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► **To cite this version:**

Chun Zhou, Serge Przedborski. Intrabody and Parkinson's disease. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, 2009, 1792 (7), pp.634. 10.1016/j.bbadis.2008.09.001 . hal-00562855

**HAL Id: hal-00562855**

**<https://hal.science/hal-00562855>**

Submitted on 4 Feb 2011

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# Accepted Manuscript

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PII: S0925-4439(08)00167-1  
DOI: doi: [10.1016/j.bbadis.2008.09.001](https://doi.org/10.1016/j.bbadis.2008.09.001)  
Reference: BBADIS 62850

To appear in: *BBA - Molecular Basis of Disease*

Received date: 15 July 2008  
Revised date: 22 August 2008  
Accepted date: 3 September 2008



Please cite this article as: Chun Zhou, Serge Przedborski, Intrabody and Parkinson's disease, *BBA - Molecular Basis of Disease* (2008), doi: [10.1016/j.bbadis.2008.09.001](https://doi.org/10.1016/j.bbadis.2008.09.001)

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**Intrabody and Parkinson's disease**

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**Abstract**

The intrabody technology has become a promising therapeutic avenue for a variety of incurable diseases. This technology is an intracellular application of gene-engineered antibodies, aimed at ablating the abnormal function of intracellular molecules. Parkinson's disease (PD) is a common neurodegenerative disease with no cure. Recent studies have explored possible intrabody applications against alpha-synuclein ( $\alpha$ -syn), whose misfolding is believed to cause a familiar form of PD. Here, we review the origin, production, and therapeutic mechanisms of intrabodies and the potential of intrabody protection against  $\alpha$ -syn toxicity. Furthermore, we propose possible intrabody applications against leucine-rich repeat kinase 2 (LRRK2), whose mutations are the most frequent known cause of familial and sporadic PD.

## Introduction

Recently, the intrabody technology has emerged as a novel tool to modulate the function of intracellular proteins [1]. Moreover, this technology has been applied in various research settings that model complex human pathological conditions such as Parkinson's disease (PD), the second most common neurodegenerative disorder after Alzheimer's dementia [2]. The pathological features of PD include a loss of dopaminergic neurons in the substantia nigra pars compacta [3] and the formation of the Lewy body (LB), a cytoplasmic proteinaceous inclusion composed principally of alpha-synuclein ( $\alpha$ -syn) [4]. Currently, while medicines such as Levodopa may help to manage symptoms, to date, no treatment has been shown to slow or stop the progression of PD. Although PD arises essentially as a sporadic condition, it is occasionally inherited [5]. In the past decade, studies on various proteins whose genetic mutations cause familial PD, such as  $\alpha$ -syn, parkin, DJ-1, PTEN-induced putative kinase 1 (PINK1), and leucine-rich repeat kinase 2 (LRRK2), have largely improved our understanding of possible pathogenic mechanisms of PD and have provided us with potential therapeutic targets for PD.

Based on a paucity of available intrabody studies in PD, the intrabody therapy for this common disorder has unambiguously a long path to explore. However, it holds one of the promises of a cure for PD since it aims to remedy the pathogenic abnormalities at the molecular level. In this review, to provide a general background, we will first discuss the origin, production and therapeutic mechanisms of intrabody. Then, we will discuss intrabody studies in PD, with focus on  $\alpha$ -syn. Finally, we will propose potential intrabody applications to prevent the cellular toxicities caused by mutant LRRK2, shedding light on intrabody studies in new PD-related scenario.

## Antibody and scFv

Antibodies, or immunoglobulins, are one of the major protein effectors of an immune system. A typical antibody

molecule consists of two heavy chains and two light chains, connected by disulfide bonds. Compared to the constant regions ( $C_H$  and  $C_L$ ), which share high homology among different antibodies, the variable regions ( $V_H$  and  $V_L$ ) are extremely variable, forming a unique three-dimensional structure, the antigen-binding site or Fv (Fig. 1). The Fv binds a structurally complementary molecule, known as an antigen (Ag). The high variability of Fv makes it possible for a natural antibody pool to recognize a broad range of antigens.

Antibodies have been broadly used for disease diagnosis and therapy mainly due to their high specificity and high affinity. An antigen determinant, also termed epitope, is the portion of an antigen that binds to an antibody. Antibody specificity is the property that enables antibodies to discriminate between two different antigen determinants. The strength with which an antibody binds to an antigen determinant is called affinity, usually described by the dissociation constant,  $K_D$  (mol/L, M). Since Kohler & Milstein described the monoclonal antibody (MAb) technology in 1975 [6], an ideal MAb with both high specificity and high affinity has been believed to be a “magic bullet” for target therapy. Although initial clinical trials with MAbs showed some promising antitumor effects [7], MAb therapy is hindered by the following major obstacles: human antimouse antibody (HAMA) responses generated by the immunogenicity of mouse MAbs, difficulties in identifying specific targets such as tumor-specific antigens, and the low efficacy of antibody enrichment at affected tissues [8].

To overcome the above issues, *in vitro* antibody display technologies have been developed to display a large repertoire of gene-engineered antibodies ( $>10^9$  members) on the surfaces of phage, yeast, or others, for rapid screening of a specific antibody of interest [9-14]. Gene-engineered antibodies refer to recombinant antibody molecules that are generated by expressing the cDNA of engineered antibody fragments, such as “ $V_H$ -linker- $V_L$ ” (Fig. 1). Usually, the engineered antibody fragments contain at least one complete or partial antigen binding site to retain antigen-binding function. Gene-engineered antibodies consist of many types of antibody molecules,

such as single-chain Fv (scFv, ~25 kDa) and Fabs (antigen binding fragments, ~55 kDa) (Fig. 1). Here, throughout this review, for the simplicity of demonstration, we use scFv to represent all other gene engineered antibodies.

ScFv, a key molecule of gene engineered antibodies, is composed of the  $V_H$  and  $V_L$  regions, which are connected by a flexible short linker with a major form of  $(Gly_4Ser)_3$  (Fig. 1) [15]. ScFv is attractive due to the following advantages. First, it has a small size of ~25 kDa (vs ~150 kDa of a typical antibody), yielding rapid renal elimination and better tumor tissue penetration [16-18]. Yet, it retains good antigen-binding specificity because the antigen-binding site is unaltered. Second, to generate a scFv, the coding gene segments of  $V_H$  and  $V_L$  regions are isolated either from a hybridoma expressing the MAb with desired specificity [19], or from a large antibody library with more than  $10^9$  members of diverse scFvs; the latter gives the potential for screening scFvs against virtually any possible antigen in a short time [20]. Third, most antibody libraries contain scFvs as human proteins, which do not induce HAMA responses, because these scFvs are made from the coding genes of  $V_H$  and  $V_L$  regions from human lymphocytes. Fourth, optimized scFv functional characteristics, such as high affinity, long retention time (the capability to stay in antigen-binding status), and enhanced protein stability, can be achieved by further mutagenesis of the scFv cDNAs and by further selection from sub-libraries containing these mutated scFvs [21]. Clearly, scFvs and other gene-engineered antibodies represent a type of promising reagent for targeted therapy. Furthermore, innovative technologies have largely improved the likelihood of an antibody being a “magic bullet” and are still undergoing further development, especially in the improvement of minimizing immunogenicity, in increasing specificity, affinity, and stability, and in employing new application scenarios. For example, recently, the application of scFv inside of human cells has become a completely novel type of molecular therapy, intrabody therapy.

## Intrabody

Intrabodies, or intracellular antibodies, here refer to gene-engineered antibodies (mainly scFvs) that are expressed intracellularly to modulate the function of their intracellular targets. Usually, cell transfections or infections with scFv cDNA-containing plasmids or viruses are the means to express scFvs intracellularly. Clearly, the intrabody technology is an intracellular application of gene-engineered antibodies.

### *Challenges*

To use scFvs intracellularly, a major obstacle is the fact that the cytoplasmic reducing environment prevents the formation of disulfide bonds. As shown in Fig. 1, disulfide bonds are necessary for optimized folding of the antigen binding site of scFv [22]. Thus, for most scFvs isolated from antibody libraries or from hybridomas, the antigen binding capacities are more or less decreased or even completely lost when these scFvs are expressed as intrabodies in the cytosol (Fig. 1) [23,24]. For this reason, to select the scFvs that can bind their antigens inside cells, researchers have developed the following two strategies: a) *in vitro* screening of scFvs from antibody libraries followed by further intracellular validation in mammalian cells [25,26]; b) intracellular scFv screening to identify intracellular functional scFvs directly [27-29]. As a result, the most critical parameter of a successful intrabody is its intrinsic folding capacity under a reducing environment, rather than its *in vitro* affinity [30]. In addition, there are other important challenges for intrabody therapy, such as the efficacy of gene delivery, which are not in the scope of this review.

### *Production of intrabodies*

As mentioned above, intrabodies can be generated by using direct intracellular scFv screening methods [27-29]. However, while these methods are waiting to be widely applied and tested, the following strategy remains as a feasible and widely used method to generate functional intrabodies (Fig. 2).

First, antigens are usually produced as recombinant proteins by expressing the antigen cDNAs in bacteria, followed by protein purification. Of note, some eukaryotic proteins require post-translational modifications for maximal folding/activity, whereas bacteria do not have the machinery required to accomplish complex modifications. Therefore, when these modifications are necessary, eukaryotic cells, such as yeast cells and insect cells, can be used for the production of recombinant proteins. In the cases that recombinant proteins are not readily available, or scFvs against specific epitopes are needed, synthesized peptides are often used as antigens. Numerous antibody libraries are available for scientific studies, such as synthetic phage libraries (the Tomlinson I and J antibody libraries, MRC Center for Protein Engineering, Cambridge, England) and naïve yeast libraries [11]. With the antigen and antibody libraries available, one can screen a set of scFvs specifically against an antigen according to standard protocols [9,11]. Once a set of binders are selected, it is important to characterize their *in vitro* features, including specificity, affinity, and epitopes. From this *in vitro* characterization, a set of scFvs with good antibody features usually can be acquired. However, only the scFvs with functionally intracellular binding to their specific targets can be used as intrabodies (see later discussion in the section of *in vitro* versus *in situ* characterization of scFvs).

### *Therapeutic strategies*

Since 1988, when a study showed that intracellular expression of the cDNAs of antibody heavy and light

chains against yeast alcohol dehydrogenase I successfully inactivated the enzyme *in vivo* [31], the intrabody technology has been broadly employed in the studies involving human diseases, mainly AIDS and cancer. The following strategies are used for intrabodies to ablate or restore the function of intracellular molecules. First, intrabodies can block the interaction between their target proteins and other molecules, such as proteins and nucleic acids. For example, human immunodeficiency virus type1 (HIV-1) protein Tat functions through binding with human cyclinT1 to promote the transcription elongation of HIV-1. By disrupting the molecular interaction between HIV-1 Tat and human cyclinT1, anti-human cyclinT1 intrabodies effectively block HIV-1 replication without causing cellular toxicity [32]. Second, by retaining their targets in a particular subcellular compartment, such as endoplasmic reticulum (ER), intrabodies can prevent their target molecules from being at their physiological locations to function [33]. In fact, by attaching an organelle-targeting sequence to an intrabody, the expression of intrabodies at specific subcellular compartments can be achieved, such as in nuclei, ER and lysosomes [34]. For instance, tagged with the ER-retention signal (SEKDEL), an anti-gp120 intrabody has been used to trap gp120, an envelope protein of HIV-1, inside the lumen of ER to prevent the assembly of new infectious virions [35]. Third, intrabodies can restore normal protein function that has been lost due to mutagenesis. For instance, p53 is a tumor suppressor gene that is mutated in ~ 50% of cancers. Mutation of p53 results in a loss of function of p53 protein by diminishing its DNA binding activity, which is required for its tumor suppressor function. An anti-p53 intrabody has been shown to restore the normal conformation and DNA binding activity of p53 and thus to suppress tumor progression [36]. Fourth, intrabody can prevent aberrant protein conformation and protein aggregation. This aspect will be discussed later in the section related to  $\alpha$ -syn.

### *Advantages*

Obviously, intrabodies are valuable for both functional studies of a given intracellular molecule and the development of novel therapeutics. In fact, compared to other similar technologies aimed at blocking protein function, such as RNA interference (RNAi), intrabody technology has the advantage of modifying certain domains of a protein without affecting other domains because intrabodies have domain-specific binding capabilities. In addition, intrabodies can also modify the three-dimensional structure of a protein and thus are important for both stabilizing active protein conformations and correcting aberrant protein conformations. The abnormal protein conformations are closely related to the pathogenic mechanisms of neurodegenerative diseases, such as PD. Moreover, for proteins with relatively long half-lives or for proteins whose mRNAs have short half-lives, RNAi may be less effective while intrabodies can still function effectively through direct binding to the proteins.

### *Efficacy in animal studies and clinical trials*

Geared toward a molecular therapy for human diseases, the intrabody technology has been applied in animal studies and clinical trials. For instance, the neutralizing anti-Ras intrabody Y13-259, which induces *in vitro* transient phenotypic reversion of rodent fibroblasts transformed by *ras*, an *oncogene*, has been shown to invoke sustained tumor regression in nude mice [37]. Moreover, an ER-targeted intrabody against Tie-2, a receptor that is up-regulated in capillaries during neovascularization processes produced in tumors, has been shown to significantly inhibit tumor growth in mouse models [38]. More recently, the intrabody EM48 has been shown to suppress neuropil aggregates in a mouse model of Huntington's disease (HD) [39], thus indicating promising applications of intrabodies in neurodegenerative diseases. In the Phase I clinical trial using an ER-targeted

anti-erbB-2 intrabody, which had been previously shown to prolong survival in human ovarian cancer xenograft models [40], of 13 ovarian cancer patients with erbB-2 overexpression, 5 (38%) had stable disease and 8 (61%) had evidence of progressive disease [41]. Except for showing that the intrabody approach is feasible for human application with very limited side effects, this clinical study indicates that compared to cellular and animal studies, the intrabody technology, including gene delivery methods, needs to be further developed to achieve a dramatic effect for the therapy of human diseases.

### **Intrabody and PD**

In light of the initial successful application of intrabodies in the studies of AIDS and cancer, the intrabody technology has been employed in the studies of neurodegenerative diseases with the hopes of preventing abnormal protein aggregation. For instance, in HD, the polyglutamine expansion in the huntingtin (htt) protein causes htt aggregation, which is thought to be toxic to cells. It has been shown that anti-htt intrabodies can inhibit mutant htt aggregation and thus rescue cells from death [42,43] and the anti-htt intrabody C4 shows *in vivo* efficacy of protection in a *Drosophila* model of HD [44]. Moreover, the intrabody EM48 has been shown to suppress neuropil aggregates in a HD mouse model [39]. Another example in the study of Alzheimer's disease is the use of intrabodies to inhibit the generation of the toxic amyloid beta-peptide, by blocking the beta-secretase cleavage site or by retaining the beta-amyloid precursor protein in ER [45].

In the following sections, we will discuss the intrabody application in PD. Although the intrabody technology can be used for studying all PD-related proteins, here, we focus on  $\alpha$ -syn and LRRK2, two proteins that cause autosomal dominant PD, to demonstrate a proof of principle of existing and future intrabody applications in PD.

### *$\alpha$ -Syn*

$\alpha$ -Syn is a 140 amino acid protein that belongs to the synuclein protein family, which also includes  $\beta$ -syn and  $\gamma$ -syn. Previous studies using both optical methods and NMR have shown that  $\alpha$ -syn exhibits a random-coil structure (or natively unfolded structure) under physiological conditions [46,47]. Mutations in  $\alpha$ -syn cause a rare form of PD. The fact that  $\alpha$ -syn is the major component of LB, a hallmark of PD, has put  $\alpha$ -syn under intense study that eventually led to the consensus that protein aggregation is one of the major pathogenic mechanisms of PD. Although it is debatable whether LBs are toxic or protective and which forms of aggregated  $\alpha$ -syn such as oligomers versus fibrils mediate toxicity, it is clear that  $\alpha$ -syn is a natively unfolded protein that may acquire a gain of toxic function due to self-aggregation [48-51]. Therefore, the development of anti- $\alpha$ -syn intrabodies may be potentially useful in blocking  $\alpha$ -syn aggregation and therefore protect cells from toxic  $\alpha$ -syn aggregates.

Natively unfolded proteins have been categorized as a novel protein family – naturally unfolded (or intrinsically unstructured) protein family. It is hypothesized that these natively unfolded proteins often acquire ordered three-dimensional structures upon binding to their partners, such as proteins and nucleic acids, and modify the function of their partner molecules [52,53]. Based on the available knowledge of natively unfolded proteins and previous studies on  $\alpha$ -syn, we propose a simplified paradigm of possible  $\alpha$ -syn structure-function relations as a working hypothesis for developing potential protective intrabodies against PD (Fig. 3). In this schematic,  $\alpha$ -syn exists physiologically as a natively unfolded protein with the potential of transforming into the following three ordered structures. The available experimental evidence shows that the N-terminal sequence of  $\alpha$ -syn binds to acidic lipids and forms an  $\alpha$ -helical structure. One hypothesis is that  $\alpha$ -syn functions on cellular membranes once it acquires an ordered structure through binding to the membranes. In addition, like other natively unfolded proteins,  $\alpha$ -syn may also bind to other multiple targets to play an important role as a modifier of

various cellular pathways. While  $\alpha$ -syn may utilize its unfolded structure to adopt several possible ordered structures to function, this possible biological benefit comes with the following potential downside:  $\alpha$ -syn can form a stable  $\beta$ -sheet structure by self-binding under certain conditions. The latter include increased  $\alpha$ -syn protein levels exceeding a threshold, genetic mutations which cause potency for self-oligomerization, changes in physiological conditions including oxidative stress, and post-translational modifications including dopamine- $\alpha$ -syn adducts. As a result, when the protein quality control system of a cell becomes impaired, the physiological balance that favors the formation of beneficial  $\alpha$ -syn structures may abnormally favor  $\alpha$ -syn self-aggregation. Such self-aggregation may in turn underlie the formation of  $\alpha$ -syn aggregates, fibrils and LBs.

Based on the above model, it is tempting to develop intrabodies that can specifically block the self-binding of  $\alpha$ -syn without affecting the binding of  $\alpha$ -syn to its physiological partner molecules (Fig. 3). In the pioneer studies aimed at this goal, various scFvs against  $\alpha$ -syn have been generated and we have listed the majority of these in Table 1. In the following, we will discuss several critical issues based on the available studies of anti- $\alpha$ -syn scFvs and intrabodies to shed light on future studies.

*Source of intrabodies:* As potential therapeutics for human diseases, intrabodies are often generated by screening scFvs from human antibody libraries rather than by cloning scFv gene segments from mouse hybridomas. Current available anti- $\alpha$ -syn scFvs/intrabodies are selected from three phage libraries and one yeast library (Table 1). ScFvs F8 [54], D10 [25], 6E [55], D5 [56] are selected from two synthetic libraries that contain synthesized variable regions of scFvs. On the other hand, scFvs NAC3, NAC24, and NAC32 [26] are selected from a yeast library that is composed of scFv gene segments from the mRNA pool of 58 human adult spleens and lymph nodes. In addition, scFvs 3, 14 and 15 [57] are from a phage library generated using the mRNAs from human peripheral blood lymphocytes of a large (>100) pool of donors. Thus, all the scFv libraries

currently used for generating anti- $\alpha$ -syn scFvs are naïve libraries in which scFv gene segments are from unimmunized individuals or are artificially synthesized. This suggests that, despite the unique unfolded structure of  $\alpha$ -syn, it is feasible to screen anti- $\alpha$ -syn scFvs from current available large scFv libraries, no matter what display platform a library adopts, or what screening method a library employs. For example, phage libraries use a panning protocol in which immobilized antigens are coated on a solid surface, while yeast libraries use a flow cytometry sorting protocol with free antigens in solution (see [9] and [11] for detailed method descriptions). Meanwhile, it also indicates that the initial screened scFvs from these naïve antibody libraries may need to be engineered further to improve their affinities for the natively unfolded protein  $\alpha$ -syn, or to increase their thermal stabilities and antigen-binding specificities.

*Antigens used for screening scFv libraries:*  $\alpha$ -Syn contains an N-terminal amphipathic region, a central hydrophobic region known as the nonamyloid component (NAC, aa 61-95), and a C-terminal acidic tail. It has been reported that the NAC region is the core sequence responsible for  $\alpha$ -syn aggregation [58,59]. The N-terminal sequence has a propensity to bind with acidic lipids whereas the C-terminal region appears to be involved in  $\alpha$ -syn aggregation [60,61]. Currently, the accurate molecular mechanisms of  $\alpha$ -syn self-binding and the formation of stable oligomers and fibrils are unclear. Therefore, there are no clear strategies for predicting antigenic epitopes to produce anti-aggregation scFvs, but to explore all of the possibilities. That is to use  $\alpha$ -syn peptides,  $\alpha$ -syn monomers, and  $\alpha$ -syn conformers (including  $\alpha$ -syn oligomeric aggregates and fibrils) as antigens. With these antigens, it is hopeful that scFvs may be generated against two kinds of potentially useful epitopes to inhibit  $\alpha$ -syn aggregation. One type of useful epitope is the linear  $\alpha$ -syn sequences that are the self-binding sites of two or more  $\alpha$ -syn monomers. Among scFvs that can bind to these sequences, it is reasonable to select those that can block the self-binding sites and thus inhibit  $\alpha$ -syn oligomer formation. The identified sequences in the

NAC region (aa 68-76 or aa 71-82), which are required for  $\alpha$ -syn aggregation, are the most promising candidates in this aspect. Indeed, scFv NAC32 is selected against a peptide containing the amino acids 53-87 of  $\alpha$ -syn. When tested in mammalian cells as an intrabody, it inhibited  $\alpha$ -syn aggregation and reduced the cytotoxicity caused by mutant  $\alpha$ -syn. However, we cannot exclude other N-terminal or C-terminal sequences as useful antigenic epitopes, since we do not know how these two regions play their roles in  $\alpha$ -syn aggregation. Another type of useful epitope is the three-dimensional structures that are important for aggregation formation. ScFvs against this kind of epitope are also expected to block  $\alpha$ -syn self-aggregation. For example, scFv D10 recognizes a conformational epitope, inhibits the formation of a high molecular weight  $\alpha$ -syn species and rescues impaired cell adhesion caused by overexpressed  $\alpha$ -syn. For these available anti- $\alpha$ -syn scFvs, a detailed description of the relationship between the epitopes and their scFvs' capacity to inhibit  $\alpha$ -syn aggregation can be found in Table 1.

*In vitro versus in situ characterization of scFvs:* As we mentioned before, antigen-binding efficiencies of an scFv between *in vitro* and intracellular conditions are often not consistent due to the reducing environment of the cytosol. For example, both scFv F8 and scFv D10 are selected against recombinant monomeric  $\alpha$ -syn from a human synthetic antibody library. ScFv F8 shows high affinity ( $K_D = \sim 10^{-8}$  M) whereas scFv D10 has only low affinity ( $K_D = \sim 10^{-6}$  M). However, when expressed intracellularly, scFv F8 is very unstable whereas scFv D10 is quite stable. Surprisingly, scFv D10 is able to bind  $\alpha$ -syn inside of mammalian cells and, further, to inhibit the formation of a high molecular weight  $\alpha$ -syn species. The phenomenon of low-affinity scFvs being functional intrabodies might be addressed by two explanatory models of antigen-antibody interaction, the “lock-and-key” and “induced fit” models. In the “lock-and-key” model, the structures of the antigen-binding site and its corresponding antigen are highly complementary. Therefore, only minimum conformational changes are required when the antibody binds to its antigen, yielding high affinity. On the other hand, in the “induced fit” model,

antigens, antibodies, or both, undergo conformational adjustments to facilitate antibody-antigen binding, thus resulting in low affinity [62]. It is likely that due to high intracellular stability, some low affinity scFvs, such as scFv D10, can accumulate to a certain amount in the cytosol and bind their antigens through an “induced fit” mechanism. Consistent with this hypothesis, other studies also revealed that the stability is much more critical than the *in vitro* affinity for an scFv to be a functional intrabody [29]. Therefore, while *in vitro* characterization is helpful for identifying scFv features, it is critical to assess the performance of scFvs inside of mammalian cells in order to generate functional intrabodies.

*Intracellular characterization of intrabodies:* From the above, it is apparent that intracellular stability and “intracellular affinity” are two critical aspects for evaluating the potential of an scFv to be a functional intrabody. To evaluate intracellular stability of scFvs, the pulse-chase method is a classic way to determine protein half-lives. In general, an scFv with a half-life of over 8 hours would likely have a high intracellular steady state level in order to be a functional intrabody. Meantime, it is also important that a stable scFv can fold correctly in the cytosol to maintain its antigen-binding efficiency in a reducing environment, i.e. “intracellular affinity”. Unfortunately, unlike *in vitro* affinity assays, such as BIAcore, a direct intracellular affinity assay is currently not available. Therefore, two intrabody binding assays have been established to test indirectly for binding efficacy between intrabodies and their antigens. One strategy is to express scFvs tagged with organelle-targeting sequences to gather scFvs at certain subcellular compartments, such as nuclei. If an scFv binds to its intracellular antigen efficiently, it can convey its antigen to the same organelle. As a result, both the scFv and its antigen can be found to be enriched and colocalized in the same organelle by immunocytochemistry. For example, co-overexpression of D10-NLS (nuclear localization signal) and  $\alpha$ -syn causes the enrichment and co-localization of both proteins in the nuclei [25]. Of note, in scFv/antigen co-transfection experiments, the degree of accumulation of the scFv/antigen to a

certain organelle varies from cell to cell. Thus, a strict quantification of the percentage and degree of the colocalization would be helpful. For this, one can use subcellular fractionation with Western blotting to assess the colocalization efficacy, which can be quantified with image quantification softwares, such as Scion Image and NIH Image. Another method to show the binding between intrabodies and their antigens is the co-immunoprecipitation assay. In this case, intrabodies and their antigens will be considered just as two proteins overexpressed intracellularly. Co-immunoprecipitation is used to evaluate whether or not intrabodies and their antigens bind to each other inside of cells. For instance, the D10 intrabody shows dose-dependent co-immunoprecipitation with  $\alpha$ -syn [25]. In general, when the above two strategies are applied, they may reveal whether an scFv can bind its antigen effectively as an intrabody. For the future, a direct and real-time binding assay is needed to assess the “intracellular affinity” of intrabodies in a more accurate way so that an “intracellular  $K_D$ ” and a retention time can be given. Such elegant intracellular quantification assays will improve significantly the evaluation of intrabody characters.

*Evaluation of anti- $\alpha$ -syn intrabody protective effects:* Once efficient anti- $\alpha$ -syn intrabodies have been identified, it is critical to evaluate whether or not these intrabodies are protective. Given that the physiological function of  $\alpha$ -syn is still unclear, it is necessary to test the protective effect of intrabodies on both of the inhibition of  $\alpha$ -syn aggregation and rescue from cytotoxicity caused by  $\alpha$ -syn, which model the pathogenic hallmarks of PD --- Lewy body and neuron death. The thioflavin T aggregation assay and atomic force microscope (AFM) imaging are widely used for *in vitro* study of  $\alpha$ -syn aggregation. Due to its aggregation-prone feature,  $\alpha$ -syn forms self-aggregates in common solvents, such as phosphate buffers. By monitoring thioflavin T fluorescence intensities, the kinetics of  $\alpha$ -syn aggregation can be established which allows one to evaluate whether or not an intrabody inhibits  $\alpha$ -syn aggregation [49]. The AFM analysis, another *in vitro* tool, is often used to observe  $\alpha$ -syn

aggregates directly, allowing identification of various  $\alpha$ -syn aggregation stages, from small aggregates to filaments [49, 52]. At the cellular level, except for analyzing  $\alpha$ -syn by immunoblotting [25], fluorescence-tagged  $\alpha$ -syn may be used to model  $\alpha$ -syn aggregation. For instance, the  $\alpha$ -syn-GFP fusion protein has been used to model  $\alpha$ -syn aggregation and its abnormal effects in yeast [63]. Similarly, when a fusion protein of  $\alpha$ -syn-red fluorescent protein (RFP) is overexpressed in human neuroblastoma SH-SY5Y cells, we observed that the majority of overexpressed  $\alpha$ -syn-RFP formed perinuclear aggregates that are both  $\alpha$ -syn and RFP positive (Fig. 4A). Furthermore, fluorescent immunoblotting shows that the majority of overexpressed  $\alpha$ -syn-RFP is full-length protein (Fig. 4B), thus supporting  $\alpha$ -syn-RFP overexpression as a model of  $\alpha$ -syn aggregation in mammalian cells. Recently, cytotoxicity assays using ST14A neuronal cells have been utilized to test the protective effect of intrabodies against  $\alpha$ -syn toxicity. Along this line, other established  $\alpha$ -syn cellular models, such as inducible  $\alpha$ -syn overexpression in PC12 cells [64] and overexpression of  $\alpha$ -syn in primary cultured dopaminergic neurons [65], may also be useful for the evaluation of the key issue --- the protective effects of anti-  $\alpha$ -syn intrabodies against neuronal death. Perhaps, the more challenging tests are whether anti- $\alpha$ -syn intrabodies can perform protective functions *in vivo*, especially in  $\alpha$ -syn models in mice [66], *Drosophila* [67], *Caenorhabditis elegans* [68], etc.

*Intrabody optimization:* It is worth pointing out that after the initial identification of functional intrabodies that can bind to their antigens intracellularly, certain optimization strategies can be applied to obtain robust intracellular stability and “intracellular affinity” (Fig. 2). For example, the affinity of scFvs derived from naïve antibody libraries often can be further increased by random mutagenesis. Such mutagenesis can create sub-libraries with  $\sim 10^6$  mutated scFvs. If sub-libraries are screened against the same antigen, a set of scFvs with higher affinities can be obtained [69]. Recently, it has been shown that using yeast scFv display, scFvs with high

thermal stability can also be achieved by mutagenesis [70]. With these further steps, intrabodies with optimized intracellular stability and affinity can be achieved eventually. Such robust intrabodies should be ready for further *in vivo* applications, including clinical trials.

*Summary:* As discussed above, anti- $\alpha$ -syn scFvs/intrabodies have been generated and tested for their potential protective effects against  $\alpha$ -syn malfunction. While these intrabodies show promising protective effects, more extensive studies are needed in the following two areas. First, robust intrabodies with optimized intracellular stability and “intracellular affinity” are needed in order to make intrabody as an intracellular “magic bullet”. Currently, only two anti- $\alpha$ -syn scFvs, D10 and NAC32, have shown promising effects as intrabodies in inhibiting  $\alpha$ -syn aggregation and in rescuing  $\alpha$ -syn toxicity. While both scFvs show relative intracellular stability, they all have low affinity. Importantly, the production of anti- $\alpha$ -syn scFvs with high “intracellular affinity” is more likely to provide robust intrabodies as possible therapeutics for PD. To achieve this goal, as we proposed, it would be helpful to establish real-time analyses to evaluate intracellular intrabody- $\alpha$ -syn dynamic interaction. That way, the intracellular binding efficacy of anti- $\alpha$ -syn intrabodies can be quantified and further used to generate high “intracellular affinity” through mutagenesis. Currently, since such “intracellular affinity assays” are unavailable, it may be important to generate the scFvs that have both intracellular stability and *in vitro* high affinity, with the assumption that given the intracellular stability, the *in vitro* affinity may reflect a certain degree of “intracellular affinity”. A strategy for generating high affinity scFvs with high intracellular stability is given in Fig. 2. In addition, the specificity of anti- $\alpha$ -syn intrabodies for other synuclein family proteins, such as  $\beta$ -syn and  $\gamma$ -syn, need to be tested. For instance, the D10-NLS construct failed to convey  $\beta$ -syn to the nucleus, thus indicating its relative specificity for  $\alpha$ -syn inside cells. This specificity issue also applies to other available anti- $\alpha$ -syn scFvs listed in Table 1. The highly specific scFvs against  $\alpha$ -syn, but not its homologous proteins, are more likely to avoid

disturbing other normal cellular functions. Second, the current studies of anti- $\alpha$ -syn intrabodies are pioneer work for demonstrating proof of principle. To generate valuable anti- $\alpha$ -syn intrabodies as therapeutics for PD, in-depth studies are needed to answer the following questions: how intrabodies inhibit  $\alpha$ -syn aggregation; whether the binding of intrabodies with  $\alpha$ -syn can change  $\alpha$ -syn degradation; whether or not and how intrabodies can provide significant protection against dopaminergic neuron death; etc. Furthermore, to overcome the intrinsic limitation of studies using cellular models, *in vivo* intrabody effects on  $\alpha$ -syn pathology could provide critical insight into the potential of intrabody therapy for PD.

### *LRRK2*

In the following, we will discuss briefly a possible use of intrabodies as potential therapeutics against cellular toxicity caused by LRRK2. Recently, the importance of LRRK2 in PD has been highlighted by the fact that LRRK2 is currently the most frequent genetic cause of all PD cases.

LRRK2 is a large complex protein of 2,527 amino acids. It contains several predicted functional domains: Roc (Ras in complex proteins), COR domain (C-terminal of ROC), a leucine-rich repeat (LRR), a protein kinase domain, a WD40 domain and an ankyrin domain. The ROC domain has GTPase activity. Currently, the functions of some of these domains, such as Roc, LRRs, Ankyrin, and WD40 domains, are unknown.

Certain PD-linked LRRK2 mutations, such as G2019S and I2020T, increase LRRK2 kinase activity, thus leading to neuronal toxicity [71-73]. Accordingly, abolishing the kinase activity attenuates cytotoxicity caused by mutant LRRK2, confirming that the kinase activity is likely a prerequisite for the toxic effects of mutant LRRK2 [74]. Therefore, blocking LRRK2 kinase activity by intrabodies could be a potential therapeutic strategy against aberrant LRRK2 toxicity, especially when using kinase domain-specific intrabodies, which do not affect the

function of other LRRK2 domains.

In addition, it has been shown that LRRK2 exists as a protein complex, including homodimers. LRRK2 homodimerization is dependent upon the interaction of the ROC GTPase domain and several other domains, such as the LRR and WD40 domains [75]. Interestingly, it has also been found that LRRK2 can form aggregates under certain circumstances. For example, overexpression of mutant LRRK2 has led to the formation of cytoplasmic aggregates or inclusions in various cell types, suggesting that LRRK2 mutations promote protein misfolding [74,76,77]. Therefore, intrabodies that can block LRRK2 misfolding may be protective.

Except for self-binding, LRRK2 has been shown to interact with other proteins, such as parkin [76] and Rab5b [78]. In addition, LRRK2 may function on cellular membranes [71,79,80] or through binding to microtubules [81]. With the function and toxicity of LRRK2 being further elucidated, intrabodies may be useful for blocking specific molecular events required for the gain of toxicity of LRRK2.

## Prospectives

Clearly, intrabodies provide a useful tool to study protein function intracellularly and, more importantly, have the potential to serve as therapeutics for PD. Further development of intrabody technology, such as new approaches to generating robust intracellular stability and high “intracellular affinity”, novel intrabody-effector fusion proteins, organelle-specific intrabodies, and protein domain-specific intrabodies, will maximize intrabody therapeutic efficacy. In-depth studies of the intrabody characters and their anti-PD effects and mechanisms in various PD models, from *in vitro* to *in vivo*, will provide solid evidence for exploring the potential of intrabody therapeutic application. The development of gene delivery methods, which are the means of expressing intrabody in target cells, will increase the *in vivo* therapeutic efficiency of intrabodies. In addition, since the

studies on the molecular mechanisms of PD pathology are advancing quickly, newly identified “toxic” molecules will provide additional targets for the intrabody therapy. Together, multi-field technological and knowledge advances related to the intrabody therapy hold one of the promises for the cure of PD.

**Acknowledgements.** We thank Drs. Anne Messer (Wadsworth Center, New York State Department of Health) and Howard Federoff (Georgetown University) for their insightful comments on the manuscript. The authors are supported by grants from the US National Institutes of Health (NS062180, NS11766-27A1, AG21617-01A1, NS38370-09), the US Department of Defense (DAMD 17-03-1-02), the Parkinson’s Disease Foundation, the Hartman Foundation, the Muscular Dystrophy Association/WOW.

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## FIGURE LEGENDS

Figure 1. Schematic representation of antibody, scFv, and intrabody molecules. A typical antibody structure consists of two heavy chains and two light chains, which contain disulfide bond linked domains. Each chain has a variable region ( $V_H$  or  $V_L$ ) and a constant region ( $C_H$  or  $C_L$ ). While the antigen binding site, Fv, refers to the structure formed by  $V_H$  and  $V_L$  regions, a Fab molecule contains a light chain ( $V_L$  and  $C_L$ ), a  $V_H$  region and the first domain of a  $C_H$  region, linked by disulfide bond. The  $V_H$  and  $V_L$ , linked by a flexible linker peptide, form scFv, a single valent antigen-binding molecule. In the cytosol of mammalian cells, based on its intrinsic folding property, scFvs have various stabilities in terms of forming functional antigen-binding molecules.

Figure 2. Schematic representation of the production of scFvs of interest, identification of functional intrabodies, and further optimization of intracellular stability and affinity. This approach applies with various antibody display system, including phage display and yeast display antibody technologies, toward yielding robust intrabodies as therapeutics.

Figure 3. A central molecular hypothesis for the intrabody therapy against the  $\alpha$ -syn pathology. By blocking  $\alpha$ -syn self-aggregation, intrabodies restore the balance between the natively unfolded structure of  $\alpha$ -syn and its ordered philological structures, rendering therapeutic effects.

Figure 4. An  $\alpha$ -syn aggregation model in human neuroblastoma SH-SY5Y cells. (A) While cells expressing RFP show homogenous distribution of RFP (red signals) without apparent protein aggregates,  $\alpha$ -syn-RFP transfected cells have perinuclear RFP-containing protein aggregates (red signals). Nuclei are shown with DAPI labeling (blue signals). In addition, the RFP-positive protein aggregates (red signals) are also  $\alpha$ -syn positive by the immunostaining with a mouse monoclonal anti-  $\alpha$ -syn antibody (BD Biosciences, San Jose, CA) (blue signals). The protein aggregates indicated by arrows are shown at higher magnification in the insets. Scale bar,

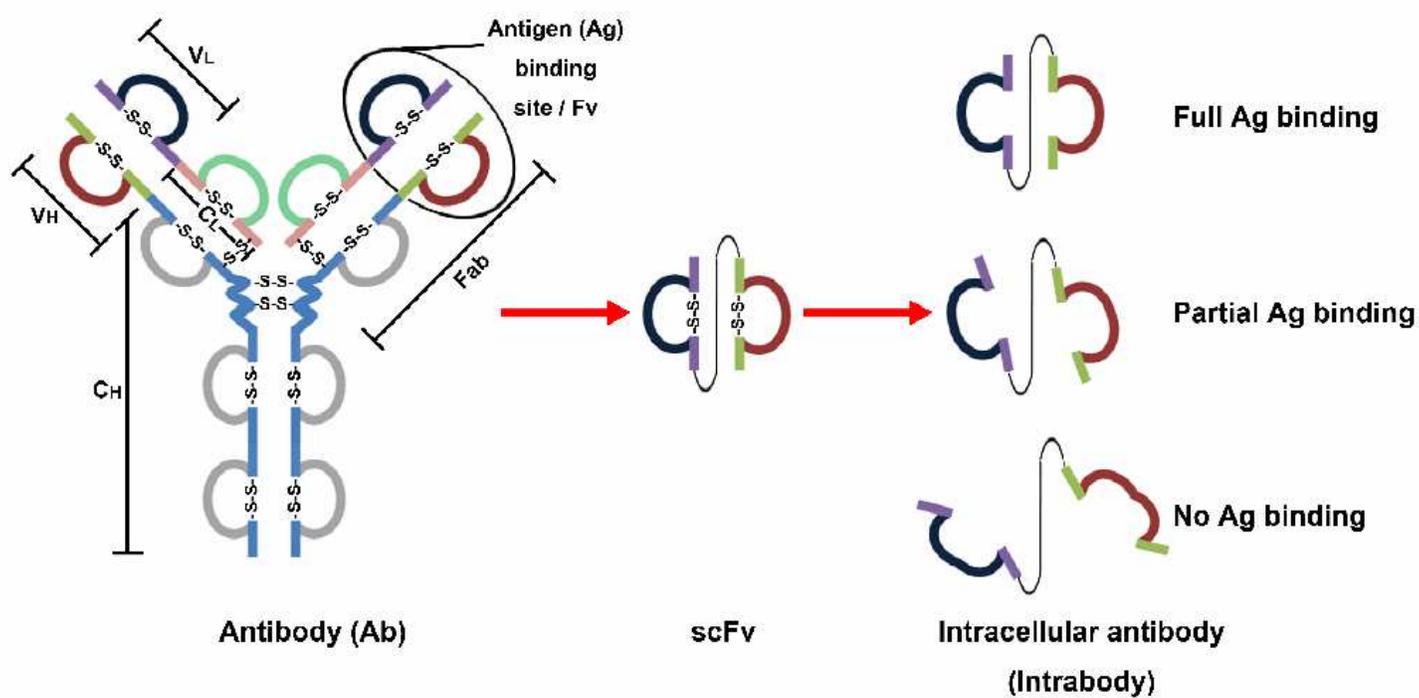
5  $\mu$ m. (B) The whole cell lysates extracted from RFP or  $\alpha$ -syn-RFP transfected cells are subjected for fluorescent immunoblotting [82].  $\alpha$ -Syn is detected by the anti- $\alpha$ -syn antibody from BD Biosciences (green signals) and RFP is detected by a rabbit polyclonal anti-RFP antibody (Clontech Laboratories, Mountain View, CA) (red signals). The merged image shows that the ~45 kDa protein species is both  $\alpha$ -syn and RFP positive, thus indicating that the majority of overexpressed  $\alpha$ -syn-RFP is full-length (~45 KD) and further supporting that the perinuclear protein aggregates, shown in (A), contain full-length  $\alpha$ -syn-RFP.

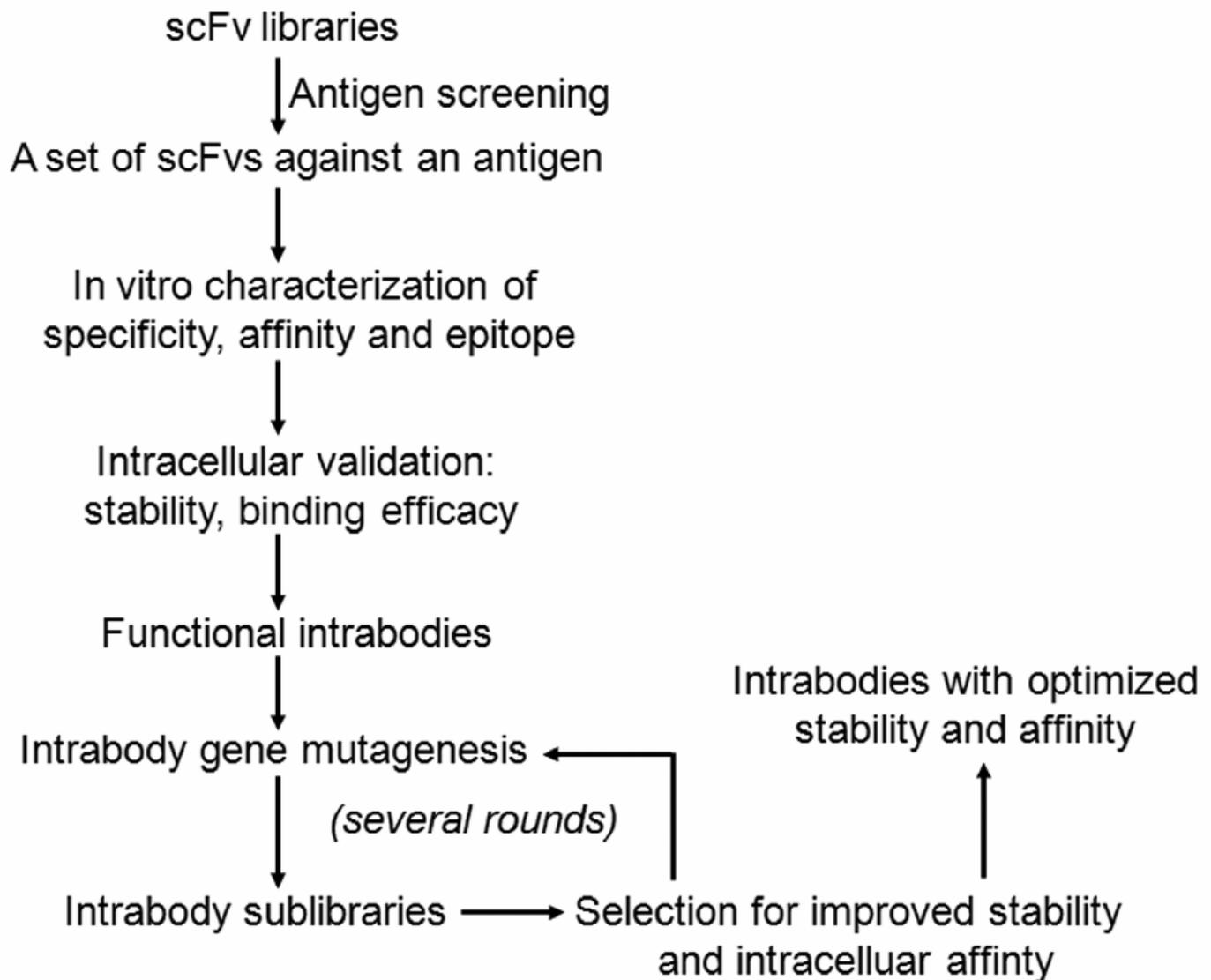
**Table 1.** Published scFvs against  $\alpha$ -syn

Name	Source library	Epitope (a.a.)	Binding to. $\alpha$ -syn aggregates	Affinity ( $K_D$ , M)	<i>In vitro</i> effects	Intracellular effects
F8 <sup>[49]</sup>	Phage	101-111/27-37	?	$10^{-8}$	Inhibit aggregation	Unstable expression
D10 <sup>[25]</sup>	Phage	*	yes	$10^{-6}$	?	Inhibit $\alpha$ -syn aggregation and rescue impaired adhesion
6E <sup>[50]</sup>	Phage	*	yes	?	?	?
3 <sup>[52]</sup>	Phage	71-85	yes	?	?	?
14 <sup>[52]</sup>	Phage	106-120	yes	?	?	?
15 <sup>[52]</sup>	Phage	117-131	yes	?	?	?
D5 <sup>[51]</sup>	Phage	*	yes	?	Inhibit aggregation	?
NAC3 <sup>[26]</sup>	Yeast	61-78	?	?	?	No significant protection
NAC24 <sup>[26]</sup>	Yeast	61-78	?	?	?	Unstable expression and no significant protection
NAC32 <sup>[26]</sup>	Yeast	53-87	?	?	?	Inhibit $\alpha$ -syn aggregation and rescue cytotoxicity

?: not reported.

\*: The scFvs D10, 6E, and D5 recognize  $\alpha$ -syn conformational epitopes, although the amino acid sequences that constitute the epitopes are not identified.





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