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An ScFv Intrabody Against the Non-Amyloid Component of Alpha Synuclein Reduces Intracellular Aggregation and Toxicity

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Summary

Prevention of abnormal misfolding and aggregation of alpha-synuclein (α -syn) protein in vulnerable neurons should be a viable therapeutic strategy for reducing pathogenesis in Parkinson's disease (PD). The non-amyloid component (NAC) region of α -syn shows strong tendencies to form β -sheet structures, and deletion of this region has been shown to reduce aggregation and toxicity *in vitro* and *in vivo*. Binding of a molecular species to this region may mimic effects of such deletions. Single-chain Fv antibodies (scFvs) retain the binding specificity of antibodies, and when genetically manipulated to create high-diversity libraries, allow *in vitro* selection against peptides. Accordingly, we used a yeast surface display library of an entire naïve repertoire of human scFv antibodies to select for binding to a NAC peptide. Candidate scFvs (after transfer to mammalian expression vectors), were screened for viability in a neuronal cell line by transient co-transfection with A53T mutant α -syn. This provided a ranking of the protective efficacies of the initial panel of intracellular antibodies (intrabodies). High steady-state expression levels and apparent conformational epitope binding appeared more important than *in vitro* affinity in these assays. None of the scFvs selected matched the sequences of previously-reported anti- α -syn scFvs. A stable cell line expressing the most effective intrabody, NAC32, showed highly significant reductions of abnormal aggregation in two separate models. Recently, intrabodies have shown promising anti-aggregation and neuroprotective effects against misfolded mutant huntingtin protein. The NAC32 study extends such work significantly, utilizing information about the pathogenic capacity of a specific α -syn region to offer a new generation of *in vitro*-derived antibody fragments, both for further engineering as direct therapeutics and as tools for rational drug design for PD.

Keywords

alpha-synuclein; non-amyloid component; engineered antibody; aggregation; Parkinson's disease

Introduction

Misfolding and aggregation of proteins, both intracellularly and extracellularly, have been implicated in pathogenesis of all of the most common neurodegenerative diseases ¹. Parkinson's disease (PD), the second most prevalent neurodegenerative disease following

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Alzheimer's Disease, affects approximately 2% of the people over the age of 65, representing an important component of this class of disorder, with both common and disease-specific features. PD results in disturbances in motor function characterized by tremor, rigidity and bradykinesia following a 50 to 70% loss of dopaminergic neurons in the substantia nigra and in other regions of the nervous system². Lewy Bodies, intracellular protein inclusions composed of a dense core of filamentous and granular material coated with radially oriented filaments; and Lewy neurites, which contain filaments that are structurally and immunologically similar to those found in Lewy bodies³, are the classic neuropathologic hallmarks of this disease. The protein α -syn is a major component of these Lewy bodies and neurites^{4; 5}. Furthermore, although most cases of PD are sporadic, three point mutations in the α -syn gene have been correlated with PD^{6–8}. Expression levels of α -syn may contribute to PD, as increased gene expression of wild type α -syn is correlated with early onset cases of PD⁹. Two other mutant proteins correlated with PD – parkin and ubiquitin C-terminal hydrolase L1 – are involved in the ubiquitin-proteasome pathway, a clearance system that fails in PD, resulting in the accumulation of heavily ubiquitinated α -syn aggregates¹⁰.

Considerable evidence from human and model system studies of PD, shows that the intracellular protein α -syn is not merely present, but plays a major role in pathogenesis of both sporadic and inherited PD^{11–14}. This intrinsically unstructured small protein, thought to acquire an alpha-helical configuration upon normal association with membranes, abnormally self-associates forming aggregates which are finally deposited into the Lewy inclusions. These inclusions may represent a cellular protective feature to sequester precursor forms of misfolded α -syn, and/or they may contribute directly to cellular dysfunction and death. The α -syn molecule consists of an N-terminal repeat and a C-terminal acidic region flanking a hydrophobic region known as the non-amyloid component (NAC) (Fig 1A, residues 61–95), which was originally identified as a component of Alzheimer amyloid plaques^{15–17}. Using deletions, this region has been shown necessary for aggregation *in vitro*¹⁸, and toxicity to dopaminergic neurons in a *Drosophila* model¹⁹; underscoring the region's suitability as a therapeutic target.

Immunotechnology based upon the application of recombinant DNA methods to immunology permits facile engineering of the antibody molecule, and genetic selection of specific human antibody binding sites, through phage and yeast surface display libraries^{20; 21}. ScFv (V_H -linker- V_L) and single-domain (V_H or V_L separately) fragments retain the affinity and specificity of binding associated with antibodies. Once expressed intracellularly, the antibody fragment (now termed **intrabody**) can alter the properties of its target upon binding, and can also be further engineered to increase its affinity, expression, and stability²². In order to apply this powerful cancer-derived technology to neurodegenerative diseases, intrabodies against misfolded neurodegeneration proteins have been recently selected, engineered, and tested^{23; 24}. Use of intrabodies to alter the protein context of the huntingtin (htt) polyglutamine has successfully counteracted its length-dependent aggregation and toxicity in several transient and stable cell culture and yeast models^{23; 25–27}; showed functional protection in organotypic slice cultures²⁸; and slowed *in vivo* neurodegeneration with improved survival of a Huntington Disease (HD) *Drosophila* model²⁹.

ScFvs selected against α -syn monomers³⁰ and oligomeric/fibrillar forms³¹ can alter α -syn aggregation when expressed as proteins *in vitro*. Since α -syn is thought to act primarily intracellularly, these proteins must either be delivered using some form of protein transduction, or expressed directly inside the target cells from genes²³. A first-generation anti-monomeric α -syn scFv intrabody selected from a phage display library improved cell adhesion properties, stabilized detergent-soluble monomer, and decreased the amount of detergent-insoluble monomeric α -syn in HEK293/ α -syn cells³². This provided proof of concept; however, more domain-specific and readily engineered species that show neuronal efficacy are desirable.

Optimized criteria for using yeast display library technology for neurodegenerative disease target validation and therapeutics have not yet been defined.

Here we show that *in vitro* selection from a publicly-available yeast surface display library for a new set of unique scFvs specific to the NAC region, followed by a neuronal cell-protection screening process, successfully identified a novel intrabody that can counteract pathogenic aggregation. Such intrabodies can serve as direct PD molecular therapeutics or as tools for further drug development. Both the general approach against specific epitopes within misfolding proteins, and the screening criteria for moving from *in vitro* testing to functional intrabodies, should be generally applicable to multiple neurodegenerative diseases.

Results

Selection of a panel of single-chain variable fragment (scFv) antibodies against α -syn NAC peptide

NAC, a 35-amino acid peptide, comprising amino acids 61–95 of the α -syn sequence, was the primary target for this set of studies (Fig 1(a)). Due to the difficulty in isolating scFvs against aggregation-prone peptides, a biotin-labeled peptide spanning amino acid 53 to 87 of α -syn with improved solubility properties was used as the antigen. To ensure the scFv specificity to NAC peptide, rather than to biotin, we tested our final products against biotin-labeled syn-2–16 peptide, which gave a negative cross-reaction (Fig 1(b)).

Single-chain antibodies against syn-53–87 were isolated from a yeast-displayed non-immune human scFv library, by sequential magnetic bead enrichment and flow cytometric sorting²¹. This library had a displayed diversity of 10^9 tagged scFvs on the yeast cell surface, allowing us to screen specific binders to syn-53–87. Screening was performed using 500 nM antigen, as screening attempts at lower antigen concentrations were unsuccessful, a fact which demonstrates the difficulty of isolating non-structure aggregation-prone peptides from a naïve scFv library.

Magnetic enrichment reduced the pool of positive yeast cells to levels that allowed rapid flow sorting. This selected pool was double-stained with biotin-labeled syn-53–87 and mouse anti-myc antibody, followed by SAPE and Alexa 633 conjugated anti-mouse antibody. FACS sorting allowed selection of syn-53–87 specific binders (Fig 1(b)) using biotin labeled syn-2–16 as a control to set the sorting gate.

Characterization of individual positive yeast clones

A set of syn-53–87 positive yeast clones was subcultured and confirmed for binding specificity. Among these specific binders, six clones showed relatively higher binding to the antigen. As shown in Fig 1(c), the positive clones demonstrated specific antigen affinity with binding percentages varying from ~30% to 60%. The lack of binding of these clones to the control peptide syn-2–16 was verified (data not shown), thus excluding the possibility of non-specific binding to biotin.

These six clones from the panel of antibodies specific for syn-53–87 were sequenced, and each was found to be unique. The germline usage and complementarity determining regions (CDRs) are shown in Fig 1(d). Four clones (NAC3, NAC6, NAC24, and NAC32) were full-length scFvs, heavy plus light chains, while NAC1 and NAC14 were heavy chain only. Single-domain antibody fragments are not uncommon in this library. There appears to be high sequence homology among the scFvs within the CDR H2 (heavy chain variable) region (Fig 1(d)) suggesting that this region has strong dominance in the syn-53–87 binding paratope. The sequences were also compared to that of our previously reported scFv, D10 which was selected

against the full-length monomeric α -syn,³² and against a recently reported anti α -syn oligomer selected scFv³¹; none were identical.

To further define the epitopes of the six positive clones, we tested each clone's capacity to bind to each of the three smaller peptides spanning the whole sequence of syn-53–87. As shown in Fig 1(c), NAC3, NAC6, NAC14, and NAC24 showed high specific binding to the linear epitope syn-61-78. NAC32 showed lower binding, and NAC1 did not appear to bind this epitope under our *in vitro* conditions.

Cellular assays identify the most promising intrabody for therapeutic use

Screening of intrabodies for efficacy using transient transfections in neuronal cells—Since intracellular efficacy is only partially predicted by *in vitro* binding, the protective capacity of each of the six clones was evaluated in the progenitor neuronal cell line ST14A from embryonic rats. This cell line was chosen for the initial screening on the basis of preliminary experiments to optimize a combination of neuronal characteristics, robust cellular changes in response to overexpressed α -syn, and high enough transient transfection efficiencies to allow statistically significant screening to occur without having to produce stable cell lines or viral vectors expressing the entire series of candidate scFvs. ST14A cells have many neuronal characteristics³³, and show ~ 70% transfection efficiency in our protocols. Co-transfection of these cells with mutant α -syn A53T and empty pcDNA 3.1(–) vector elicited a reduction of cell proliferation and viability as measured by the MTT and LDH assays, clearly demonstrating the toxic effects of the α -syn construct. Co-transfection of mutant α -syn A53T with the panel of anti-NAC intrabodies identified NAC32 as exhibiting the highest degree of rescued cell viability, and a reduction of 75% in cytotoxicity as compared to cells transfected with the A53T construct and empty vector (Fig 2(a)). NAC1, NAC3, and NAC24 each seemed to have little effect on cell viability. As a control for non-specific effects on heterologous aggregation-prone neurodegeneration proteins, the MTT assay was performed with the mutant huntingtin Exon1 fragment, htt-72Q-GFP, which is also toxic in ST14A cells.

NAC1 and NAC14, which are heavy chain only, and did not appear more robust than the full scFv, were deferred from further screening. On further analysis of cell lysates by Western blotting (Fig 2(b); anti-c-myc), it appears that the null effect of NAC24 could have been the result of low expression or stability levels in the intercellular milieu, as compared to *in vitro* conditions, as only NAC3 and NAC32 were expressed consistently. Low stabilities can compromise molecular structure, weaken binding affinities, and hence lower efficacy in blocking the α -syn aggregation and consequent toxicity.

Confirmation of intracellular binding and re-targeting of α -syn by NAC32—To verify specificity we conducted more comprehensive tests on NAC32, since this was the most promising clone. The intracellular *in situ* interactions of antibody to antigen can be assessed using an intrabody-NLS (nuclear localization signal) re-targeting construct. Although increasing nuclear localization is not therapeutically desirable, this experiment is the classic test of the capacity of an intrabody to bind its target intracellularly. Analysis by immunocytochemistry 48 h post-transfection demonstrated the extranuclear localization of A53T α -syn transfected alone. NAC32 and htt-72Q-GFP also remained extranuclear when transfected alone (Fig 3(a)), as is characteristic of many intrabodies^{27; 32}. NAC32-NLS alone shows the expected nuclear localization (Fig 3(a)). Co-transfection of A53T α -syn with NAC32-NLS but not NAC32 showed nuclear displacement of the antigen-antibody complex in almost half of the cells, demonstrating the ability of the intrabody to bind to its α -syn target intracellularly (Fig 3(b)). Similar nuclear displacement was also demonstrated in ST14A cells stably expressing the wild-type construct WTsyn-EGFP, 48 h after being transfected with NAC32-NLS (data not shown). Co-transfection of NAC32-NLS with the mutant htt protein

failed to displace it to the nuclear compartment, confirming specificity of NAC32 for the α -syn construct.

Synuclein aggregation reduction in a stably transfected ST14A/NAC32 cell line

—The stable cell line ST14A/NAC32 was generated to enable a direct comparison with the parent cell line, minus the variability arising from transient co-transfections. Immunocytochemical data were then used to show the efficacy of the stable line NAC32 against an aggregation model previously used^{34; 35}, in which a truncated version of WTsyn-EGFP was shown to form aggregates detectable by antibodies against α -syn, but not by antibodies against GFP. This indicated that the α -syn species in the aggregated form had been cleaved within the EGFP. The aggregation-prone α -syn species, termed synT, has a MW of about 27. After being co-transfected with synphilin (a protein colocalized with α -syn aggregates in inclusion bodies physiologically³⁶) aggregates were observed in ca. 60% of ST14A cells (Fig 4). This percentage was reduced substantially, to ca. 25%, in the NAC32 stable line. A parallel reduction from 30% to ca. 8% aggregation was observed in the A53T/synphilin co-transfections (Fig 4(b)).

Discussion

Significant evidence points to the NAC sequence of α -syn as a critical region for abnormal aggregation and subsequent toxicity. The elimination of all or part of the NAC from α -syn prevented both the protein's aggregation *in vitro*¹⁸, and its toxicity to dopaminergic neurons in an α -syn overexpression *Drosophila* model¹⁹. The amino-acid sequence of this region diverges considerably from parallel sequences in the β - and γ -synucleins which are otherwise highly homologous to α -syn, but are not as prone to aggregation (although it can be forced *in vitro*), and they can themselves inhibit α -syn fibrillation^{37; 38}. In the *in vitro* work¹⁸, the NAC appeared to be localized within the core of α -syn aggregates, since its sequence in this form was protected against proteinase treatment as compared to its monomeric forms. Successful binding to, and blockage, of the NAC region of α -syn intracellularly therefore constitutes an important therapeutic goal. This study demonstrates conclusively that anti-NAC intrabodies can be used as a deliverable therapy to mimic NAC deletions.

Yeast surface display libraries offer both selection and direct affinity maturation of a human repertoire. The library that was used here is a non-immune human library, which is publicly available²⁰, facilitating follow-up work. Primary selection produced a range of scFvs and single-domain antibodies with affinities for syn-53–87 (a major component of the NAC) that were appreciably higher than background (Fig 1(c)). *In vitro* affinity assays should give some indication of intracellular ability of scFvs to target the antigen. In order to characterize the binding efficacy of these clones, we titrated NAC14 heavy chain (which had the highest binding affinity among set of clones tested) against peptide syn-53–87. In this yeast display assay, the affinity to antigen syn-53–87 was approximately 3 μ M (data not shown), a value which seems rather low. In similar work²⁵ a case was described where a variable light chain (V_L) selected against a htt fragment showed an *in vitro* affinity > 10 μ M; nevertheless, when this V_L was expressed intracellularly, it inhibited aggregation to the same extent as one with an *in vitro* affinity of about 30 nM, a result apparently explicable through the loss of the stability of the latter in the reducing environment of the cytoplasm. Thus, although the scFvs here showed relatively low affinities *in vitro*, the cell culture assays demonstrated that intracellular properties are still promising. The fact that the intracellular affinity for α -syn is not extremely high could even be beneficial therapeutically, since binding of a moderately expressed low-affinity intrabody to over-expressed or excess α -syn might serve to protect the normal cellular function(s) of the target during the “off” time.

Attempts to characterize the specific linear binding epitopes of the positive clones indicated that four of them showed strong binding to the (presumed) linear epitope syn-61–78 while NAC32 showed only weak binding. Given that this scFv showed the highest intracellular efficacy, the *in vitro* binding assay suggests that either the NAC32 epitope may bridge two or more of the tested sequences, or that the target being recognized is a critical conformational structure in this region. This is especially valuable, as it may allow the use of scFv NAC32 in a competition screen for protective small molecule drugs.

An analysis of the differences between the *in vitro* binding and the *in vivo* efficacy highlights the additional steps that must be taken when translating from the primary screen display library data to *in vivo*. We have shown that NAC32, when cloned into mammalian expression vectors and expressed as an intrabody, shows significant steady-state levels in lysates of transiently transfected cells; we have also shown this intrabody to maintain a useful degree of stability and affinity in the cytoplasmic environment. The decision to use mutant A53T α -syn in initial intracellular survival screening stemmed from the observation that this mutant was more toxic to the ST14A neuronal cells than either wild-type or other α -syn mutants. (Apparently A53 is not a critical part of the binding epitope to the intrabody, a reasonable assumption given that this is the first amino acid position of the sequence). Somewhat unexpectedly, given its relatively low *in vitro* binding activity, NAC32 proved to be the most effective among the clones as a protective intrabody when co-transfecting test intrabodies plus A53T. This efficacy appears to be due at least in part to moderately high steady-state levels of expression. However, some of the other clones, e.g. NAC3 and NAC24 were expressed in appreciable amounts although inconsistently, yet appeared to have no potency. The data on the single-domain V_H NAC14 clone suggests that it could be a promising candidate as well, since it does show some protective capacity. Based on a combination of all factors, this would be the next choice for follow-up, subsequent to the fusion of a myc tag onto the heavy chain for detection.

The classic test of the capacity of an intrabody to bind an otherwise cytoplasmic target protein intracellularly is to fuse the intrabody sequence to a NLS peptide. Given some disparity between the *in vitro* binding and *in situ* efficacy data, these experiments were especially valuable in the present study. Although α -syn has been demonstrated to be nuclear-localized when subjected to a toxic insult i.e. injections of herbicide³⁹, it is usually expressed in the cytoplasm. Transient co-transfection of α -syn A53T plus intrabody, fixed and examined with immunocytochemistry, clearly shows that a significant fraction of the α -syn is translocated to the nucleus when the NLS, but not the unfused form of the intrabody is present. Controls verified that this effect is specific, and similar experiments could be observed using live-cell imaging in an ST14A cell line that was stably transfected with WTsyn-EGFP. These experiments confirm the specificity of the *in situ* antigen-antibody binding, although they suggest that further engineering of this scFv is warranted, since re-targeting was less than 100%. As noted above, this moderate affinity is not necessarily a disadvantage, given the evidence that dose effects of α -syn are quite pronounced^{9; 40}. However, maturation of NAC32 to increase both binding affinity and intracellular stability can be done as with other scFvs²⁵.

The extent to which different multimeric forms of α -syn represent toxic vs. protective species remains controversial. Although α -syn aggregates are difficult to visualize in cell culture, the protein has a tendency to form higher MW oligomers when over-expressed or in mutant form^{31; 32; 35}, and misfolded aggregated α -syn is found in combination with other proteins in Lewy body inclusions^{11; 12; 41}. Large aggregates (possibly fibrillar) may initially be a cell-protective feature; however, their presence is an indication that some abnormal cellular event is occurring, and eventually toxicity follows. A therapeutic solution which involves prevention of aggregation can thus be deleterious if it merely interferes with the protective sequestration feature, but if in addition it delays toxicity, or increases cell viability, then the therapy would

have been successful in addressing the initial event(s) which triggered the aggregation. Most recently, inhibition of Sirtuin 2 was demonstrated to reduce α -syn-mediated toxicity in cellular and *Drosophila* models of PD. Rescue entails the enlargement of small inclusions, reducing the total surface area available for abnormal interactions⁴². However, this increase in aggregation should not be necessary if the initial oligomerization of α -syn is blocked. It will be of great interest to combine these two treatment approaches to determine whether they can operate synergistically, both from the standpoint of designing combinatorial therapies, and to dissect underlying mechanisms of protection.

The NAC32 intrabody reduces both the overall abnormal aggregation and toxicity of mutant α -syn in this neuronal cell system. Our immunocytochemical experiments directly investigated the effect of the NAC32 intrabody on visible aggregated forms of the α -syn. Quantitation of aggregates showed high variability in standard dual-transfection assays, suggesting a dependence on the final ratio of intrabody to target, which can vary substantially when separate plasmids are transiently transfected into neuronal cells. The stably transfected ST14A/NAC32 cell line was therefore created and tested to standardize intrabody levels. All aggregating α -syn combinations clearly demonstrated a significant decrease in aggregation in NAC32 stable vs parent ST14A cells, as assessed both visually and by cell counts, along with an apparent stabilization of the α -syn monomer in some experiments. These experiments using stable transfections are the closest to mimicking a scenario in which the intrabody gene is delivered to neurons by means of a gene therapy vector, with proteins continuously synthesized.

Intrabody technology has already been shown to hold promise against cancer⁴³ and HIV⁴⁴. The next step in the development of a therapeutic anti-PD strategy is to affinity-mature NAC32. This process can tighten specificity towards a specific toxic epitope⁴⁵, providing further information on α -syn structural pathogenesis, as well as stronger efficacy. Exogenous evidence on the specific toxic synuclein species, or epitope, could also be incorporated into our screens. Stability maturation is more challenging, but certainly technically feasible²⁵. The other intrabodies that demonstrated less apparent potential may also be subjected to further engineering, if a larger panel of candidates is needed for functional or structural studies. We anticipate that delivery of the intrabody genes will require gene therapy. This should not be a barrier to clinical use. There are currently three approved PD gene therapy trials ongoing in the US; and both adeno-associated virus^{46; 47} and lentiviral vectors⁴⁸ have shown promising gene delivery in PD animal models. Small molecule screening for compounds that are specifically selected to block or inhibit the conformation that allows initial aggregation could also be done using the NAC32 protein, which can be readily purified as a secretory product from yeast or *E. coli*²³ in competition assays. The work described here represents a significant advance toward a molecular therapy targeted against Parkinson's Disease, and against the other neurodegenerative conditions in which α -syn aggregation constitutes a pathological feature.

Materials and Methods

Reagents and yeast strains

Yeast rich non-selective medium (YPD), selective growth medium (SD + CAA), and selective scFv induction medium (SG/R + CAA) were made as previously described (<http://www.sysbio.org/dataresources/usermanual031113.pdf>). Yeast strain EBY100 and the scFv library were provided by Pacific Northwest National Laboratory (Richland, WA).

LS Macs columns, Streptavidin-conjugated magnetic MicroBeads, Anti-Biotin magnetic MicroBeads, and a MiniMACS™ manual magnetic separator were purchased from Miltenyi Biotec Inc. (Auburn, CA). Anti-HA (12CA5) antibody and anti-c-myc antibody (9E10) were from Abcam, Cambridge, MA; goat anti-mouse Alexa 488 conjugated, goat anti-mouse Alexa

633 conjugated, goat anti-mouse PE conjugated, streptavidin-R-phycoerythrin (SAPE), streptavidin Alexa 633 and neutrAvidin Alexa 633, were from Invitrogen, Carlsbad, CA.

Biotinylated purified peptides syn-2-16, syn-53-87 (spanning most of NAC region), syn-53-63, syn-61-78, and syn-74-87 were synthesized at the Protein Core Facility, Tufts University, Boston, MA.

Yeast scFv library screening

A publicly available non-immune human scFv library with clones labeled according to previously published protocols (<http://www.sysbio.org/dataresources/usermanual031113.pdf>; ²⁰ was used. In brief, 10^{10} yeast cells were harvested from the cultured scFv yeast library with a diversity of 10^9 . Yeast cells were resuspended in S/GR+CAA medium, incubated at 20°C for 1 to 2 doublings and labeled with biotinylated syn-53-87 at 500 nM final concentration. MACS Streptavidin magnetic MicroBeads and Anti-Biotin magnetic MicroBeads were used sequentially to enrich the positive yeast clones (alternation between these bead types during subsequent rounds of selection decreases the chance of obtaining secondary reagent-specific clones).

Flow-cytometric sorting of specific scFv clones against syn-53-87 from the magnetically enriched yeast pool was performed in parallel with sorting against syn-2-16 as control. This served to eliminate possible clones with non-specific biotin binding, using a FACS Aria high-speed cell sorter (BD Biosciences, Franklin Lakes, NJ) at the Ordway Research Institute, Inc., Albany, NY. Double staining with antigen/SAPE and anti-myc/Alexa 633 was used for screening with the gating set to no positive clones with syn-2-16 peptide control. Neutravidin PE was used between rounds of flow cytometric sorting to reduce secondary reagent-specific scFv.

Individual scFv clone confirmation and epitope mapping

Syn-53-87 positive yeast clones from the library screening were validated by a binding assay with both syn-2-16 and syn-53-87. Positive yeast binding percentage was used to evaluate the capacities for binding to syn-2-16 control and syn-53-87, for each clone. To map detailed epitopes of these positive yeast clones, we synthesized three peptides spanning the whole sequence of peptide syn-53-87. Each clone was tested for the binding capability to peptides syn-53-63, syn-61-78, and syn-74-87, and positive yeast binding percentage was used to identify the epitopes.

Sequencing and sequence analysis

Plasmids were recovered from yeast using a Zymoprep kit (Zymo Research), and were sequenced by the Wadsworth Center DNA Sequencing Core facility. Sequences were analyzed using the NIH's IgBlast (<http://www.ncbi.nlm.nih.gov/igblast/>) and Vbase (<http://vbase.mrc-cpe.cam.ac.uk/>).

Plasmids constructs

ScFv genes were cloned in fusion with the c-myc tag into pcDNA 3.1(-) (Invitrogen, Carlsbad, CA), to allow expression as intrabodies. Mutant A53T α -syn in pRK5 vector ⁴⁹ was a gift from Dr. Christopher Ross, Johns Hopkins University, Baltimore, MD; synT (an aggregation-prone truncated form of WTsyn-EGFP), synphilin-1-V5 (a protein found co-localized with α -syn in Lewy bodies) in pcDNA 3.1/V5/His-Topo ³⁴ and WTsyn-EGFP-pN3 were a gift from Dr. Pamela McLean, Harvard Medical School, Boston, MA. The mutant htt-72Q-GFP was donated by Drs. Kazantsev and Houseman, MIT, Boston, MA ⁵⁰. NAC32 with a nuclear localization signal (NLS) was constructed by PCR using the forward primer 5'CCATC GATGCC ACCAT

GAGTAG CCAG GTACA GCTGC AGCAG 3', and reverse primer 5'CCGC TCGAGCTA AACCTTAC GTTCTT CTTCG GCGGA GTCAA GTC TTC TTCAG AAATAA GCTTTTG 3'. The sequence of intrabody NAC32 has been deposited in the GenBank Data library with accession number [EU079027](#).

Cell viability assays

ST14A neural progenitor cells (a gift from Dr. Elena Cattaneo, Milano, Italy) were seeded in equal numbers in a 96-well plate (Corning, Inc, Corning, NY), transfected (FuGENE 6, Roche Applied Science, Indianapolis, IN) with mutant A53T α -syn construct and the intrabody of interest, and grown in Dulbecco's Modified Eagle's medium (Invitrogen, Grand Island, NY) with 10% FBS, 100 U/ml each of penicillin and streptomycin, and 2 mM L-glutamine at 33°C, and 9.5% CO₂. The MTT test reagent (ATCC, Manassas, VA) was administered 48 h post-transfection and cell proliferation was measured at 570 nm on a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA). Eight wells were transfected for each intrabody, and the experiment was repeated twice. Cell toxicity was ascertained 48 h post-transfection, by measuring the absorbance at 450 nm of lactate dehydrogenase release into the cell culture media (LDH Cytotoxicity Assay kit II, BioVision Research Products, Mountain View, CA) with microplate reader previously mentioned. The non-specific intrabody C4 (specific for htt), was added to the experiment for comparison. Data were analyzed by ANOVA with Fisher's probability of least significant differences, using STATVIEW software v 5.0 (SAS Institute, Cary, NC).

Western blots

Cell lysates from cultures in 60-cm plates were prepared in lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris base, 1 mM EDTA, protease inhibitor (Roche, Penzburg, Germany)), and were centrifuged for 10 min at 15,000 g. The supernatants (Triton X-100 soluble) were separated by 4%–16 % gradient Tris-HEPES-SDS-PAGE (Pierce, Rockford, IL), and were blotted onto PVDF transfer membranes (PerkinElmer LifeSciences, Inc, Boston, MA). Mouse α -syn antibodies LB509 (1:1000; Zymed Laboratories, San Francisco, CA) and Clone 42 (1:1000; BD Transduction Laboratories, San Diego, CA), and mouse monoclonal anti-actin (1:1000; Sigma, St. Louis, MO) were used. Intrabodies were probed with either rabbit anti-c-myc (A-14) or mouse anti-c-myc 9E10 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactivity was detected after 1 h of incubation with goat anti-mouse (or anti-rabbit) IgG-horseradish peroxidase (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA) conjugate in 5% milk TBS-T buffer, using the Western Lighting Chemiluminescence kit (PerkinElmer LAS, Inc, Boston, MA) with 30 sec exposure, except as noted.

Immunocytochemistry and aggregation assay

All stable ST14A cell lines were generated according to the Invitrogen pcDNA 3.1 vector Manual v.1 protocol http://www.invitrogen.com/content/sfs/manuals/pcdna3.1_man.pdf using Geneticin G418 selection. Equal numbers of ST14A and ST14A/NAC32 (low-passage stably transfected ST14A cells expressing the NAC32 intrabody) cells plated in 12-well dishes onto poly-L-lysine 12 mm cover slips (BD Biosciences, Bedford, MA), were transfected (JetPEI™, Polyplus Transfection Inc., New York, NY) with the plasmid constructs of interest, fixed after 48 h with 4 % paraformaldehyde at room temperature, and permeabilized with 0.1 % Triton X-100 blocking solution in 10 % horse serum for 1 h. Cultures were labeled with primary antibody mouse LB509 overnight (1: 100; Zymed Laboratories, San Francisco, CA), and donkey anti-mouse Alexa 488 (1:1000; Molecular Probes, Eugene, Or) was used as the secondary antibody for α -syn detection. Slides were viewed with VectaShield mounting medium with DAPI (Vector Labs, Burlingame, CA) at 40X magnification. Cells from at least six fields of view were counted either as non-aggregated, or else as aggregated if at least one

visible aggregate was present. For cellular localization experiments either mouse anti-c-myc conjugated to FITC (9E10-FITC, 1:200; Santa Cruz Biotechnology, Santa Cruz, CA), or rabbit anti-c-myc (A14, 1:500) followed by donkey anti-rabbit Alexa 488 (1:1000) were used to detect intrabody expression, whereas mouse anti- α -syn (Clone 42, 1:500) followed by donkey anti-mouse 594 (1:1000) were used to detect α -syn expression. Goat anti-GFP and anti-goat IgG-FITC (Santa Cruz Biotechnology, Santa Cruz, CA) were used to detect mutant htt-72Q-GFP.

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Figure 1.

(a) Amino acid sequence of α -syn. The region comprising the non amyloid component (NAC) of α -syn originally identified in Alzheimer's plaques is underlined. Syn-2-16 sequence is in blue and syn-53-87 sequence is in red. (b) Clones of yeast-displayed scFvs are specific for biotin-labeled syn-53-87, rather than biotin-labeled syn-2-16. The pool of scFvs isolated from a yeast non-immune scFv library by sequential magnetic enrichment and FACS sorting against syn-53-87, with syn-2-16 as negative control. Yeast cells were labeled by both antigen/SAPE (FL-2) and anti-myc/Alexa 633 (FL-4). The FL-4 channel shows the expression level of C-terminal c-myc tagged scFv on the yeast surface, labeled with mouse 9E10 anti-c-myc monoclonal antibody and Alexa 633-conjugated goat anti-mouse polyclonal antibody. The FL-2 channel shows antigen binding, detected by streptavidin conjugated to PE. A gating box to sort yeast cells that express scFv and bind antigen is shown. In the control syn-2-16, there were no yeast clones in the gating box. (c) The percentage of yeast clones binding to 1 mM syn-53-87 peptide. Six yeast clones showed, via surface displayed scFv, a capability of binding syn-53-87. Data represent the average $\pm$ standard error of three separate experiments. Epitope mapping. The three peptides, syn-53-63, syn-61-78, and syn-74-87, were labeled with biotin and used to detect the binding capabilities of the six yeast clones. Data represent the average $\pm$ standard error of three separate experiments. (d). Germline usage and sequences of complementarity determining regions (CDRs) of identified scFvs selected against syn-53-87. Germline sequences shown were determined using IgBlast and Vbase. The Germline sequence of scFv D10 is shown for reference. CDR H1 refers to the first CDR of the heavy chain, etc., and CDR L1 denotes the first CDR of the light chain etc.

Figure 2.

Comparison of intrabody efficacies when screened by co-transfection with A53T α -syn in ST14A rat neural progenitor cells. **(a)** Effect of various intrabodies on proliferation and viability of ST14A cells when transiently co-transfected with mutant A53T α -syn, after 48 h of transfection (MTT assay; LDH cytotoxicity assay). Cells were cultured in 10% FBS medium, at 10 % CO₂ and 37°C. Comparison of intrabodies against the empty vector control was performed by ANOVA with Fisher's probability of least significant differences; *, $P < 0.05$. A control experiment (MTT assay) against toxic mutant huntingtin protein (htt-72Q-GFP) fails to show any efficacy of the NAC intrabodies against this amyloidogenic species. C4 intrabody specific for htt was included for comparison. **(b)** Western blots of transient co-transfections of

A53T α -syn plus the scFv clones. Exposure time was 30 sec, except as noted. Anti-c-myc identifies intrabody steady-state expression levels, showing stability only for NAC3 and NAC32, and occasionally for NAC24 as shown. (Heavy chain clones NAC1 and NAC14 were omitted from further screening).

Figure 3.

Immunocytochemistry of transiently transfected species into ST14A cells showing the cellular localization. All cells were fixed 48 h post-transfection. **(a)** Cellular localization of overexpressed proteins, mutant α -syn A53T, red channel; mutant htt-72Q-GFP, green channel; NAC32, green channel, NAC32-NLS, green channel. Blue nuclear DAPI stain indicates that NAC32 localization is extranuclear, whereas NAC32-NLS re-locates to the nucleus. **(b)** Co-transfection of mutant α -syn A53T (red channel) with NAC32 (green channel) shows cytoplasmic co-localization, whereas co-transfection of mutant α -syn A53T (red channel) with NAC32-NLS (green channel) shows intrabody relocation of mutant α -syn to the nucleus. **(c)** Control co-transfections with toxic high poly-Q mutant huntingtin Exon1 (htt-72Q) does not display similar nuclear relocation.

Figure 4.

Effect of NAC32 stably-transfected intrabody on aggregation in ST14A cells transfected with mutant A53T α -syn, and synT. **(a)** Immunocytochemistry was performed 48 h post-transfection. Visual comparison of ST14A cells with ST14A/NAC32 stable cells transfected with A53T mutant, and comparison of ST14A cells with ST14A/NAC32 stable cells, transfected with synT mutant, alike show diminished aggregation behaviour. **(b)** Cell number counts with aggregates in both A53T and synT transfections are significantly reduced in NAC32 cell lines.