsiflex-C3) with lysis buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA, 1 mM PMSF), 20 mM per 1 L of cell culture. The lysate was then heated up to 96 °C for 30 min in a water bath and centrifuged afterwards for 30 min at 4 °C with 22,000 × g (Beckman Coulter, JA-25-5 rotor). Streptomycin was added at a final concentration of 10 mg/mL and incubated for 15 min. Following another centrifugation step, the soluble protein was precipitated by ammonium sulfate for 15 min at 4 °C. Protein pellet was dialyzed overnight against 25 mM Tris-HCl, pH 7.7 and loaded on an anion exchange column (GE Healthcare, Mono Q 5/50 GL). Protein was eluted with 300 mM NaCl. Monomeric haSyn purity and was achieved by size-exclusion chromatography (GE Healthcare, Superdex 75 10/300 GL) using 50 mM HEPES, pH 7.4, 100 mM NaCl, and 0.02% NaN\(_3\). The protein was sterile filtered (0.22-μm) and stored at 1 mM at ~80 °C. Monomeric haSyn in 50 mM HEPES, pH 7.4, 0.2% NaN\(_3\), and 0.02% cationic detergent was centrifuged at 84,000 × g at 4 °C. The supernatant was filtrated through 0.22 μm ULTRAFREE-MC centrifugal filter units (Millipore) and adjusted to 0.25 mM protein concentration. Aggregation was performed for 10 days at 37 °C with constant stirring at 200 rpm. Progress of fibril formation was monitored with a Thioflavin T fluorescence assay\(^59\). The fibrils were finally collected by ultracentrifugation at 20 °C, washed twice with 50 mM HEPES, pH 7.4, 100 mM NaCl and quantified by subtracting the amount of monomeric haSyn in the supernatant from the total protein used for aggregation. Prior to their use, fibrils were resuspended in the washing buffer at 0.1 mM protein concentration.

#### Proteasome inhibition

MG132 (Sigma-Aldrich, St. Louis, MO, USA) was administered at different concentrations (Supplementary Fig. 2) or at 1 μM to the culture medium for different time intervals (Fig. 2).

#### CSF samples

Study participants consisted of individuals who were in treatment at the Paracelsus Elena Klinik, Kassel, Germany, and had been diagnosed with a...
variety of neurological disorders related to αSyn aggregation disorders. The study cohort consisted of 23 females and 19 male individuals with a mean age of 70.95 ± 1.51. For a detailed presentation of demographic and clinicalfeatures of participants please see Supplementary Table 1. CSF samples from all individuals were collected after the informed consent of the participant at the Paracelsus Elena Klinik in accordance with the principles of Declaration of Helsinki and following identical standard operating procedures. In brief, CSF was collected by lumbar puncture in the morning with fasting patients in a sitting position. The samples were centrifuged at 2000 x g for 10 min at room temperature (RT). The concentrations of αSyn in CSF samples were measured with a validated sandwich ELISA system (mSA1/Syn1-BB; 384 well plate format)72. Aliquots of the supernatant were incubated in the range of 20–30 min and stored at ~80 °C until their use. The cell count was established in tube (1 mL). Samples with erythrocyte counts >50 cells μL−1 were excluded from all analyses. The use of the CSF samples in this study was approved by the ethical committee of the Medical Center Göttingen with the approval numbers 36/7/02 and 97/04.

Plasmid electroporation of Xenopus levis tadpole. All procedures for animal handling were approved by the governmental animal care and use office (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, Oldenburg, Germany, Az.12/0779) and were in accordance with the German Animal Welfare Act as well as with the guidelines of the Göttingen University Committee for Ethics in Animal Experimentation.

Primary hippocampal neurons. Postnatal (P1–P2) pups from Wistar rats were decapitated and the brains were extracted. The hippocampi were isolated, washed in Hank’s balanced salt solution (HBBS; Invitrogen, Darmstadt, Germany) and incubated in HBBS supplemented with 10% FBS, 2 mM l-glutamine and 600 U/ml penicillin-streptomycin (Lonza). Reporter-cells (HEK293 stably expressing TetON-NbSyn87-EGFP-P2A-mCherry) and hαSyn cells (HEK293 stably expressing TetON-hαSyn) were grown in DMEM supplemented with 10% FBS, 2 mM l-glutamine supplemented with 0.5 µg/ml puromycin (InvivoGen, San Diego, CA, USA). Both, Reporter-cells and hαSyn cells were produced by Biogen Biologics (Lone Tree, CO, USA). TetON Induction was performed using 0.5 µg/ml doxycycline (Sigma-Aldrich, St. Louis, MO, USA) at least 12 h before their use.

Cell lines. Wild-type HEK293 were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, 4 mM l-glutamine and 600 U/ml penicillin-streptomycin (Lonza). Reporter-cells (HEK293 stably expressing TetON-NbSyn87-EGFP-P2A-mCherry) and hαSyn cells (HEK293 stably expressing TetON-hαSyn) were grown in DMEM supplemented with 10% FBS, 2 mM l-glutamine supplemented with 0.5 µg/ml puromycin (InvivoGen, San Diego, CA, USA). Both, Reporter-cells and hαSyn cells were produced by Biogen Biologics (Lone Tree, CO, USA). TetON Induction was performed using 0.5 µg/ml doxycycline (Sigma-Aldrich, St. Louis, MO, USA) at least 12 h before their use.

Cellular uptake of recombinant huSyn from the culture medium. Purified synuclein proteins were introduced to the medium of (i) neurons cultured on 96-well plates 6 days post infection with AAV NbSyn87-mCherry and NLS (ii) Reporter-cells cultured on 24-well plates 48 h (~70,000 cells per well) post-seeding. hαSyn (monomers or fibrils) and hβSyn proteins were diluted in Opti-MEM and incubated with Lipofectamine RNAiMax (Invitrogen, Thermo Fisher Scientific, 2 and 0.3 µl per well for Reporter-cells and neurons, respectively) for 20 min and then added to the culture medium. The concentration of the reporter cells was prepared with minor modifications from the original protocol72. Five days after plating, neurons were infected with an AAV (88e5 Tu AAV/5 fl) containing the sequence for NbSyn87 fused to mCherry and NLS sequences (NbSyn87-mCherry-NLS).

Detection of huSyn species in CSF samples. Wild-type HEK293 and Reporter-cells were seeded (~14,000 per well) and induced in a PLL-coated 96-well plate with glass bottom (SensoPlate, Greiner Bio-One International Gmbhl, Kremsmünster, Austria). After 24 h of induction, the cells were exposed to CSF samples. In brief, the medium was partially eliminated leaving 25 µl per well, and supplemented with 50 µl of CSF or culture medium as negative control. Cells were further incubated for 15 min. After washing the neurons with HBBS, the hippocampi were incubated in inactivation solution for 15 min. Another washing step in Neurobasal A, neurons were mechanically dissociated by pipetting. In all, 15,000 neurons per well were added to the plating medium (MEM, 10% horse serum, 3.3 mM glucose, 2 mM glutamine) in PLL-coated 96-microwell glass bottom well plates (SensoPlate, Greiner Bio-One International Gmbhl, Kremsmünster, Austria) and kept at 37 °C, 5% CO2. After ~1 h, when the neurons adhered to the glass bottom, the plating medium was exchanged with 100 µl Neurobasal A and plates were further cultured at 37 °C, 5% CO2. To maintain healthy cultures, 50 µl of medium was removed every second day and replaced with 50 µl of fresh Neurobasal A. The primary rat hippocampal neuron cultures were prepared with minor modifications from the original protocol72. Five days after plating, neurons were infected with an AAV (88e5 Tu AAV/5 fl) containing the sequence for NbSyn87 fused to mCherry and NLS sequences (NbSyn87-mCherry-NLS).

Imaging. Conventional epifluorescence images of the Reporter-cells were obtained with an Olympus IX71 microscope equipped with a 0.5 NA>/>/20 dry UPlanFl N objective and captured with an Olympus F-View II CCD camera (Olympus, Hamburg, Germany). Experiments with CSF samples and neurons on 96-well plates were acquired using a Biotek Cytation 3 Imaging Reader (BioTek Instruments, Winooski, VT, USA) equipped with a >/>0 Plan Fluorite WD 6.6 NA 0.45 objective, a 465 nm LED cube (Cat# 1225001), EGFP Filter cube (Cat# 1225010), 525 nm LED cube (Cat# 1225003), RFP filter cube (Cat# 1225103) and a 16-bit monochromatic CCD camera (pixel size 6.45 µm × 6.45 µm).

In vivo multiphoton imaging of the Xenopus olfactory system. For in vivo imaging, we anesthetized the electroporated tadpoles in 0.02% MS-222 (ethyl 3-aminobenzoate methanesulfonate; Sigma-Aldrich; pH: 7.6) for 3 min until complete immobility and irresponsiveness. Subsequently, the animal was transferred to a moistened dish under a stereomicroscope with brightfield and fluorescent illumination (Olympus SZX16; light source: X-Cite Series 120, Lumen dynamics). The glass pipettes filled with plasmid solution/s were mounted to a micromanipulator connected to a FemtoJet injection setup (Eppendorf). The microinjection was carefully penetrated into the olfactory mucosa at three to five different locations without injuring major arteries. Up to five pressure pulses of 250–1000 hPa (1 x each) were applied per site. Once homogeneous blue fluorescent signal was visible throughout the olfactory mucosa, an external electric field was applied to the olfactory mucosa using an electroporation setup79. One of the 0.2 mm platinum wire electrodes was positioned in the water-filled nostril, the other one in contact to the skin, laterally to the olfactory nerve. Trains of three square pulses (20 V, 500 ms duration, and 25 ms delay) were applied four times in alternating polarity (ELP-01D, NI Electronics; additional capacitor connected in parallel: Domnopt, 3 µF). The entire procedure was performed in a calm and quiet room to grant proper doses of the dissipation of plasmid solution before electroporation. Following electroporation, animals were put into water until they woke from anesthesia. After assessment of normal swimming the larvae were left in their aquaria to recover for at least 24 h. Individual nostrils were electroporated sequentially, with a 1 h recovery period in between. We injected and electroporated two plasminids in the left nostril: expressing FluorOSyn and the other hαSyn, both under the CMV promoter. In the right nostril, only the FluorOSyn expressing plasmid was electroporated.
Western blotting. Reporter-cell lysates were thawed on ice and diluted according
their total protein (determined using BCA assay (Merck)) to load the same
total protein concentration in each lane. Samples were mixed with pre-heated 5x
Laemmli buffer (50 mM Tris-HCl, 4% sodium doeduced sulfate (SDS), 0.01% Serva
Blue G, 12% glycerol, pH 6.8, 50 mM DTT) to be further boiled at 95 °C for 10 min,
centrifuged and then loaded into previously casted 10–12% PAGE. After the SDS-
PAGE run was completed, proteins were transferred to a nitrocellulose membrane
in wet transt blot cell (BioRad) with 400 mA for 2 h at 4 °C while stirring the transfer
buffer (25 mM Tris, 192 mM glycine, pH 8.3 and 20% methanol, and 0.04% SDS). The
membrane was incubated for 1 h in blocking buffer (5% Nonfat Dried Milk, 0.1% Tween20 in PBS and then was further incubated with a mouse mononclonal anti-
EGFP antibody (1:500; Cat. No. A11212, Invitrogen, Thermo Fisher Scientific)
or a rabbit polyclonal anti-β-Actin-Cy5 antibody (1:1000; Cat. No. 251033, SySy,
Göttingen, Germany). Primary antibody incubations were performed overnight
at 4 °C with constant shaking. The following day, the membrane was washed thor-
oughly in blocking buffer and incubated with the fluorescein labeled secondary
donkey polyclonal anti-mouse antibody (1:1000; Cat. No. 715-175-150, Dianova)
for 1 h. After washing with PBS, the membranes were dried, it was blocked with
2% FBS or BSA, 5% Nonfat Dried Milk, 0.05% Tween20 in PBS for 1 h under agitation. This was followed by incubation with the fluorescein labeled
monobody NSyn87-Alexa467 for 1 h. Unbound monobodies were washed away by
several thorough washing steps with 0.05% Tween20 in PBS for a total duration of
1 h. Finally, images of the membranes were taken with an Amersham Imager 600
(GE Healthcare Life Sciences, Little Chalfont, UK) to detect the NSyn87-Alexa467
signal.

Data analysis and statistics. Image analyses of experiments presented in Figs. 1, 4,
5, Supplementary Fig. 1 and Supplementary Fig. 3 of this manuscript or mCherry
(Fig. 7) signals. The average signal intensity within a cell was calculated and
corrected for the background intensity by subtracting the background region
of interest from the average signal intensity.

Cells were considered as positive if their background-corrected GFP (Figs. 1, 4,
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Author contributions
C.G., T.O., and E.F.F. designed and conducted experiments, analyzed and interpreted data, and contributed with the writing of the manuscript. N. Waal and H.V. designed and performed experiments, analyzed and interpreted data. N. Wender initially characterized the sensor, designed the Reporter-cell lines, designed and performed experiments, analyzed and interpreted data, contributed to the supervision of the study and scientific discussions. C.T. and B.M. provided the human CSF samples. T.S., M.Z., and S.B. prepared and provided αSyn fibrils. S.O.R. analyzed and interpreted the data, contributed to the supervision of the study and scientific discussions. F.B.B. designed and performed experiments, analyzed and interpreted the data, supervised the study and wrote the manuscript. F.O. conceived the project, designed and performed experiments, analyzed and interpreted the data, supervised the study and wrote the manuscript.

Competing interests
F.O. and S.O.R. are shareholders of NanoTag Biotechnologies GmbH. All other authors declare no competing interests.

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