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REVIEW

Intrabodies as Neuroprotective Therapeutics

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Abstract The process of misfolding of proteins that can trigger a pathogenic cascade leading to neurodegenerative diseases largely originates intracellularly. It is possible to harness the specificity and affinity of antibodies to counteract either protein misfolding itself, or the aberrant interactions and excess stressors immediately downstream of the primary insult. This review covers the emerging field of engineering intracellular antibody fragments, intrabodies and nanobodies, in neurodegeneration. Huntington's disease has provided the clearest proof of concept for this approach. The model systems and readouts for this disorder power the studies, and the potential to intervene therapeutically at early stages in known carriers with projected ages of onset increases the chances of meaningful clinical trials. Both single-chain Fv and single-domain nanobodies have been identified against specific targets; data have allowed feedback for rational design of bifunctional constructs, as well as target validation. Intrabodies that can modulate the primary accumulating protein in Parkinson's disease, alpha-synuclein, are also reviewed, covering a range of domains and conformers. Recombinant antibody technology has become a major player in the therapeutic pipeline for cancer, infectious diseases, and autoimmunity. There is also tremendous potential for applying this powerful biotechnology to neurological diseases.

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Biological Antibody Therapies for Misfolding Proteins

The problem of misfolding proteins appears to underlie a significant fraction of neurodegenerative diseases. Protein homeostasis, often referred to as proteostasis, is an especially critical process in postmitotic neurons. The balance of pathways for protein folding, interactions, intracellular localizations, and degradation is a complex one, and can be dysregulated by combinations of mutations, environmental stressors, and aging. We have taken the neurotherapeutic approach of normalizing or manipulating proteostasis using engineered intracellular antibody fragments—intrabodies—that bind with high specificity to selected targets. These intrabodies can be selected and manipulated as genes, allowing the full spectrum of genetic engineering to produce multifunctional constructs that can alter the folding, interactions, intracellular localization, and turnover kinetics/ levels of the target protein. Intrabodies are also valuable molecular tools, which can be used to dissect the functional and pathogenic motifs in these proteins and that could be useful for the development of alternative therapeutics. In this review, we will cover the development of this approach for Huntington's disease (HD), which has a well-described target, evaluating effects in several cell culture and *in vivo* systems with strong readouts for therapeutic efficacy, and advances in engineering anti-HD intrabodies. We also cover a small, but growing, literature on engineering antibody therapies that target alpha-synuclein (α-Syn) for Parkinson's disease (PD)/other synucleinopathies, and the potential interactions of HD and PD targets.

Biological therapies, many of them antibody-based, currently represent 30 % of the new drugs in development, a figure that is increasing yearly. Clinical trials using passive or active antibody therapeutics for Alzheimer's disease (AD) have

given mixed results, probably owing to the initiation of the therapy at a stage where the pathogenic process was already substantially advanced in most cases. For HD, gene-positive individuals would have an option to begin therapies at much earlier premanifest points. For "sporadic" neurodegenerative diseases, combinations of clearer understanding of genetic risk factors, whole genome sequencing, and biomarkers may offer much earlier intervention options to enhance the potential of the class of neurotherapeutics covered in this review.

What is an Intrabody?

An intrabody is a small antibody fragment that targets antigens intracellularly. The Fv variable regions responsible for antibody specificity can be expressed separately from the full-length immunoglobulin, retaining many of the advantages of conventional antibodies, including high specificity and affinity for target epitopes. These fragments are only 140–250 amino acids (AAs) in length, and they can be manipulated and delivered as genes or as proteins. Critically, they lack the potentially inflammatory Fc region. Intrabodies were first reported in 1988 [1], and they have been studied extensively as potential therapeutics for infectious diseases [2–5] and cancer [6–8]. Our work and that of others has recently exploited the specificity and affinity characteristics of intrabodies to combat neurodegenerative diseases that share the cellular and molecular features of protein misfolding and aggregation [9–13].

In single-chain Fv (scFv; sometimes also abbreviated as sFv) intrabodies, the variable heavy (VH) and light chain (VL) binding domains of an antibody are genetically linked using a flexible hinge sequence, generally $(Gly_4Ser)_3$ or $(Gly_4Ser)_5$ [14] (Fig. 1). Antigen binding sites were initially thought to reside in the pocket between the VH and VL chains; however, examples of antigen binding that is exclusively on one or the other chain are also relatively common, with the second chain serving to stabilize the folding of the first. Single-domain antibodies/nanobodies (VH or VL) may have additional advantages as they are even smaller and less bulky than scFvs. Camelid nanobodies are small heavy-chain-only antibody fragments (VHH) from naturally-occurring heavy-chain-only antibodies made in alpacas, llamas, and camels [15] (Fig. 1). A high fraction are extremely stable, can fold correctly under a wide range of conditions, and are being tested as gene and protein therapeutics [16, 17]. A phage display synthetic library derived from such fragments yielded a VHH specific to Aβ fibrils [18]. Recently, a llama-derived VHH against misfolded mutant PABPN1, a protein implicated in oculopharyngeal muscular dystrophy, was reported to suppress muscle degeneration in *Drosophila* [19].

There are many considerations in considering the choice of selection methods, epitope conformations in context,

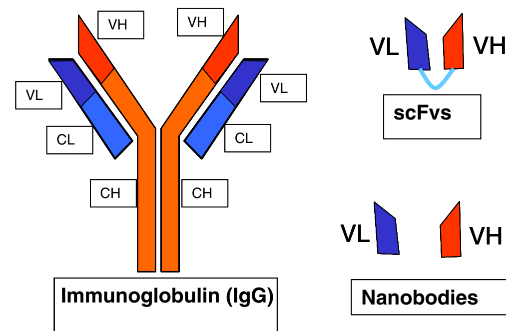

Fig. 1 Schematic of antibody and intrabody/nanobody structures. VH and VL are the heavy and light chain variable regions containing the specific antigen binding sites. CL and CH are the constant regions that mediate interactions. The single-chain Fv (scFv) is produced recombinantly by adding a flexible linker between the VH and VL chains

solubility (particularly with single domains), and whether the highest affinity is optimal for the application. These are covered in detail in Butler et al. [20]. In summary, *in vitro* and *in situ* selection conditions will, of necessity, differ from intracellular conditions, particularly when cells are stressed. However, judicious initial screening has the potential to generate valuable lead candidate intrabodies that can be further characterized *in situ* [21]. Lower affinities may be sufficient for many applications, and may be preferable when the goal is intrabody recycling or for use as a non-perturbing probe. Antibody engineering can be used to modulate a wide range of characteristics.

HD and Targets

Human Disease

HD is a progressive autosomal dominant neuropsychiatric disorder characterized neurologically by unsteady gait and uncontrolled movements, called chorea. There are also personality changes/mood disorders, sometimes quite severe, that can be the first presentation, particularly in younger patients. Progression generally proceeds for >15 years, with total care required at later stages. Neuropathologically, the most severe atrophy is seen in the corpus striatum and regionally in the cortex [22]. Gene-positive individuals show region-specific imaging changes in brain volume years in advance of clear clinical symptoms. At the cellular level, HD is characterized by large fibrillar aggregates that stain for both abnormal mutant huntingtin protein (mHTT) and ubiquitin. When observed in non-degenerated cells at autopsy, these are most commonly neuronal nuclear inclusions. HD was among first human genes mapped and later cloned, as the critical confirming example of CAG trinucleotide repeat expansions, leading to proteins containing excess polyglutamine (polyQ) tracts that could rapidly misfold [23]. Some similarities in the

pathogenic cascade for this family of diseases may lead to more rapid development of treatments.

Age of onset of symptoms corresponds roughly and inversely to the size of this repeat, with repeats >36 showing adult disease and repeats >60 showing juvenile onset [24]. The encoded protein, huntingtin (HTT), is 3188 AAs, with 67 exons. The first 17 AAs are followed by a variable polyQ tract, followed by a polyproline-rich region. This N-terminus of the protein, Exon1, is found as a cleavage product of the mutant protein, and data support its identification as the pathogenic species. While abnormal cleavage is not the dominant determining feature of HD in the same way that amyloid precursor protein cleavage is for AD, the HTT Exon1 fragment alone is more rapidly toxic than full-length protein in all models. HTT Exon1 is therefore a valid neurotherapeutic target. It should be noted that the germline repeat number may not accurately reflect the actual CAG or polyQ number found in HTT in the brain or other cells. The mutant CAG repeat number expands in post-mitotic cells with time, in both human HD and mouse HD models, with particular DNA mismatch repair enzymes as a major controller [25, 26]. This is an important consideration, both for modeling the size and aggregation kinetics of the protein that needs to be corrected, and as a potential source of a drug target.

HD Models for Testing

The identification of the gene and its truncated pathogenic product has allowed the creation of cellular and animal models of the disease, which are critical for therapeutic testing. As severity tracks with repeat number, it is also possible to accelerate the process tremendously by using very large numbers of repeats and including only HTT Exon1. There is a small number of human cases with such large repeats, confirming that very early and severe HD exists clinically, and that models using long repeats are not simply artifacts. Cultured cell lines transfected with HTT Exon1-polyQ-green fluorescent protein (GFP) show length-dependent formation of intensely fluorescent sodium dodecyl sulfate (SDS)-insoluble aggregates, with moderate levels of cell death over 48–72 h. This has been confirmed in multiple neuronal and non-neuronal lines, with varying levels of transcription/ translation of the mutant protein product. A conditionally immortalized rat embryonic striatal cell line, ST14A [27], provides an *in situ* test system with acceptable delivery and robust readouts [28, 29].

Candidate intrabodies are delivered as transgenes. HD *Drosophila* models have provided a valuable test system (reviewed in Butler et al. [20]). The most extensively utilized HD animal models are transgenic mice. Crook et al. [30] have comprehensively reviewed HD mouse models, phenotypes, advantages, and disadvantages. The five transgenic fragment models, starting with the R6 transgenic lines [31], are the most common for intrabody testing as there is onset of symptoms in 6–12 weeks, and there is morbidity in 12–30 weeks in 3 models. However, studies of delivery to full-length models based on the human mHTT expressed from yeast (YAC) or bacterial artificial chromosomes (BAC), such as YAC128 and BAC97 [32, 33], or knock-in, for example Menalled et al. [34], are also very valuable, despite a lengthy timecourse.

Intrabody Approach

Given the prominence of misfolded mHTT in human and model system pathogenesis, therapies directed against this protein seem most promising. Although the precise structure of the pathogenic species is still a matter of debate, intrabodies offer the option to target a therapy to a very early step in intracellular pathogenesis, prior to any misfolding [35]. Binding to mHTT has the potential to alter kinetics of misfolding and turnover, access of the misfolded species to partner proteins, and subcellular localization of pathogenic protein (e.g., not in the nucleus) [36]. Engineered intracellular antibody fragments, known as intrabodies or nanobodies, can also facilitate critical target validation and rational drug design.

Specific Anti-HTT Intrabodies

Given that expression of HTT Exon1 with an expanded polyQ stretch is sufficient to elicit multiple aspects of the HD phenotype across diverse models of the disease, multiple recombinant scFv antibody fragments targeting domains of mHTT Exon1 have been selected and examined (Fig. 2). Candidate clones have been isolated from phage or yeast surface-display libraries, as well as from hybridoma cell lines. The initial logical target appeared to be the expanded polyQ tract. However, we now recognize that anti-fibrillar intrabodies can increase both the size and the toxicity of misfolded expanded polyQ HTT [28, 37]. Both N- and C- domains adjacent to the polyQ have therefore served as targets. The N-terminal 17 AA (N17) forms a highly conserved amphipathic alpha helix, and exhibits post-translational modifications that can affect the intracellular localization and toxicity. The proline-rich region that is C-terminal to the polyQ affects the folding of the polyQ, and also appears to modulate overall toxicity of mHTT. PolyQ tract aggregation is known to be sensitive to flanking domains [38, 39]; binders to both these domains can alter protein context to reduce misfolding and/or may minimize abnormal protein-protein interactions by the misfolded species.

Anti-N17 Intrabodies

C4 scFv

The first anti-HTT intrabody, C4 scFv, was selected from a naive human spleen scFv phage-display library by panning in

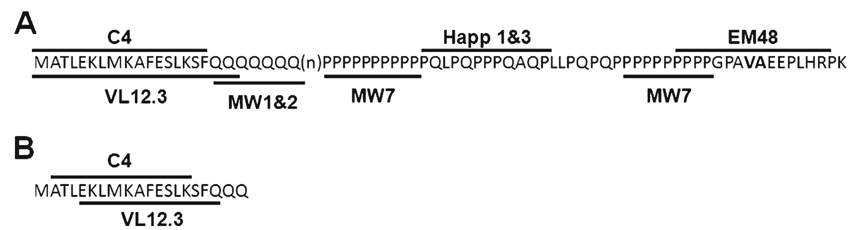

Fig. 2 Recombinant antibody fragments targeting domains of mutant HTT Exon1. (**a**) Antigen used in biopanning and or/ immunization. (**b**) Binding sites determined by crystallography

solution with a peptide of the N-terminal amino acid residues 1–17 of HTT [40]. Although many scFv fragments fold poorly in the reducing environment of the cytoplasm, this construct displayed excellent intracellular folding properties, which, in retrospect, is likely owing to its negative charge at neutral pH [21]. *In vitro* affinity is high (~8 nM), and this construct can counteract *in situ* length-dependent mHTT Exon1 aggregation and toxicity in several cell lines, as well as organotypic slice cultures [28, 29, 40, 41]. Critically, this intrabody preferentially binds soluble mHTT Exon1 fragments, with much weaker affinity for endogenous full length HTT [29]. This should enhance its long-term safety profile, given that conditional deletion of wild type HTT in forebrain neurons of transgenic mice elicited a progressive neurodegeneration [42].

C4 scFv Correction of the HD Fly Phenotype

HD *Drosophila* model [43] intrabody experiments have provided *in vivo* proof of concept of the protective effects of C4 scFv [44]. However, flies still died prematurely, with adults building up aggregates and neurodegeneration. As an early preclinical approach to combinatorial therapies with intrabodies, we therefore tested several combinatorial approaches to improve phenotype. HSP70 plus C4 scFv showed additive effects on survival, but not aggregation [45]. The small molecule cystamine showed a complex pattern of neuronal protection and survival, depending on dose and time of administration [46]. It might be worthwhile to consider follow-up combinatorial studies with this drug plus intrabody in mice, as progression through the larval stages may correlate with early presymptomatic treatment in mice. An additional compound which showed promising preliminary results in flies was nicotinamide. This was moved directly into mice for small molecule testing, where it improved motor deficits and up-regulated *PGC-1alpha* and *BDNF* gene expression in HDR6/1 mice [47]. The doses used did not, however, appear to confer long-term neuroprotection. The small molecule complementation of the intrabody effects provides additional information on classes of therapies that may be worthwhile to use in combination in the clinic.

C4 scFv in Mouse Models

An assessment of mammalian brain efficacy and safety of C4 scFv used adeno-associated viral vectors (AAV2/1) for intrastriatal delivery of C4 intrabody genes into inbred B6.HDR6/1 mice. Quantitatively, both the size and number of HTT aggregates were significantly reduced at early-to-middle stages of disease [48]. Confocal imaging with an anti-HA antibody, used to identify the presence of intrabody, confirmed that most transduced cells lack HTT aggregates initially. However, with time, aggregates build up again. We hypothesize that the kinetics of aggregation are such that a small fraction of the mutant protein can misfold during the time that the intrabody is dissociated from its target, and that over a period of months this “escaped” fibrillar species becomes insoluble and resistant to further intrabody correction. A bifunctional fusion construct that can immediately increase the turnover of bound protein is one approach to solving this problem, as described in the following. Vector spread in these initial experiments was also suboptimal. Newer delivery vectors, and combinatorial therapies based on the fly data, are being used to follow up on these partial corrections. Nanobodies with extremely high affinity are also being considered.

VL12.3

A second intrabody to the region N-terminal to the polyQ was selected from a nonimmune human yeast surface display library, using HTT AA1-20 rather than AA1-17 as a target. An scFv intrabody was shown to have full binding activity with only the light chain, and was therefore carried forth as a variable light chain only single-domain intrabody (V_L). This V_L intrabody product of affinity maturation and binding site analysis on the yeast cell surface modestly inhibited mHTT Exon1 aggregation [49]. To enhance biological functionality in the cytoplasm, the V_L intrabody was then engineered to fold in the reducing environment of the cell via a series of substitutions of cysteine with hydrophobic residues, followed by additional rounds of affinity maturation [50]. The initial *in vivo* testing of this new intrabody in the fly paradigm mentioned earlier was disappointing. Eclosion rates of HD flies expressing V_L12.3 were only improved to 73 % compared with nearly 100 % in the HD + C4 scFv flies, and adult survival was not significantly improved. Furthermore, attempts to increase expression of V_L12.3 in the presence of HTT Exon1 proved to be toxic (unpublished results.).

Southwell et al. [51] delivered VL12.3 using AAV in both the R6/2 transgenic mice and a model made by

injecting mHTT Exon1 using a lentivirus. In the latter model, with very high expression of mHTT Exon1 and co-administration of the toxic protein and its binder, VL12.3 improved behavior and neuropathology [51]. However, VL12.3 binding appears to block cytoplasmic retention of HTT resulting in higher levels of antigen-antibody complex in the nucleus of transduced cells [52], This may explain the modest increase in toxicity in the HDR6/2 mouse model [51]. Because VL12.3 causes nuclear retention of mHTT, it may need to be delivered prior to the onset of aggregation of mHTT Exon1 fragments to have a therapeutic effect.

Comparing the Two Intrabodies

The difference in the *in vivo* efficacy of the two anti-N-term antibody fragments offers an opportunity to fine-tune our concept of epitope dominance, antigen choice, and target validation. C4 scFv was selected in solution against the highly-conserved AAs 1–17.

A recent crystal structure of C4 scFv in complex with the first 17 AAs of HTT Exon1 shows that the first 15 HTT residues are in intimate contact with the intrabody (De Genst, et al. unpublished). V_L12.3, however, was selected against a peptide that included AAs 1–20, and then highly engineered using yeast surface display. The contact residues have been identified structurally as including AAs 5–18 [53]. The two intrabodies may therefore differentially affect the kinetics of post-translational modifications of the HTT Exon1 at Thr-3 and Ser-16, which have been implicated in subcellular localization and toxicity [43, 54, 55]. As we learn more about the function and importance of specific domains and AAs on targets, *in silico* evaluation and ranking of current and future intrabody candidates will become more efficient. Structural studies to establish precisely how the intrabodies are interacting with their target proteins could save a great deal of time and effort in the long run.

Intrabodies That Target HTT Exon1 Regions C-terminal to the PolyQ

Happ

The proline-rich region immediately C-terminal to the polyQ domain of HTT Exon1 contains two pure polyproline (polyP) series, interrupted by a unique proline-rich stretch. If this domain is not included in Exon1 fragments, aggregation is accelerated [56, 57]. The Patterson laboratory has investigated a series of intrabodies against this region C-terminal to the polyQ. The first generation was an scFv cloned from the variable domains of the monoclonal antibody MW7, which binds to the two polyP stretches [58]. scFv MW7 has been shown to reduce mHTT-induced aggregation and enhance survival in an HEK293 culture model of HD [37]. Intracellular solubility may have been limiting, and this intrabody has the potential to bind other proteins containing a polyP stretch. Another set of antiproline-rich domain intrabodies (Happ1 and Happ3) was selected from a nonimmune human recombinant scFv phage library [52]. Similar to VL12.3, these Happ intrabodies bound via a VL single light-chain domain. Doses to prevent aggregation and toxicity were effective at a 2:1 ratio of intrabody to mHTT Exon1-103Q for Happ1 versus a 4:1 ratio for scFv MW7 [52]. All three intrabodies accelerated the clearance of this very rapidly-aggregating mHTTExon1 in HEK293 cells, presumably by allowing the bound target to remain soluble and therefore subject to normal turnover mechanisms [52]. It appears that the mechanism of Happ1-induced clearance of mHTT is owing to enhanced calpain cleavage of the first 15AA of mHTT followed by lysosomal degradation [59].

Intrastriatal AAV delivery of Happ1 was found to be beneficial in a variety of *in vivo* assays in diverse mouse models, including an acute unilateral lentiviral model, and R6/2, N171-82Q, YAC128, and BACHD transgenic HD mouse lines [51]. In all five mouse models, intrastriatal AAV delivery of Happ1 showed improvement of the neuropathology, as well as correction of a variety of motor and cognitive phenotypes [51, 59]. The N171-82Q model also showed increased body weight and a 30 % increased lifespan. It would be extremely interesting to combine these treatments with intrabodies targeting the N-terminal region of HTT Exon1, and/or combinatorial therapies with small molecules.

scFvEM48

mEM48, a mouse monoclonal antibody that recognizes a human-specific epitope in the C-terminus of HTT Exon1, is a valuable immunocytochemical reagent for following aggregated protein in mice expressing human HTT. When cloned as an scFv and expressed as an intrabody, scFvEM48 suppressed the cytoplasmic, but not nuclear, toxicity of mHTT Exon1 in HEK293 cells, possibly via increased ubiquitination and degradation of cytoplasmic mutant HTT [60]. Delivery into the striatum of transgenic N171-82Q HD using adenovirus, which primarily transduces glia, led to reduced neuropil aggregate formation and motor deficits of N171-82Q during an 8-week test [60]. Longer testing periods, and delivery to other cell types, should be very interesting.

PD and α-Syn as a Target

PD is associated with a relatively selective loss of dopaminergic neurons in the substantia nigra pars compacta and presence of α-Syn-containing lesions, Lewy bodies and Lewy neurites [61], and progressive development of motor symptoms, such

as bradykinesia, hypokinesia, and resting tremor [61, 62], plus non-motor symptoms that are a serious problem for many PD patients. Not all dopaminergic neurons are affected to the same extent in PD, and not all affected neurons are dopaminergic [63]. PD appears in both familial and idiopathic forms; however, such distinctions are becoming blurred, as low-penetrance genetic risk factors are uncovered [64]. Currently, therapies do exist for the treatment of PD, such as dopamine replacement, administration of dopaminergic agonists, and deep brain stimulation [65]. However, these are only symptomatic in nature. They are focused on promoting dopaminergic neurotransmission, and do not address the progressive neurodegeneration associated with PD.

For neuroprotection, α-Syn is a logical target. It is the major protein component in Lewy bodies. Three separate point mutations in the *α-Syn* gene give rise to autosomal dominant PD. Duplication and triplication of wild type *α-Syn* can also give rise to inherited PD, in which age of onset is inversely proportional to the dose of α-Syn. Genome-wide association studies have also indicated that single nucleotide polymorphisms in the *α-Syn* gene promoter may increase the risk of PD [66]. *LRRK2*, another major risk factor gene, may be an upstream regulator of *α-Syn*, potentially influencing α-Syn deposition through autophagy [67, 68]. In patients with Gaucher disease, with lysosomal dysfunction, the risk of developing PD is substantially increased. Recently, Gaucher mutations were shown to lead to increased accumulation of α-Syn [69]. An α-Syn pathogenic cascade may include excess monomeric α-Syn interference at the synapse [70], and accumulation of stress-inducing phosphorylated, truncated, nitrated, or dopamine-conjugated α-Syn [71, 72]. Excess α-Syn may also lead to misfolded α-Syn with increased potential to aggregate into intra- and extracellular toxic conformations. Because α-Syn appears to play a central role in the pathogenesis of PD, therapies that reduce intracellular levels to prevent the abnormal misfolding, aggregation, and toxicity in vulnerable cells may be valuable for slowing the progression of the disease.

α-Syn is a small 140-AA protein that is natively unfolded and unstructured in isolation, with many conformational forms upon interacting. The protein has an amphipathic N-terminal and acidic C-terminal, with a hydrophobic central domain (AAs 61–95) termed the nonamyloid component (NAC) [73]. The NAC domain is aggregation-prone and can form beta sheets by self-association, and is found to be part of amyloid plaques of AD [63]. A transgenic *Drosophila* model expressing wild type α-Syn without the NAC domain was shown to lead to significantly reduced toxicity. Similarly, deletion of the NAC led to decreased aggregation *in vitro*. The hydrophobic NAC domain of α-Syn may thus be important in α-Syn aggregation and toxicity, making it an attractive target for treatment such as recombinant antibody technology [73–75].

Intrabodies/Nanobodies Targeted for α-Syn Monomer

Multiple α-Syn-specific intrabodies, summarized in Table 1, have been selected from either phage display (D5, D10) or yeast surface display libraries (VH14; NAC32). Using phage display libraries, D10 scFv was selected for strong binding to monomeric α-Syn, while the NAC32 scFv target was AAs 53–87, part of the NAC region of α-Syn. VH14 is an antiNAC-α-Syn-monomeric heavy chain only human nanobody derived from a nonimmune yeast surface display library [74]. Functionally, D10 stabilized soluble monomeric α-Syn, inhibiting formation of detergent-insoluble α-Syn. It also corrected the α-Syn-induced reduction in cell adhesion. scFv NAC32 reduced the toxicity of A53T mutant α-Syn [74, 76]. Attaching non-conventional secretary signal to D10 and D5 led to removal of α-Syn from cells and scFv D5 with secretary signal targeting oligomeric α-Syn provided protection against α-Syn-induced toxicity [77]. This approach suggested that these target-specific intrabodies can themselves be targeted to specific cellular compartments or the extracellular space by fusing an appropriate signal sequence. However, many of these constructs are still suboptimal owing to misfolding in the cell cytoplasm. For long-term use, they may also allow the kinetic escape and target protein accumulation found in our HD studies.

Vuchelen et al. [78] reported a camelid VHH nanobody, NbSyn2, which bound to the C-terminal of α-Syn. This nanobody, obtained by immunization followed by phage display, has a high affinity for monomeric α-Syn (Kd~ 10 nM). Nuclear magnetic resonance spectroscopic methods and determination of the crystal structure of the nanobody both confirmed that NbSyn2 bound to the C-terminal four residues of the α-Syn peptide [79]. The aggregation behavior and morphology of α-Syn was not affected by binding of this nanobody to α-Syn, although the nanobody bound to both monomeric and aggregated α-Syn with high affinity, suggesting that the C-terminal of α-Syn may not be involved in the aggregation reaction of α-Syn. Further *in situ* and *in vivo* testing will serve both to determine protective effects of binding in this region and as validation of specific C-terminal targets.

Intrabodies Targeted to Conformational α-Syn Epitopes

α-Syn is a natively unfolded protein, but, in the cell, it may adopt different conformations when partnered and/or aggregated. *In vitro*, conditions such as low pH, presence of metal cations, or pesticides, have been shown to change the conformation of α-Syn to the aggregation-prone beta-sheet conformation. *In situ*, oligomers, protofibrils, and fibrils can form when the levels of α-Syn increase inside cells. Intrabody approaches can therefore include intrabodies targeted to reduce the monomeric α-Syn or those targeted to a particular

Table 1 Published anti-alpha-synuclein (α-Syn) intrabodies

Intrabody	Type	Target	Source
D5E [80, 90]	Human scFv	Oligomeric α-Syn	Tomlinson I and J antibody library
10H [81]	Human scFv	Oligomeric α-Syn	Tomlinson I and J antibody library
D10 [76]	Human scFv	Pan-specific	Human scFv phage Griffin I library
VH14 [74]	Human nanobody	Part of hydrophobic NAC of α-Syn	Human nonimmune yeast surface display library
NAC32 [74]	Human scFv	Part of hydrophobic NAC of α-Syn	Human nonimmune yeast surface display library
6E [28]	Human scFv	Fibrillar α-Syn	Tomlinson I and J antibody library
NbSyn2 [79]	Camelid VHH	Monomeric α-Syn	Phage display

ScFv = single chain Fv; VHH = small heavy-chain-only antibody fragments; NAC = nonamyloid component

neurotoxic conformation of aggregates. Emadi et al. [80, 81] have combined phage display and atomic force microscopy technique to isolate intrabodies that bind to different types of oligomeric α-Syn targets. Atomic force microscopy was used to ascertain the morphology of α-Syn target and to monitor the process of panning. scFv D5 has a high affinity for the oligomeric form of α-Syn and was obtained from panning Tomlinson I and J antibody libraries against specifically oligomeric form of α-Syn. This intrabody decreased the toxicity and aggregation of α-Syn *in vitro* [80]. Another intrabody, 10H, recognized a later-stage oligomer and inhibited toxicity induced by extracellular α-Syn, and also inhibited *in vitro* aggregation of α-Syn [81]. Recently Guilliams et al. [82] reported additional anti-α-Syn nanobody, NbSyn87, obtained by immunization of llama with α-Syn. NbSyn87 bound residues 118–131 of α-Syn with nanomolar affinity. This nanobody bound both monomeric and fibrillar forms of α-Syn, indicating that this epitope is accessible to nanobody binding in the fibrillar state. The length of time of fibrillization and the fibrillization conditions influenced the apparent affinities and thermodynamic parameters of fibril and nanobody binding. This indicated that the α-Syn C-terminal domain environment is modified in the fibrillization process and nanobodies can target different, potentially pathogenic, forms of α-Syn.

Bifunctional Intrabodies to Enhance HD and PD Intrabody Function

An advantage of recombinant antibody fragments is that they can be engineered to enhance efficacy using genetic fusions, which are then expressed as multifunctional proteins. Fusions can be used to alter intracellular localization, improve cytoplasmic solubility, and, potentially, allow cell permeation of protein products.

HTT

A major problem that became evident from the fly and mouse *in vivo* data is that protein is only prevented from misfolding during the time that it is actually bound to the intrabody. Aggregates can therefore build up over weeks or months, even in the presence of high levels of intrabody. Intrabodies typically have a neutralizing function via specific binding to the targeted molecule. To add additional functionality during the bound phase of the C4 scFv intrabodies, we added a targeting signal to induce rapid turnover of bound HTT Exon1. Proteins containing a PEST motif—an enriched region of AAs Proline (P), Glutamic Acid (E), Serine (S), and Threonine (T)—typically have a short half-life and are degraded by the proteasome [83]. Recently, our laboratory fused the mouse ornithine decarboxylase PEST motif (AAs 422–461) to anti-HTT C4 scFv, leading to significant turnover of antigen, while not changing solubility of the intrabody [84]. It is important to note that C4 scFv maintains mHTT in a monomeric soluble conformation, which allows mHTT Exon1 to enter the proteasome and undergo degradation. Aggregated mHTT fragments have been reported to be resistant to degradation by the ubiquitin proteasome system (UPS) [85], and fusion of the PEST motif to an anti-fibrillar scFv, 6E, failed to enhance clearance of the already misfolded conformer of the mHTT protein. This underlines the role of the target antigen conformation for potential use of the intrabody-PEST approach. Levels of aggregated mHTT can be reduced by induction of autophagic/lysosomal pathway of intracellular protein degradation [86–88]. It may be possible to use a fusion construct similar to that employed by Bauer et al. [89] to target intrabody–mHTT Exon1 complexes to the lysosome for degradation by chaperone-mediated autophagy.

SYN

Most of the anti-α-Syn constructs fold very suboptimally in the cytoplasm, a problem that is exacerbated in cells already under proteostatic stress. Addition of the acidic PEST peptide to four anti-α-Syn intrabodies improved the solubility for all 4, while still retaining sufficient intracellular levels of intrabody-PEST protein. In addition, the highest affinity, but least soluble of the parent constructs, the NAC-specific

human heavy-chain only nanobody, VH14, acquired the capacity to degrade α-Syn~GFP [90]. Thus, this bifunctional approach to the intrabody engineering can be used successfully to target and direct the molecule of choice to the required compartment, and shows great promise of targeting the earliest stages of neurodegeneration.

Interactions Between Misfolded Proteins

The reach of proteostasis is broad. A series of new studies has highlighted the possibility that there are important interactions between misfolded HTT and misfolded α-Syn. Aggregates of HTT have also been shown to stain for α-Syn [91]. It appears that this is not owing to heterologous oligomers, but to smaller aggregates of mHTT and oligomeric (or fibrillar?) α-Syn sticking to each other to create larger aggregates. This process may be more prevalent with the non-nuclear aggregates. In culture models, the presence of overexpressed α-Syn leads to a statistically significant increase in the proportion of larger HTT aggregates when compared with cells without excess α-Syn [92]. This could be owing to an overstressed folding/ chaperone system that exacerbates the effects of any metastable proteins, similar to what has been described in *Caenorhabditis elegans* models. In one cell culture model, co-transfection of mutant HTT and α-syn led to increased aggregation, but not an increase in cell toxicity [92]. However, cell death over the limited timeframe of the cellular experiments may mask an increased toxic effect. *In vivo*, there are clear effects on the onset and progression of HD with manipulations of α-Syn levels. HTT transgenic mice show an earlier onset and death when crossed to α-syn overexpression transgenic mice, while expressing the HTT transgene in mice that lack the *α-Syn* gene slows the HTT phenotype [91, 93]. Although there is still much work to be done with the genetic epidemiology to determine if known *α-Syn* promoter polymorphisms are modifiers for the human HD phenotype, and if the size of the CAG repeat in HTT and additional trinucleotide repeat proteins can affect PD risk, these emerging data suggest that there may be an option for using therapies directed against one misfolding protein to modify the disease course in another. This concept can also be extended to using intrabodies against one disease protein to modify another, for proteins such as Tau and Aβ.

Intrabody Versus Messenger RNA Knockdown

An alternative to antibody-based strategies for knocking down the levels of proteins prone to misfolding is to target the messenger RNA. For HD, AAV-mediated delivery of a microRNA [94] and striatal infusion of small interfering RNA [95] showed localized protective effects in overexpression fragment models. HTT antisense oligonucleotides (ASOs) delivered via transient infusion into the cerebrospinal fluid of symptomatic HD mouse models also showed efficacy and no apparent toxicity [96]. These studies confirm the value of knocking down HTT as a model therapeutic strategy. However, there is still concern that targeting both the mutant and the wild type forms of the protein may have long-term consequences that are not readily predicted from the existing animal studies, as conditional deletion of wild type HTT in forebrain neurons of transgenic mice did show a progressive neurodegeneration, suggesting the potential for selective long-term vulnerability [42]. Allele-specific targeting offers a potentially safer option [97], but will require optimization based on the kindred, and requires the use of targets that are far downstream. Antibody-based approaches to clearance based on the protein product have a much higher level of specificity, and could offer a higher margin of safety in the clinic. The longevity of the effects of a transient reduction of HTT that were demonstrated with the ASOs do suggest that neither treatment needs to be continuous, and it is not impossible that RNA and protein clearance approaches could be combined to cover a larger number of cells and targets.

The Challenge of Delivery

Delivering intrabodies to neurons or other cells within the brain remains a challenge. For continuous production of intrabodies, viral delivery of genes encoding intrabodies has been shown to be effective in a fraction of cells, with persistence of signal over weeks-to-months [48, 51]. Newer vectors may offer better spread and more efficient transduction, although localized and systemic immunogenicity remain concerns. There may also be a role for protein delivery, particularly for pulsed treatments.

Next Directions in Gene Delivery

As recombinant antibody fragments, intrabodies can be delivered as genes. Bowers et al. [98] have published a review of vectors, including their respective advantages and disadvantages for different neurologic disease applications. AAV currently appears to be the safest human gene therapy vector for central nervous system clinical trials as it transduces nondividing cells without genomic integration, leading to long-term expression, and there are multiple different capsid variants available to improve cell-specific transduction [99]. Genes encoding intrabodies are small enough (750 nucleotides for scFvs and 360–420 for single-domain nanobodies) that the coding regions can be readily accommodated within either single or double-stranded AAV vectors [100], leaving space for bispecific or bifunctional constructs. A localized immune response to AAV9 that induced a response against the transgene was recently

reported in rats [101]. Immunogenicity will be minimized by the use of human antibody fragments for human therapies, or by camelid scaffolds that closely mimic an optimized human scaffold. However, preclinical testing using assays developed to measure immune responses in the vaccine field will be necessary to ensure safety.

Multifunctional Constructs to Allow Protein Delivery

Direct protein delivery will be a short-term treatment. For HD, and probably for PD, giving the brain a "holiday" from the toxic protein may be sufficient to reset the clock, as was recently demonstrated for an ASO anti-HD therapy [96]. Given that engineered antibodies can be synthesized to be bifunctional or bispecific, linkage to cellular transduction domains is feasible. Both naturally-occurring autoantibodies, and those selected against cell surface receptors or channels can be internalized and used to deliver cargo proteins, including other antibodies. In a stroke model, an scFv derived from a mouse anti-DNA autoantibody, 3E10, was used to ferry HSP70 into neuronal nuclei via the equilibrative nucleoside transporter [102]. For HD and PD, this bispecific construct would be suboptimal, as it targets its cargo to the nucleus; however, similar antibody constructs may be developed for cytoplasmic delivery. Bispecifics incorporating anti-transferrin receptor antibodies are appealing owing to possible up-regulation of this system on compromised cells, and chimeric antibodies using this approach were recently described as potential AD therapeutics [103, 104].

Future Intrabody Directions

We have reviewed the existing literature on HD and PD intrabodies/nanobodies. Targeting of misfolding protein before it can be aggregated and/or secreted should be a viable strategy for almost all neurodegenerative diseases characterized by abnormal intracellular structures at some stage of pathogenesis, including AD and amyotrophic lateral sclerosis. Antibody specificity can also recognize abnormally structured RNA, and binding could be engineered to induce turnover, block sequestration of RNA binding proteins, and/or reduce anomalous translation from repeat sequences [105, 106]. The intrabody approach can therefore be expanded to an additional cohort of neurodegenerations in which toxic RNA is a primary pathogenic molecule. The use of intrabodies for neurologic diseases is quite new, and the extensive antibody engineering technology that is being aggressively developed for biological therapies for cancer and infectious diseases will allow use of these reagents for neurodegenerative diseases to move forward rapidly. Multifunctional antibody constructs have been used extensively for other diseases, and are in the very early stages of exploration for HD and PD targets. Enhanced delivery methods will come from the fields of both gene and protein therapies. Combinatorial therapies, such as combining the systemic symptomatic relief of a small molecule [47] with potentially neuroprotective effects of targeting the pathogenic protein, hold great promise for the future.

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