

# CANADIAN ASSOCIATION OF NEUROSCIENCES REVIEW: Polyglutamine Expansion Neurodegenerative Diseases

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**ABSTRACT:** Since the early 1990s, DNA triplet repeat expansions have been found to be the cause in an ever increasing number of genetic neurologic diseases. A subset of this large family of genetic diseases has the expansion of a CAG DNA triplet in the open reading frame of a coding exon. The result of this DNA expansion is the expression of expanded glutamine amino acid repeat tracts in the affected proteins, leading to the term, Polyglutamine Diseases, which is applied to this sub-family of diseases. To date, nine distinct genes are known to be linked to polyglutamine diseases, including Huntington's disease, Machado-Joseph Disease and spinobulbar muscular atrophy or Kennedy's disease. Most of the polyglutamine diseases are characterized clinically as spinocerebellar ataxias. Here we discuss recent successes and advancements in polyglutamine disease research, comparing these different diseases with a common genetic flaw at the level of molecular biology and early drug design for a family of diseases where many new research tools for these genetic disorders have been developed. Polyglutamine disease research has successfully used interdisciplinary collaborative efforts, informative multiple mouse genetic models and advanced tools of pharmaceutical industry research to potentially serve as the prototype model of therapeutic research and development for rare neurodegenerative diseases.

**RÉSUMÉ:** Expansion de polyglutamines dans les maladies neurodégénératives. Depuis le début des années 1990, on a découvert qu'une expansion de répétitions de triplets d'ADN était la cause d'un nombre de plus en plus considérable de maladies neurologiques d'étiologie génétique. Un sous-groupe de cette grande famille de maladies génétiques possède une expansion d'un triplet CAG dans le cadre de lecture ouvert d'un exon codant. Cette expansion de l'ADN s'exprime au niveau de la protéine atteinte par une expansion de la séquence répétée d'un acide aminé, la glutamine, ce qui a donné lieu au terme de maladies à polyglutamines, terme qui s'applique à cette sous-famille de maladies. Jusqu'à maintenant, ce phénomène a été observé dans neuf gènes différents en relation avec des maladies à polyglutamines dont la maladie de Huntington, la maladie de Machado-Joseph et l'amyotrophie spino-bulbaire ou maladie de Kennedy. La plupart des maladies à polyglutamines se classent au point de vue clinique parmi les ataxies spino-cérébelleuses. Nous discutons des découvertes récentes et des progrès de la recherche sur les maladies à polyglutamines et nous comparons ces différentes maladies qui ont un défaut génétique commun au point de vue biologie moléculaire. Nous traitons également de « drug design » pour une famille de maladies pour lesquelles plusieurs nouveaux outils de recherche ont été développés. La recherche sur les maladies à polyglutamines a bénéficié d'une collaboration interdisciplinaire et a utilisé avec succès plusieurs modèles génétiques de souris très informatifs ainsi que des outils de pointe de la recherche pharmaceutique et servira potentiellement de prototype en recherche et développement thérapeutique pour les maladies neurodégénératives rares.

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Since the early analysis of the DNA sequence of the human genome, trinucleotide repeats have been noted and were thought to be common sequences found scattered throughout chromosomal DNA.<sup>1,2</sup> Modern genetic approaches to disease gene linkage and cloning led to the first discovery of CAG triplet repeat expansion in the reading frame of the androgen receptor, the disease gene for x-linked spinobulbar muscular atrophy (SBMA, or Kennedy's disease).<sup>3</sup> The next few years led to the discovery of CAG repeats and polyglutamine expansion in Huntington's disease<sup>4</sup> and several spinocerebellar ataxias.<sup>5,6</sup> The exact mechanism of CAG triplet repeat expansion is unknown, but can be recapitulated in vitro with a C-G rich triplet repeat sequence,<sup>7</sup> suggesting that the primary defect is dictated by the DNA structure of these sequences (Figure 1).

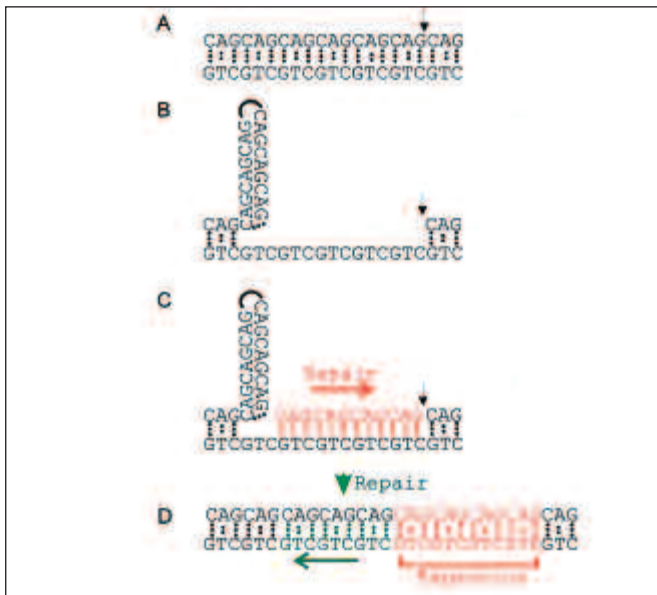
There exists some commonality, as well as many differences

between these triplet repeat diseases. Triplet repeat expansions show both somatic and germ-line instability,<sup>8,9</sup> where the sequences can expand in subsequent generations. This expansion of CAG repeats leads to earlier age of onset in subsequent generations of an affected patient's family, a phenomenon known

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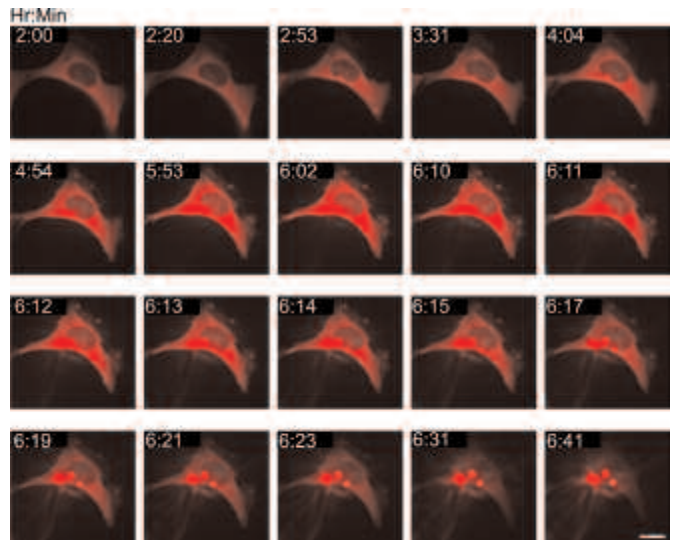
**Figure 1:** Model of CAG DNA repeat triplet expansion. A. CAG tract DNA is nicked on the phosphate backbone, leading to the formation of stable stem-loop structure due to high G-C content (B), with the base of the stem forming an important C-G pairing. C. Single-stranded DNA is filled in by the DNA repair machinery and DNA polymerase I, starting at the first C, hence maintaining the triplet codon frame. D. The DNA mismatch repair machinery fills in the bottom strand to result in the net expansion of the CAG tract.

as genetic anticipation.<sup>10</sup> For most of these polyglutamine diseases, polyglutamine length varies inversely with age of onset, but not always with disease severity. The additional genetic factors and disease modifiers that affect disease severity are the recent focus of groups revisiting genetic family data.<sup>11,12</sup> Anticipation can be affected by the parental origin of the mutant expansion, with paternal transmission leading to higher risk.<sup>13</sup> Further genetic studies in human and mouse models reveal that most polyglutamine diseases are autosomal dominant, displaying a “gain-of-function” phenotype, where the function of the wild-type protein can be carried out by the mutant protein, but an additional polyglutamine-dependent function is involved in triggering pathology.<sup>14</sup>

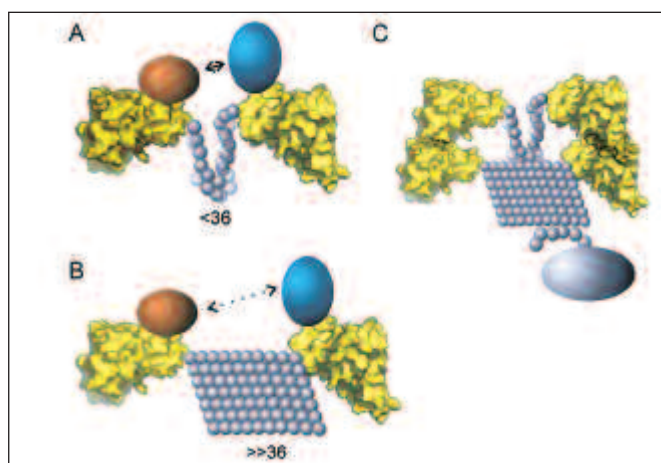
Research into polyglutamine diseases has led to close scrutiny by other genetic disease researchers. Polyglutamine diseases such as Huntington’s disease (HD) were among the first to have accurate genetic tests to predict the likelihood of disease and the age of onset, leading to studies of genetics and ethics of testing.<sup>15–17</sup> Within a few years of discovery, transgenic mouse models were constructed that recapitulated several aspects of the human disease,<sup>18</sup> and in some cases, multiple sophisticated models have been constructed to test hypotheses of pathogenicity.<sup>19–22</sup> Recent techniques of pharmaceutical industry-type high-throughput chemical compound screens have been applied in attempts to find potential drug leads.<sup>23,24</sup>

Since the discovery of multiple polyglutamine disease proteins, this research has bifurcated into two paths: determining if there is a single, common gain-of-function of polyglutamine

tracts in proteins, and the specific effects of polyglutamine tracts on the normal biological functions of these disease proteins. In 1993, the Nobel Laureate, Max Perutz, presented a biochemical theory of polyglutamine polar side group “zipper” interactions that would lead to intermolecular interactions and aggregation of protein.<sup>25</sup> Indeed, protein aggregates of several polyglutamine disease proteins were detected in some patient brain samples,<sup>26</sup> huntingtin mouse models,<sup>27,18</sup> and more recently in real time using methods of live cell microscopy (Figure 2).<sup>28</sup> One hypothesis is that aggregates of polyglutamine-expanded proteins lead to a universal toxic gain-of-function, such as the sequestration of critical transcription factors in the nucleus,<sup>14</sup> or the immobilization of the polyglutamine disease protein into insoluble protein precipitates. These observations have led to the suggestion that polyglutamine diseases may be considered misfolded protein neurodegenerative diseases, analogous to misfolded proteins detected in Alzheimer’s, Parkinson’s and transmitted spongiform encephalopathy diseases. This has led to cross-talk in research between fields dedicated to “amyloid-like” neurodegenerative diseases.<sup>29</sup> Another hypothesis is that normal, short polyglutamine tracts in proteins are normally unstructured and flexible components of a protein “scaffold”, where the protein makes more than one protein-protein interaction and in turn, these interacting proteins can interact with each other, analogous to construction workers on a scaffold. In polyglutamine disease, the expanded glutamine tract takes on



**Figure 2:** Dynamics of Polyglutamine Aggregation Initiation and Polymerization. Live cell time course fluorescence microscopy of huntingtin Q138 Exon1 (from juvenile-onset patient)-mRFP fusion expressed in STHdh<sup>Q7/Q7</sup> cells. Over four hours, soluble huntingtin fragment accumulates in the cell until a minimum threshold concentration is reached, then rapid formation of inclusions is seen within minutes, and subsequent sequestration of remaining soluble Q138 huntingtin exon1. In this cell line, the formation of mutant huntingtin exon1 inclusions is seen to delay cell death over cells where no inclusions have formed. Scale bar is 10  $\mu$ m. Time in hours, minutes is in the top left corner of each frame. Full video can be viewed and downloaded at [www.raytruantlab.ca](http://www.raytruantlab.ca)



**Figure 3:** Models of Polyglutamine Induced Toxicity in Polyglutamine disease Proteins. A. Normal length polyglutamine tracts act as flexible components of a protein scaffold, a platform for important regulatory interactions between proteins involved in cell signaling or the normal biological function of the protein. B. Polyglutamine disease, the expanded polyglutamine tract gains structure, and can perturb the normal communication between bound proteins, leading to a perceived gain-of-function, or, allows the formation of new interactions with cellular factors. C. Potential mechanisms of genetic dominance over the wild-type allele protein, sequestration of the wild-type protein into mutant inclusions, and/or sequestration of glutamine-rich transcription factors essential for cell health as gain-of-function.

structure,<sup>30</sup> disrupting the protein scaffold, leading to a gain-of-function by impeding important protein intramolecular interactions leading to a molecular switch of regulation stuck in either the “on” or “off” position (Figure 3). This second scaffold-disruption model may also apply to other genetic diseases in which other amino acid tracts are expanded, such as polyalanine expansions of PABP2 in oculopharyngeal muscular dystrophy<sup>31,32</sup> the HOXA13 protein in hand-foot-genital syndrome<sup>33</sup> or within FOXL2 in Blepharophimosis syndrome.<sup>34</sup>

This review will summarize recent advancements, successes and controversies of this class of neurodegenerative diseases. Most advances in polyglutamine disease research have come from attempts to understand the normal biological function of the affected proteins, which help to infer the pathogenic trigger caused by the polyglutamine expansion. In the case of Huntington’s disease, the research funded between government agencies and private charities has led to a new model of non-profit pharmaceutical research and development towards the goal of a therapy.

#### HUNTINGTON’S DISEASE OR HUNTINGTON’S CHOREA IN THE CLINIC

Huntington’s disease (HD) is characterized by a triad of cognitive, behavioral and motor changes, usually with onset around middle age and progression over 15-20 years to death. Clinical features related to cognition and behavior include: subcortical dementia; personality changes such as irritability, increased aggression, or apathy, often accompanied by mood

disorder and less commonly psychosis. Those features related to motor changes include extrapyramidal movement disorders typified by dyskinesias, motor incoordination, and postural instability that later evolves to severe bradykinesia and rigidity.<sup>35-37</sup> Clinical manifestations result from selective neuronal dysfunction and loss, mainly of the striatum medium-sized spiny neurons in caudate and putamen nuclei; lesser and to a lesser extent in certain layers of the cortex, CA1 of the hippocampus, and some subcortical nuclei.<sup>37,38</sup> Currently, therapy in HD is limited to treatment of symptoms, since no therapy has yet been proven to slow progression or delay onset of the disease. Agents that inhibit dopamine transmission are generally effective for suppressing chorea, but side effects such as sedation, bradykinesia and rigidity (both typical and atypical neuroleptics), or depression (for the dopamine-depleting agent tetrabenazine) limit their use. Mood and obsessive-compulsive disorders are treated with typical drugs.<sup>39</sup> A deeper understanding of the normal function of the huntingtin protein, as well as the molecular mechanisms underlying pathogenesis of Huntington’s disease, will facilitate development of therapies targeted toward modifying the disease course.

The typical length of the polyglutamine tract of the HD *IT15* gene product in normal individuals is about 9-15 in length, and similar lengths are seen in normal alleles of SCA1,3, and DRPLA.<sup>40</sup> For late-onset HD, disease is seen with polyglutamine expansions in the 36 or higher repeat range, and earlier onset is seen with longer expansions of 60-85 in length, where this expansion length can be used as an accurate predictor of age of onset.<sup>17</sup> Juvenile cases can express huntingtin protein with very long repeats in excess of 100 polyglutamines.<sup>41</sup> However, what is not known about HD and some other polyglutamine diseases is the significance of the 36 repeats of polyglutamine being the threshold of disease.<sup>42</sup> This may relate to the biophysical and biochemical properties of 36 or more glutamines that are different from lower repeat lengths.

#### THE HUNTINGTON’S DISEASE PROTEIN, HUNTINGTIN: CLUES FROM HIPs, HAPs AND HYPs

One of the typical questions from clinicians interested in the molecular biology of Huntington’s disease is: what is the normal function of huntingtin protein? The answer is only being elucidated recently, through the use of protein-protein interaction studies with huntingtin, focusing on the biological functions of huntingtin-interacting proteins. Early studies defined unique huntingtin interacting proteins, such as the Huntingtin Interacting Proteins (HIPs),<sup>43</sup> the Huntingtin Associated Proteins (HAPs),<sup>44</sup> and Huntingtin Yeast-interacting Proteins (HYPs),<sup>45,46</sup> but with a lack of known biological functions for most of these proteins. However, as more information was being uncovered about these interacting proteins, overlap in functions started appearing.<sup>47-51</sup> Huntingtin is now seen as a protein whose biological functions include involvement in vesicular trafficking, i.e. the transport of lipid vesicles (endocytic,<sup>52</sup> synaptic,<sup>53</sup> lysosomal<sup>54</sup>) along microtubules, or the cell skeleton, via an energy-dependent motor machinery.<sup>55,56</sup> At the early endosome, this interaction requires HAP40.<sup>57</sup> Huntingtin has also been seen at the endoplasmic reticulum, and in the nucleus,<sup>58,59</sup> and can dynamically traffic between these two compartments.<sup>28</sup> While this may seem like many functions for one protein, the huntingtin



protein is 350 KDa in size, and is one of the larger non-transmembrane proteins known in the human proteome. The large size of huntingtin has been a major contributing factor to the technical difficulties in studying this protein, making expression and biochemical studies very challenging. The immediate goal of this line of HD research is to understand which of these activities is most important to triggering pathology in HD. Within the nucleus, huntingtin is thought to be involved in transcription modulation in response to the brain-derived neurotrophic factor, BDNF,<sup>60-64</sup> and the cyclic AMP response element binding protein, CREB,<sup>65,66</sup> thus implicating huntingtin's potential role in signal transduction from the cytoplasm to the nucleus. In the cytoplasm, huntingtin has also been seen to be directly involved in the transport of BDNF-containing vesicles.<sup>67</sup>

The connection between huntingtin and neurotrophic factors is important to understanding the molecular pathology of Huntington's disease. Huntingtin has been shown to be involved in programmed cell death, or apoptosis,<sup>68,69</sup> and is proteolytically cleaved by the carboxy aspartyl proteases, or caspases involved in apoptosis.<sup>70</sup> This work has suggested that striatal cells are triggered for death by mutant huntingtin by a molecular "suicide" mechanism gone wrong. In the brain, striatal cells do not produce significant quantities of BDNF, but rely on input of this critical factor from the cortical cells. Lack of transport of BDNF out of cortical cells, or lack of uptake via endocytosis by striatal cells, suggests that the alternative, "murder" hypothesis of the striatal cells by the cortical cells may induce striatal loss.<sup>71</sup> One of the striking features of autopsied late-stage HD patient brains is the tremendous loss of the striatum, and up to 30% of total brain mass.<sup>37</sup> These hypotheses of lowered BDNF levels are leading to radical gene therapeutic approaches in attempt to restore BDNF levels in HD-affected brains.<sup>72</sup>

#### HUNTINGTIN AND SMALL MOLECULE SCREENING: ON THE PATH TO A THERAPY

Since 1999, many projects on huntingtin and polyglutamine diseases have been using modern state-of-the-art techniques employed successfully by the pharmaceutical industry, including high-throughput screening (HTS) of small chemical compounds. High-throughput screening involves simple assays that can be scaled to very large repetitive levels and hundreds of thousands of samples that can be performed continuously and robotically. The successes of this approach have led to screens for several diseases, and Food and Drug Administration (FDA) approved therapeutic drug compounds such as cyclooxygenase 1,2 inhibitors, (Rofecoxib, Etoricoxib),<sup>73-74</sup> antiviral compounds,<sup>75,76</sup> and the Bcl-Abl tyrosine kinase inhibitor for the treatment of chronic myelogenous leukemia and gastrointestinal stromal tumors, Gleevec.<sup>77</sup> Unlike these success stories however, polyglutamine disease researchers do not yet have a defined target to do precise screening and drug design. This is a tremendous challenge, as high-throughput screening with very large chemical libraries can be logistically difficult even when a very specific target is known, i.e. the ATP-binding pocket region of a kinase protein.

Initial screens against HD were targeted at compounds that prevent aggregation of polyglutamine in biochemical assays.<sup>23</sup> These screens have led to the identification of small molecules

and polypeptides that could inhibit polyglutamine-mediated aggregation in vitro, with a few that worked in vivo, but at relatively high effective concentrations.<sup>23,78,79</sup> The problem with chemical compound screens as the starting point to drug discovery is that considerable time and finances must be in place to bring a compound to FDA approval. Pharmacological obstacles such as compound toxicity, solubility, stability and the ability to cross the blood/brain barrier are necessary considerations while the chemical derivatives must still maintain the desired activity. Modern numbers for lead compounds development to a drug are in the hundreds of millions of dollars and over a decade in time, which are difficult resources to obtain for such relatively rare diseases.

An alternative approach to broad chemical compound screens is to screen previously FDA-approved molecules for novel activities, or from libraries of known biologically active compounds. One such screen defined a single hit from this library of 2800 molecules: the p160ROCK rho associated kinase inhibitor, Y-27632, a molecule that could inhibit polyglutamine mediated aggregation of huntingtin protein fragments.<sup>80</sup> This intriguing result suggested that factors in addition to polyglutamine expansion may have an effect on aggregation of huntingtin. Additionally, recent work in cell biological studies and the use of intracellularly expressed antibodies or "intrabodies" suggest that the polyproline region adjacent to the polyglutamine tract in huntingtin may be important in triggering aggregation.<sup>81,82</sup> Another screen identified several pre-approved compounds, but at effective concentrations too high for practical use and are therefore characterized as potential lead compounds.<sup>83</sup> Screens in *Drosophila* fly models identified the histone de-acetylase (HDAC) inhibitor family of compounds as suppressors of polyglutamine toxicity, implying a potentially important role of huntingtin activity in the nucleus at the level of transcription regulation. Histone de-acetylase inhibitors, also used as anti-tumor agents, show efficacy in HD fly models, primary neuronal cultures and mouse models.<sup>84-86</sup> Another promising lead compound is C2-8, a sulfobenzoic acid derivative that shows neuroprotection against huntingtin toxicity at nanomolar concentrations.<sup>24</sup> While the exact mechanism on C2-8 activity is not known, rather than preventing the onset of aggregation, C2-8 prevents the growth of small polyglutamine aggregates into large protein inclusions (Figure 2).<sup>24</sup> A converse approach is to force mutant huntingtin into inclusions by the use of pro-inclusion compounds, such as B2 and B5, which do show protection against toxicity by mutant huntingtin and alpha synuclein in Parkinson's Disease cell models, but at high concentrations.<sup>87</sup> While polyglutamine and its biophysical properties are a logical target for targeted drug design in polyglutamine diseases, successful therapies for other diseases often focus on signaling molecules, typically small, soluble molecules with kinase activity, amenable to inhibition by deliverable small chemical compounds.<sup>88</sup>

#### INTRACELLULAR SIGNALING AND HUNTINGTON'S DISEASE

If the expanded polyglutamine tract may not be the optimal target for drug design in HD and other polyglutamine diseases, well-established practical targets of drug design in disease which may lead to a combination therapy approach are signaling molecules such as serine, threonine or tyrosine kinases involved

in sensor activation pathways, or nuclear hormone receptors.<sup>77</sup> A good example of dedicated efforts in this respect are inhibitors of nuclear hormone receptors with broad applications in cancer treatment, cardiac disease, angiogenesis and asthma.<sup>89-93</sup> Understanding the biological function of huntingtin means understanding any role of huntingtin in intracellular and intercellular signaling. The involvement of huntingtin in these signaling pathways and cascades not only elucidates the activities of normal huntingtin, but can help us understand the molecular pathological triggers of mutant huntingtin in HD. In one example, huntingtin is phosphorylated by the akt kinase/protein kinase B at serine 421. Post-translational modification of huntingtin at serine 421 can be induced by insulin growth factor 1, inhibit neuronal cell death by mutant huntingtin and inhibit huntingtin aggregation.<sup>94</sup> The switching on of akt kinase in mutant huntingtin mouse cells has been linked to the enhanced activation of the N-methyl-D-aspartate receptor.<sup>95</sup> Excessive activation of the N-methyl-D-aspartate receptor is a classic observation in HD leading to the theory of excitotoxicity, or over-activation of glutamate-type receptors leading to cell death.<sup>96-98</sup> Huntingtin affects the signaling of the mGluR1 glutamate receptor through an associated HYP protein, Optineurin.<sup>99</sup> The intriguing commonality of some of these experiments, and those previously mentioned for the p160ROCK inhibitor,<sup>80</sup> is that kinase activators or inhibitors appear to be able to modulate polyglutamine-dependant aggregation of huntingtin, again suggesting that additional factors other than polyglutamine tract expansion may influence the presence of aggregated huntingtin protein.

Mutant huntingtin has also been seen to activate signaling by I kappa B kinase,<sup>100,101</sup> and activate the JNK terminal kinase, an important step to triggering apoptosis.<sup>102</sup> Calcium-mediated signaling is disrupted in HD mouse models, but is not restricted to neurons and can be detected in mutant huntingtin lymphoblasts, suggesting that huntingtin's involvement in calcium signaling may be a universal function in all cells.<sup>103-105</sup> Disrupted calcium signaling is seen in another polyglutamine disease, spinocerebellar ataxia type 6, where the polyglutamine expansion is in the alpha 1A voltage-dependent calcium channel.<sup>106</sup> Unlike most other polyglutamine diseases, the polyglutamine tract expansion in SCA6 is thought to result in a loss of function of the alpha 1A voltage-dependent calcium channel.<sup>107</sup>

The inter-regulatory pathways that typically connect signaling cascades in cells provide several potential therapeutic targets for HD. The challenge to cell biologists will be to determine which of the signaling kinases, involved with huntingtin modification, are the result of cell stress due to mutant huntingtin expression, or direct pathways triggering mutant huntingtin-mediated cell death. Targeted inhibition of a signaling pathway critical to HD could prevent mutant huntingtin from triggering pathology by keeping it inactive. This concept has seen fruition in the case of spinal and bulbar muscular atrophy (SBMA), caused by polyglutamine expansion in the well-characterized androgen receptor.

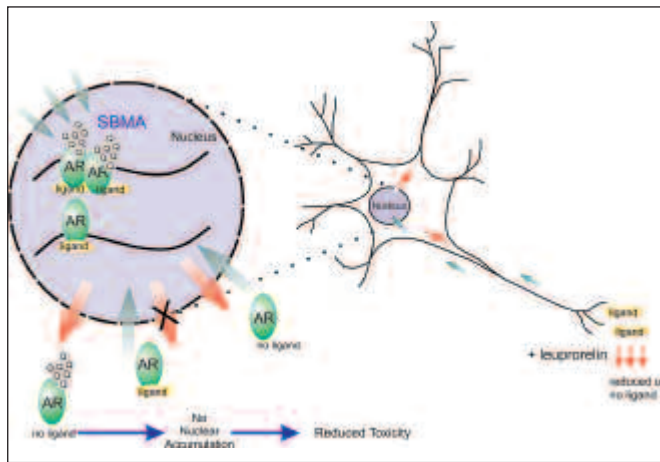
## SPINAL AND BULBAR MUSCULAR ATROPHY (SBMA): THE ANDROGEN RECEPTOR. A MODEL FOR POLYGLUTAMINE DISEASE THERAPY?

Spinal and bulbar muscular atrophy (or Kennedy's Disease) is a late-onset progressive neurodegenerative disease affecting lower motor neurons and manifesting with proximal limb muscle weakness, fasciculations and bulbar movement. The late result of this disease is a progressive atrophy of muscle fibres and replacement with adipose tissue. Until recently, no specific treatment was available. Unlike the other polyglutamine diseases, SBMA primarily affects males, following a mode of X-linked inheritance. Females typically exhibit a reduced pathology. The molecular basis for this disease at the genetic level became apparent when the gene mutation was identified as a CAG expansion in exon 1 of the androgen receptor (AR).<sup>3</sup>

In contrast to the other polyglutamine disease proteins, the exact biological function of the AR is well characterized. The AR is a steroid nuclear receptor, a ligand-dependent nuclear transcription factor that is essential for the development of the male fetus, male sexual characteristics and the maintenance of spermatogenesis.<sup>108</sup> Like huntingtin, the AR has the ability to enter the nucleus. The precise genes that are activated and the co-factors required for transcription activation with the AR are well known. Also as with huntingtin, and other polyglutamine diseases, polyglutamine expansion in the AR exhibits a gain-of-function, in that loss of the AR in males leads to feminization, but no neuropathy, in a disease termed Androgen Insensitivity Syndrome.<sup>109</sup> This indicates that the polyglutamine expanded AR is capable of all the functions of the normal AR, but with an additional toxic function.<sup>14</sup>

The function of the AR as a transcription modulator involves both on and off mechanisms. These mechanisms include ligand binding and nuclear localization via a nuclear localization signal near the DNA binding domain as the "on" switch, and nuclear export via a nuclear export signal as the "off" switch. These signals are amino-acid sequences within the proteins that are used as recognition targets by other protein factors to move proteins into different cellular compartments, and are now being seen as important for regulation of transcription events and cell signaling.<sup>110</sup> The role of ligand binding, specifically testosterone, in this mechanism is to inhibit the nuclear export signal, thus allowing the AR to accumulate in the nucleus at effective concentrations to activate transcription<sup>111</sup> (Figure 4). In the absence of the ligand, the AR continuously traffics between the nucleus and the cytoplasm, but once bound to testosterone, enters the nucleus and does not exit due to inhibition of nuclear export.

In SBMA, the polymorphic polyglutamine region in the AR that is normally between 10 and 36 repeats is expanded to 40-62 repeats.<sup>3</sup> Spinal and bulbar muscular atrophy neurons are seen to contain intranuclear inclusions of the AR and elevated nuclear AR levels, like some other polyglutamine diseases.<sup>112,113</sup> The exact mechanism of toxicity of these nuclear inclusions is not clear, but as with huntingtin, it is thought to involve the sequestration of transcription factors,<sup>114</sup> or the inhibition of normal proteolytic turnover of proteins.<sup>115</sup> However, neither of these mechanisms explains the toxic specificity of polyglutamine-expanded proteins to specific neuronal



**Figure 4:** Model of the Androgen Receptor Function and Dysfunction in SBMA. The Androgen receptor is a nuclear hormone receptor, with nuclear localization and nuclear export signal sequences. The AR normally shuttles between the nucleus and cytoplasm in the absence of ligand, i.e. testosterone, but in the presence of ligand, nuclear export is inhibited, while nuclear import is unaffected, resulting in nuclear accumulation of AR to threshold levels for transcription activation of specific genes. In SBMA, polyglutamine-expanded AR is toxic, but only when retained in the nucleus by ligand, not in the absence or low levels of ligand. Testosterone release inhibitors, such as leuporelin, reduce circulating testosterone levels resulting in inhibited AR nuclear signaling and hence polyglutamine-expanded AR nuclear accumulation. In contrast, the androgen antagonist, flutamide, promotes nuclear translocation of AR, but has no therapeutic benefit in animal models of SBMA.

populations, despite ubiquitous expression, or even higher expression in cell types not lost during SBMA. In recent studies, lower motor neurons in mice were noted to have increased levels of nuclear polyglutamine expanded AR, but not necessarily in inclusions.<sup>112</sup>

There are cases where polyglutamine-expanded AR leads to an attenuated SBMA. For example, in female carriers of the polyglutamine expanded mutant AR. The main reason for disease attenuation is that females express lower levels of circulating androgens, such as testosterone. This theory was tested in animal models of polyglutamine expanded AR, where the reduction of testosterone levels by castration led to significant decreases in neurodegeneration in both fly and mouse models of SBMA.<sup>116,117</sup> This principle was carried further, by the use of a known testosterone release inhibitor, leuporelin, to significantly reduce neuropathology in mouse models. These elegant and well-controlled studies by this Japanese group also used flutamide, an AR agonist that stimulates nuclear localization of the AR, to show no therapeutic effect.<sup>118</sup> Thus, the prevention of nuclear accumulation of the polyglutamine expanded AR has led to a therapy for SBMA that is now being tested in the clinic. Leuporelin has already undergone human clinical phase III trials for treatment of certain types of cancer.<sup>119</sup>

This exciting prospect of the first clinical therapy for a polyglutamine disease may have two important lessons for polyglutamine disease and other neurodegenerative diseases. Firstly, that knowing the precise biological function of the

protein which expresses the polyglutamine tract expansion has led to faster development of a therapy; and secondly, that the therapy was targeted to the normal biological function of the protein affected by polyglutamine expansion, and not to a biophysical property of expanded polyglutamine. The success of hormone antagonists in SBMA could lead to a new paradigm of drug development for Huntington's disease and other polyglutamine diseases, and/or potentially lead to compounds that may be used in combination with those that inhibit polyglutamine-dependent aggregation. Despite the presence of an expanded polyglutamine tract, a more conventional target could be to inactivate the normal biological function of the protein by specific signaling inhibition to render it innocuous to the cell. This may be very challenging for Huntington's disease, because the normal huntingtin protein is essential for development,<sup>120</sup> and may be essential for the life of any cell, as it is ubiquitously expressed in all cell types<sup>121</sup> and is found highly conserved in all vertebrate species.<sup>122</sup> Unlike the AR, human neurons may not be able to tolerate induced loss of normal huntingtin function.

Comparative studies between the polyglutamine diseases are relatively few in the press, but are often used to test the universality of conclusions about polyglutamine expansion in huntingtin. Some of the most detailed mouse model work in polyglutamine diseases has been accomplished by two collaborating groups of Harry Orr at the University of Minnesota and Huda Zoghbi at Baylor College on the spinocerebellar ataxia type 1 protein, ataxin-1.<sup>123</sup>

#### THE SPINOCEREBELLAR ATAXIAS (SCAs), TYPES 1, 2, 3, 6, 7 AND 17.

Ataxia is a defect in motor coordination that typically affects gait and balance, but can additionally affect gaze and speech.<sup>124</sup> Ataxia can be caused by a variety of factors, including metabolic dysfunction, toxicity, autoimmunity, paraneoplasm, and genetic factors.<sup>125</sup> Over 35 loci for autosomal dominant and recessive hereditary ataxias have been identified.<sup>125</sup> Most autosomal dominant ataxias are polyglutamine diseases, with disease causing expansions beginning in the range of 35-40; collectively, the prevalence of these diseases has been estimated at ~3 per 100,000.<sup>126-129</sup> Clinically, these diseases primarily affect the cerebellum, brainstem and the spinal cord, but clinical features vary among the different SCAs and even within subtypes.<sup>129</sup> Spinocerebellar Ataxias typically present as dominantly transmitted ataxia, with additional features such as dysarthria and eye movement abnormalities related to cerebellar dysfunction as well as features that vary with SCA type, such as retinopathy, optic atrophy, slow saccades, ophthalmoplegia, dementia, epilepsy, amyotrophy, spasticity, dyskinesias, parkinsonism and peripheral neuropathy. The diagnostic pathological feature that defines SCAs is olivopontocerebellar atrophy,<sup>130</sup> except for SCA6 which manifests as pure cerebellar atrophy. Despite the common genetic mutation of an expanded polyglutamine tract in these proteins, there exist some differences in brain pathology. For example, in SCA1, 2 and 6, but not SCA3, patients have significant loss of Purkinje cells, the highly arborized cerebellar neurons with the highest number and longest input branches of any neuronal cell type. Of all the SCAs, SCA1 is perhaps the best defined in terms of molecular pathogenesis due to the extensive use of mouse genetic models.



### THE SCA1 PROTEIN, ATAXIN-1

Polyglutamine-expanded ataxin-1 has several characteristics typical of polyglutamine diseases. These include genetic anticipation, age of onset varying with polyglutamine tract length (with average onset in the fourth decade), progressive neurodegeneration, toxic gain-of-function, autosomal dominance, and the presence of well defined neuronal intranuclear inclusions.<sup>131</sup> Transgenic mouse models of SCA1 with polyglutamine expanded ataxin-1 exhibit typical ataxic phenotypes and severe loss of Purkinje cells.<sup>22</sup> Subsequent mutant ataxin-1 transgenic models have attempted to address the role of nuclear inclusions of protein by identifying the nuclear localization signal (NLS) sequence within the protein, inactivating the signal activity with specific point-mutations and assessing the NLS-defective polyglutamine expanded ataxin-1 expressed back in the transgenic mouse model. These studies demonstrated that nuclear polyglutamine expanded ataxin-1 was necessary for generation of ataxia in the mouse, and that cytoplasmic polyglutamine expanded ataxin-1 was not toxic.<sup>21</sup> This result contradicted work by others that indicated that polyglutamine expansion synthetically placed in any protein could lead to neurodegeneration, with noted intranuclear aggregates, and thus only expression of polyglutamine appeared to cause disease.<sup>132,133</sup> The NLS mutant SCA1 mouse demonstrated that the polyglutamine expansion alone in any expressed protein was not sufficient to cause disease, but that nuclear localization was required. When an analysis of potential phospho-modification and signaling of ataxin-1 was carried out, a single serine adjacent to the NLS sequence was identified as being a target of phosphorylation by akt kinase, the same signaling pathway implicated in huntingtin phosphorylation.<sup>134</sup> However, while this phosphorylation did not affect nuclear localization, transgenic mice expressing ataxin-1 with a serine to alanine mutation did not develop ataxia in a transgenic mouse, despite the presence of ataxin-1 in the nucleus. This data indicated that alone, nuclear localization of polyglutamine tracts was not sufficient for the triggering of SCA1.<sup>20</sup> These studies on the specific akt phosphorylation site in ataxin-1 pointed to a potential therapeutic target for SCA1, by the use of akt kinase inhibitors that prevented ataxin-1 phosphorylation.<sup>135</sup>

What about the normal biological function of ataxin-1 and its role in SCA1? Ataxin-1 is known to be a nucleic acid binding protein, binding directly to RNA.<sup>136</sup> Ataxin-1 nuclear inclusions contain the silencing mediator of retinoid and thyroid hormone receptors, or SMRT.<sup>137</sup> In studies where the polyglutamine tract in ataxin-1 was completely removed by mutagenesis, ataxin-1 protein still localized to nuclear inclusions, with similar characteristics to normal and polyglutamine-expanded ataxin-1.<sup>137-139</sup> When the “aggregates” of several polyglutamine disease inclusions were analyzed at the level of biophysical studies in live cells, it was found that polyglutamine-expanded ataxin-1, ataxin-3 and huntingtin proteins were dynamic and able to move between inclusions, suggesting that the inclusions in these polyglutamine diseases were not static, aggregated protein.<sup>138,140</sup> While polyglutamine was not required for the presence of nuclear inclusions, mRNA and active transcription were seen tied to the formation of ataxin-1 inclusions, and in response to cell stress, ataxin-1 could recruit a general mRNA export factor to these inclusions.<sup>139</sup> Ataxin-1 is also capable of nuclear export,

a function that is inhibited by polyglutamine expansion in the protein.<sup>139</sup> These observations of ataxin-1 in transcription modulation and RNA processing suggest that the normal nuclear function of ataxin-1, in response to akt signaling, may be as a transcription modulator at the level of gene activation, RNA export, or both. Toxic specificity of mutant polyglutamine-expanded ataxin-1 in SCA1 may therefore be at the level of a precise subset of mRNAs retained in the nucleus. In terms of drug development, these elegant transgenic mouse model studies of SCA1 point to kinase inhibitors as modulators of toxicity. Thus, as with the case of the androgen receptor and SBMA, there is a potential therapy and may not be directly targeted at expanded polyglutamine. A potential overlap in function and functional pathways with ataxin-1 may be in ataxin-2, the SCA2 disease protein.

Spinocerebellar ataxia type 2, like SCA1, shows a high level of Purkinje cell loss, as well as loss in the pontine nuclei and inferior olives. Clinical features are similar to those seen in SCA1, except that tremor, slowing of saccades and hypoflexia are more common. Unlike SCA1, SCA2 pathology also extends to the substantia nigra, which may account for some patients exhibiting features of parkinsonism without ataxia.

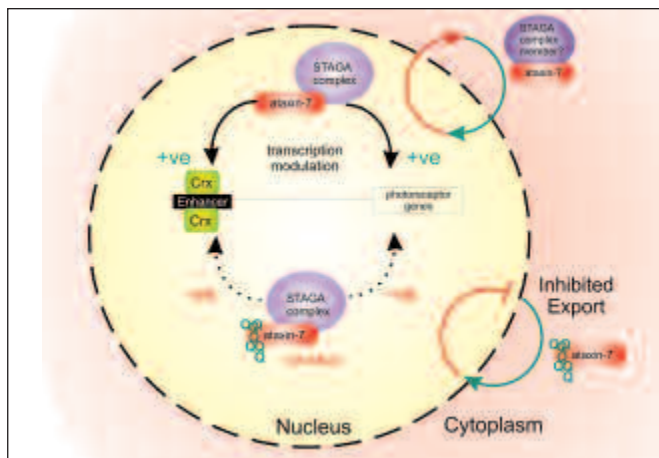
While nuclear inclusions of ataxin-2 protein are seen in 1-2% of cells in the brain, unlike the other polyglutamine diseases, ataxin-2 inclusions are not noted at the site of primary pathology, the Purkinje cells.<sup>141</sup> In a SCA2 transgenic mouse model, there is no correlation of toxicity with either nuclear localization or the presence of nuclear inclusions of ataxin-2 protein.<sup>142</sup> However, in those studies the investigators did not address the possibility that ataxin-2 may be dynamically moving between the nucleus and cytoplasm, as is seen with other polyglutamine disease proteins. Ataxin-2 interacts with RNA binding proteins,<sup>143</sup> and with RNA directly through an Lsm domain.<sup>144</sup> By integrative proteomics, ataxin-2 has been implicated in RNA processing, and can co-localize with the mRNA poly A binding protein.<sup>145</sup> Poly A binding proteins are known to dynamically shuttle between the nucleus and cytoplasm, exporting the nucleus along with RNA.<sup>146</sup> Integrative proteomics is a relatively new method of attempting to understand the biological function of a protein using homology studies to similar proteins in other species and the pathways they participate in. This technology, by taking advantage of the completed yeast, human and other genomic sequences, also defines cellular protein-protein interactions with proteins of known function in order to infer biological function.<sup>147</sup> The connection of both ataxin-1 and ataxin-2 to RNA suggests possible mis-processing or nuclear retention of RNA in SCA1, 2 diseases. RNA transport inhibition has precedent in neurologic disease: in Fragile X syndrome, loss of expression of FMR1, or mis-splicing leading to loss of a nuclear export signal-encoding exon, leads to lack of nuclear export of a family of mRNAs essential for normal brain development;<sup>148,149</sup> nova RNA-associated proteins, the paraneoplastic Ri antigen RNA binding proteins, are involved in paraneoplastic opsoclonus ataxia;<sup>150</sup> and the NTX5 RNA export factor is not expressed in some patients with X-linked mental retardation.<sup>151</sup> As with ataxin-1, ataxin-2 toxic specificity may be dictated by the specific messenger RNAs affected by the gain-of-function in polyglutamine-expanded SCA2.

## SPINOCEREBELLAR ATAXIA TYPES 7 AND 17

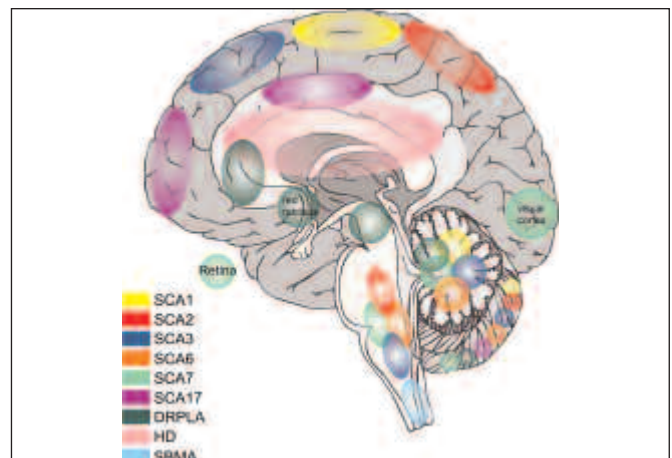
Given that so many polyglutamine disease proteins result in Purkinje cell loss and ataxia, it may not be difficult to imagine that common mechanisms related to inter-regulatory pathways important for Purkinje cell health may be affected by multiple ataxins. Some polyglutamine-expanded proteins may act as genetic modifiers for other polyglutamine diseases.<sup>152</sup> In SCA7, in addition to the typical Purkinje cell pathology of SCA1, marked degeneration is also noted in the retinal ganglia, the optic tract and the visual cortex (Figure 5). In the clinic, this leads to blindness in SCA7 patients in addition to progressive ataxia.<sup>153</sup> CAG repeat lengths can exceed 200 with infantile onset in this disease. Like ataxin-1, ataxin-7 is normally nuclear.<sup>154</sup> While SCA7 transgenic mouse models reveal a progressive accumulation of nuclear mutant ataxin-7,<sup>155</sup> Ataxin-7's biological role is being quickly elucidated.<sup>156</sup> It has been shown that Polyglutamine-expanded ataxin-7 can inhibit transcription of Crx-controlled genes. Crx is a transcription factor involved in the regulation of retinal genes.<sup>157</sup> Ataxin-7 is a member of a transcription co-activator complex known as STAGA, which includes the GCN5 histone acetyl-transferase (one target of HDAC inhibitors).<sup>158</sup> Ataxin-7's role in this complex may be to act as the molecular bridge between DNA-bound transcription factors such as Crx and GCN5 in a classic mechanism of transcription regulation by upstream DNA bound enhancers affecting RNA polymerase II mRNA transcription<sup>157</sup> (Figure 6). Recently, ataxin-7 has been found to contain a nuclear export signal and has the ability to shuttle to and from the nucleus, an activity in common with AR, huntingtin, atrophin-1, ataxin-1 and likely ataxin-2.<sup>159</sup> As with ataxin-1, polyglutamine expansion in

ataxin-7 inhibits ataxin-7 nuclear export.<sup>159</sup> Despite the accumulation of nuclear ataxin-7 during disease progression in the SCA7 mouse model, this is not due to ubiquitin-mediated proteasome inhibition by polyglutamine-expanded ataxin-7, as aggregation in SCA7 mice inversely correlates with toxicity.<sup>159,160</sup> Along with observations with SCA2, this suggests that in some protein contexts, aggregated polyglutamine protein may be either protective or innocuous. A similar conclusion was made for huntingtin aggregates based on experiments that monitored aggregate formation in live primary cultured neurons that showed a negative correlation with cell death.<sup>161</sup> Consistent with these observations, compounds that have been selected to promote the aggregation of mutant huntingtin exon1 fragment, show a protective effect against toxicity.<sup>87</sup> In addition, ataxin-7 has a short region of homology with the phospho-protein binding domain of beta-arrestins.<sup>162</sup> Together with its shuttling activity to and from the nucleus, this domain suggests that ataxin-7 may be a beta arrestin-like molecule involved in signaling.<sup>163</sup>

Downstream of ataxin-7's role as transcription co-activator, the STAGA complex interacts with the RNA polymerase II complex at the promoter. One essential factor required for transcription of most genes by RNA polymerase II is the TATA-box binding protein, or TBP. TBP is a saddle-shaped protein that acts as a DNA-bound scaffold as part of the transcription initiation complex. TBP is highly conserved from yeast to humans, with the exception of a 38-repeat glutamine tract in human TBP. While 36 repeats of polyglutamine appear to be the disease threshold for many polyglutamine diseases, the presence of a normal tract of 38 glutamines in TBP contradicts this concept.<sup>164</sup> TBP is the disease protein affected by polyglutamine



**Figure 5: Regional Pathology in the Brain of Polyglutamine Diseases.** Despite often ubiquitous expression in all regions of the brain and in all cell types, polyglutamine diseases are distinct in their pathology and regions of cell loss during disease. The spinocerebellar ataxias all commonly affect the cerebellum and most commonly the Purkinje cells. HD primarily affects the striatum, and later the cortex, with severe mass loss by death. SCA7 displays unique retinal cell loss, visual cortex loss, and resultant blindness during disease. DRPLA affects mainly the cerebellar dentate nuclei, red nucleus in forebrain, globus pallidus and subthalamic nucleus in the basal ganglia.



**Figure 6: Model of Ataxin-7 Function and Dysfunction in SCA7.** Ataxin-7 is a member of the STAGA transcription co-activator complex. The amino terminus of ataxin-7 makes interactions with upstream DNA-bound activators, such as Crx, and therefore modulates transcription of some retinal-specific genes. Ataxin-7 also shuttles between the nucleus and cytoplasm. Polyglutamine expansion in ataxin-7 leads to reduced nuclear export and accumulation in the nucleus. Nuclear accumulation of full-length mutant protein at concentrations above wild-type level of protein, may result in dominant inhibition by disruption of STAGA interactions or the inability to export a STAGA complex member.



expansion in SCA17, but only when repeats are seen at greater than 50-55.<sup>165</sup> TBP has been seen to be recruited, and likely sequestered, into nuclear inclusions of other polyglutamine disease proteins, such as ataxin-3.<sup>166</sup> SCA17 typifies the conundrum of disease specificity in polyglutamine diseases (Figure 5). TBP function is critical to the basic mechanism of RNA polymerase II transcription, and is required in every living cell, but polyglutamine-expanded TBP specifically leads to neurologic disease, with focal dystonia, ataxia and dementia.<sup>167,168</sup> As with other polyglutamine diseases, protein expression patterns of TBP do not dictate sites of pathology. TBP and huntingtin are just two examples of ubiquitously expressed polyglutamine disease proteins that lead to very specific cell death in neurons. The reasons for this toxic specificity are not understood, but may have to do with the normal functions of these proteins being more critical in neuronal subpopulations, or that contain neuronal sub-types that are particularly fragile and sensitive to the induced stresses triggered by polyglutamine-expanded proteins.

### SCA3: MACHADO-JOSEPH DISEASE (MJD) AND DIRECT PROTEASOME INVOLVEMENT

The protein affected in SCA3/MJD, ataxin-3, is the smallest of the polyglutamine disease proteins at 376 amino acids.<sup>169</sup> This disease was originally described in populations with Portuguese-Azorean ancestry, but is now known as the most common cause of dominant cerebellar ataxia, with a prevalent haplotype implying a genetic founder effect.<sup>170</sup> In SCA3, Purkinje cells are affected, but to a lesser extent than SCA1, and a large amount of degeneration is seen in Clark's column, as well as vestibular and pontine nuclei.<sup>171</sup> While inhibition of normal proteolytic turnover has been noted for some polyglutamine disease proteins,<sup>172,173</sup> ataxin-3's role in proteasome function has been recently elucidated to be more direct. Ataxin-3 has been shown to have a deubiquitylating activity on chains of four or more ubiquitins and therefore can regulate protein degradation.<sup>174</sup> Also, as with some other polyglutamine disease proteins, ataxin-3 is seen to be proteolytically cleaved,<sup>175</sup> and is found in intranuclear inclusions.<sup>113,176</sup> However, live cell protein dynamic studies using green fluorescent protein fusions to ataxin-3 demonstrated that these polyglutamine dependent inclusions are not static, precipitated protein, but areas of high local concentration that are dynamic,<sup>177</sup> as seen with ataxin-1 inclusions.<sup>138,139</sup> Therefore, it appears that the normal function of ataxin-3 as regulator of proteasome function is being perturbed by the expanded polyglutamine tract, leading to abnormally high levels of proteins whose intracellular concentrations are normally regulated by ataxin-3. It is likely that the toxic specificity seen in SCA3 is dictated by abnormally high levels of particular proteins. An intriguing observation in the CAG tracts of ataxin-3 is that this mRNA is susceptible to frame-shifting during translation, leading to the expression of polyalanine and insolubility of ataxin-3.<sup>178</sup> However, this mechanism has not yet been seen with other polyglutamine diseases.

### DENTATORUBRALPALLIDOLUYSIAN ATROPHY (DRPLA), THE ATROPHIN-1 PROTEIN

Dentatorubralpallidolusian Atrophy (DRPLA) is a very rare disease in caucasian populations, but can occur at incidences as

high as 1 in 250,000 in Japanese populations.<sup>129</sup> Dentatorubralpallidolusian Atrophy and SCA2 are two polyglutamine diseases that show marked geographic distributions of incidence, with SCA2 as high as 1 in 500 in the Cuban population due to a genetic founder effect.<sup>179,180</sup> Dentatorubralpallidolusian Atrophy patients present with ataxia, as well as features similar to those found in Huntington's disease, such as dementia and choreoathetosis (writhing or twitching involuntary movement), with severe juvenile-onset cases displaying myoclonus and epileptic seizures.<sup>181</sup> One of the traits paramount to clinical diagnosis of DRPLA is pronounced mood swings and attention deficit. The polyglutamine-expanded disease protein of DRPLA is atrophin-1 with expansions of 49 or higher associated with disease. As with huntingtin and TBP, atrophin-1 is ubiquitously expressed in all tissues, and as with several polyglutamine disease proteins, is proteolytically cleaved in neuronal cells, but not in other cells.<sup>182</sup> Atrophin-1 is differentially phosphorylated in DRPLA versus normal brains,<sup>183</sup> suggesting a signaling role for atrophin-1. As with several other polyglutamine diseases, atrophin-1 fragments are seen to accumulate in the nucleus.<sup>184</sup> The function of atrophin-1 has been suggested as a transcription factor, and polyglutamine-expanded atrophin-1 can sequester the CREB binding protein (CBP) transcription factor.<sup>185</sup> As with huntingtin, the androgen receptor, ataxin-1, and ataxin-7, atrophin-1 can shuttle to and from the nucleus. Fragmentation by proteolysis removes the nuclear export signal sequence in atrophin-1 which results in nuclear accumulation of a fragment containing a nuclear localization signal and the polyglutamine tract.<sup>65</sup> This mechanism of gain-of-function by nuclear accumulation appears to be a common observation in several polyglutamine diseases.<sup>28,139,159,186</sup>

### POLYGLUTAMINE DISEASES: THERAPY AFTER DISEASE ONSET?

Can a hypothetical polyglutamine disease therapy practically halt or reverse the neurodegenerative damage in patients and improve quality of life? The answers from sophisticated mouse genetic models are strikingly positive. In mouse genetic models in which mutant huntingtin fragment expression can be turned on or off, expression of the mutant protein results in neurodegeneration and HD-like motor dysfunction. This can be reversed as quickly as 14 days after shutting off mutant protein expression, for both huntingtin and SCA1 mouse models.<sup>19,187</sup> In these mouse brains, restoration of the Purkinje cells or complete clearance of huntingtin aggregates was seen.<sup>19,188</sup> The strong conclusions from these studies is that preventing expression of the polyglutamine disease protein will halt toxicity, and the brain's plasticity can lead to rapid recovery, even after severe disease has been triggered. Along with this approach to therapy, several groups are developing small inhibitory RNAs (siRNAs) as a very potent and specific molecular method of shutting off mutant protein expression.<sup>189</sup> In the mouse model and cell biological studies, siRNAs are being seen as effective against HD, SCA3, and SCA1.<sup>174,190,191</sup> The future of this potential route of therapy is the development of viral vectors that can introduce expression of these siRNAs into the brain or other tissues.<sup>190,192,193</sup> The other alternative approach could be to remove the mutant proteins from their normal biological functions or mutant-gained functions by the use of small molecule inhibitors.

Tremendous advances have been made in the field of polyglutamine diseases research, despite the relatively small size of this research field, in just over a decade. The use of researchers and clinicians with expertise in biochemistry, cell biology, as well as neurosciences, in addition to the power of mouse genetic models and small molecule screening has ensured that no stone will be left unturned in the expedient development of therapies for these tragic diseases. Current and future collaborations with pharmaceutical industry and the ongoing development of biomarkers for these diseases, as well as new technologies of viral-based therapies lay the groundwork for an exciting next few years on the road to therapy for several of the polyglutamine expansion neurodegenerative diseases.

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