Polyglutamine Diseases

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Abstract

Polyglutamine diseases are a collection of nine CAG trinucleotide expansion disorders, presenting with a spectrum of neurological and clinical phenotypes. Recent human, mouse and cell studies of Huntington's disease (HD) have highlighted the role of DNA repair genes in somatic expansion of the CAG repeat region, modifying disease pathogenesis. Incomplete splicing of the *HTT* gene has also been shown to occur in humans, with the resulting exon 1 fragment most probably contributing to the HD phenotype. In the spinocerebellar ataxias (SCAs), studies have converged on transcriptional dysregulation of ion channels as a key disease modifier. In addition, advances have been made in understanding how increased levels of toxic, polyglutamine-expanded proteins can arise in the SCAs through post-transcriptional and -translational modifications and autophagic mechanisms. Recent studies in spinal and bulbar muscular atrophy (SBMA) implicate similar pathogenic pathways to the more common polyglutamine diseases, highlighting autophagy stimulation as a potential therapeutic target. Finally, the therapeutic use of antisense oligonucleotides in several polyglutamine diseases has shown preclinical benefits and serve as potential future therapies in humans.

Introduction

Polyglutamine diseases are a collection of nine monogenic neurodegenerative conditions with similar aetiologies. In the disease-affected gene, expansion of a CAG trinucleotide repeat region translates into a long stretch of glutamine (Q) residues in the associated protein, initiating downstream phenotypes such as enhanced DNA damage, intracellular aggresomes, plus transcriptional and proteasomal dysregulation. These disorders demonstrate a CAG repeat length-dependent effect on age at onset (AAO), with longer CAG repeat tracts being associated with earlier disease manifestation. Expansion of the CAG repeat throughout an individual's lifetime (somatic expansion; Table 1) and between generations (germline meiotic expansion) also occurs in many of these diseases. However, certain pathological phenotypes are disease-specific, likely reflecting the expression profile of the gene of interest or the interactome of the cognate protein.

This review will encompass recent advances in our understanding of polyglutamine diseases. Significant progress has been made in the DNA repair field and mechanisms have emerged among downstream processes such as post-transcriptional splicing and ion channel dysregulation. Recent therapeutic efforts, specifically the use of antisense oligonucleotides (ASOs), will be briefly discussed.

Huntington's disease

Huntington's disease (HD) is caused by CAG repeat expansion in exon 1 of the *HTT* gene, clinically characterised by progressive chorea, cognitive and behavioural symptoms [1]. Recent work has highlighted genetic modifiers of HD pathogenesis, primarily in DNA repair, and the role of incomplete *HTT* splicing.

Genetics modifiers of Huntington's disease

The CAG repeat region in *HTT* is typically 3' capped with a CAA-CAG motif, encoding 2 additional glutamine residues. Rare variants of this 3' motif were identified by ultra-deep sequencing of *HTT* exon 1 DNA from the blood of TRACK-HD and ENROLL-HD cohorts [2]. Loss of the CAA interruption (LOI) was associated with AAO ~10 years earlier than predicted by polyglutamine length, whereas duplication of the motif was associated with a ~3-year delay. These findings were replicated within reduced penetrance samples (36-39 CAGs): LOI was linked with ~25 years earlier AAO [3]. Interestingly, 84.6% of those with extremely early onset possessed the LOI variant, suggesting this is an important prognosis differentiator within the reduced penetrance population. The LOI variant was associated with higher somatic expansion [3], which itself was associated with worse clinical outcomes such as earlier onset and faster progression [2]. Therefore, somatic expansion, modified by pure CAG repeat length, appears a significant driver of HD pathogenesis.

Several *HTT* loci were simultaneously identified in a genome-wide association study (GWAS) which, upon investigation, identified the LOI and duplication variants [4]. Encompassing over 9000 HD patients, the GWAS corroborated previous HD AAO modifier signals and identified several DNA repair genes, including *FAN1*, *MSH3*, *MLH1*, *LIG1*, *PMS1* and *PMS2* - many of which are active in mismatch repair (MMR; Figure 1). The most prominent single-nucleotide polymorphism (SNP) within *FAN1* (rs150393409) tagged the onset-hastening 15AM1 haplotype, predicted by SIFT to be deleterious. Conversely, an imputed transcription-wide association study (TWAS) using the GTEx database associated the onset-delaying haplotype 15AM2 with increased *FAN1* expression in cortex. The onset-hastening 5AM1 haplotype was similarly associated with *cis*-eQTLs for increased *MSH3* expression in blood, which correlated with increased somatic expansion. These data indicate that *MSH3* hastens HD pathology, whereas *FAN1* is protective.

The pathological role of *MSH3* had been previously indicated in HD: a locus spanning *MSH3*, *DHFR* and *MTRNR2L* was associated with a novel HD progression score in the TRACK-HD cohort [5]. The leading SNP (rs557874766) within *MSH3* was later revealed to be an alignment artefact, arising from a variant in the highly polymorphic 9 base-pair tandem repeat region of exon 1 [6]. This variant, termed '3a', was associated with delayed AAO, slower HD progression and slower somatic expansion. *MLH1*, an *MSH3*-interactor, was also reinforced as a HD-modifier by a dichotomous GWAS analysis (20% phenotypic extremes), associating a minor allele of *MLH1* with 0.7 years delayed AAO [7].

Similar to *MSH3* and *MLH1*, the HD-modifying effects of *FAN1* are believed to operate via somatic expansion [8]. FAN1$^{-/-}$ U2OS osteosarcoma cells complemented with a 118 CAG exon 1 construct demonstrate faster expansion, in addition to 109Q induced pluripotent stem cells (iPSCs) and medium spiny neurons (MSNs) with shRNA-mediated *FAN1* knockdown [9]. In agreement, another study identified 8 individuals with delayed onset who had 3 copies of the *FAN1* gene and showed that onset-hastening coding SNPs, R507H (rs150393409) and R377W (rs151322829), have reduced DNA-binding [10]. Although the mechanism by which *FAN1* stabilises the CAG repeat is unknown, it appears to be mediated by *MLH1*. Hdh$^{Q111/+}$ mice lacking *FAN1* exhibit significantly increased expansion in the striatum and liver at 5 months of age, noticeable in the latter tissue by 2.5 months [11]. However, no expansion is evident by 3 months when *MLH1* is knocked out. Thus, although *FAN1* is believed to work via the canonical nuclease activity, it requires a functional *FAN1-MLH1* genetic interaction.

Incomplete splicing of HTT

Generation of a toxic *HTT* exon 1 fragment by incomplete splicing (Figure 2) has previously been observed in all HD knock-in mouse models, however, Neueder et al. [12] provided the first evidence that this occurs in HD patients. The authors designed a novel qPCR assay for measuring truncated *HTT* transcripts. Higher expression of the exon 1 variant was observed in fibroblasts from juvenile HD patients (JHD; 60-70+ CAG), compared to those with adult-onset HD, indicating that incomplete splicing is dependent on CAG repeat length. In support of this, higher expression of the exon 1 transcript was found in the sensory motor cortex and hippocampus of JHD post-mortem tissue, with only minor increases seen in adult-onset HD samples, compared to controls. Subsequently, a human cell-based minigene system was developed to investigate the requirement of *HTT* intronic sequences for incomplete splicing [13]. The presence of a 5' 1.3kb region of intron 1 including transcription regulatory elements

was shown to greatly enhance production of the exon 1 transcript, which was CAG repeat length-dependent. Together, these data support the hypothesis that somatic expansion would exacerbate production of the toxic exon 1 fragment.

The authors also examined the effect of polyglutamine length on transcription rate by performing ChIP against phospho-Pol-II (polymerase II), and measuring occupancy along the *HTT* transcript in constructs with 7 or 100 CAG repeats [13]. Constructs with 100 CAG repeats had a slower rate of transcription which, when slowed further by tethering dCas9 at this locus, showed an increase in incomplete splicing. Slower transcription potentially allows polyadenylation (polyA) factors more time to recognise cryptic polyA sites, leading to more incomplete splicing in the presence of longer CAG repeats. The pathological importance of the exon 1 fragment was later illustrated by creating, via CRISPR, heterozygous HD (140Q) mice encoding truncated HTT protein (96 or 571 amino acids) [14]. Both genotypes, in addition to those expressing full-length HTT, exhibited similar pathology, including maturation of the exon 1 *HTT* mRNA, striatal aggregation of a ~50kDa HTT fragment (equivalent to exon 1), motor deficits at 7-9 months and transcriptional dysregulation. Therefore, despite expressing different HTT fragments, the commonality of the exon 1 protein likely dictated pathology.

Spinocerebellar ataxias

Collectively, the prevalence of the spinocerebellar ataxias (SCAs) is estimated to be ~2.7 per 100,000 but varies by disease, geographic region and ethnicity [15]. The polyglutamine SCAs are dominantly inherited and share the clinical ataxic phenotype, arising from progressive cerebellar degeneration (Table 1). Recently, pathogenic mechanisms have been established encompassing aspects of DNA repair, ion channel dysfunction and dysregulated proteostasis.

DNA repair and somatic expansion

Aside from SCA6, somatic expansion has been observed in all polyglutamine SCAs [16]. Quantification of CAG instability in post-mortem tissue of HD patients and an individual with SCA1 uncovered similar patterns of *HTT* and *ATXN1* expansion, respectively, within the brain [17]. Disease locus-independent trans factors may thus drive the regional rate of CAG expansion. Interestingly, SNPs in *FAN1* and *PMS2* have been associated with AAO in the polyglutamine SCAs [18]. These DNA repair genes are known modifiers of HD [4] and of somatic expansion [2,8,9,11,19], suggesting that the *trans* factors which govern CAG instability are likely involved in the DNA repair network.

Importantly, the tissue-specific patterns of CAG expansion do not always predict pathology. High instability was observed in the cortex and caudate, which demonstrate vulnerability in HD but are relatively unaffected in SCA1 [17]. Hence, particular cell-types appear to have unique pathogenic thresholds for different toxic species.

Ion channels

Perturbed Purkinje neuron excitability has emerged as a shared pathogenic mechanism across the polyglutamine SCAs [20]. Recent work converges on dysregulation of ion channel transcripts as the initiator of this process.

Gene expression analyses of cerebellar transcriptomes in SCA1 mice revealed a downregulation of Purkinje cell-enriched genes in ataxic mice [21]. ATXN1 was implicated as the main regulator, with an enrichment of Capicua transcriptional repressor (CIC) binding-motifs in the upstream region of downregulated genes. The ATXN1–CIC complex has shown an essential role in the developing forebrain, whereby loss-of-function leads to a spectrum of

neuro-behavioural disorders [22]. However, loss of the ATXN1-CIC complex in the cerebellum does not cause ataxia or cerebellar toxicity. Instead, a gain-of-function of the complex upon ATXN1 polyglutamine expansion is critical for cerebellar pathology and motor deficits in ATXN1 (82Q) mice [23]. In the same mouse model, this complex was later shown to act by downregulating an ion channel module (including critical genes *Kcnma1, Cacna1G* and *Itpr1*), which correlated with the onset of Purkinje neuron hyperexcitability and neurodegeneration [24].

Cacna1G and *Itpr1* encode calcium channels or their subunits, which act as sources for a calcium-activated potassium channel (BK) encoded by *Kcnma1*. A recent study in SCA7 (92Q) mice showed synergistic dysregulation of BK channels and perturbed calcium homeostasis are required to drive irregular spiking of Purkinje neurons [25], and reduced cerebellar transcripts for the same genes described in SCA1 mice [24] were observed. Abnormal Purkinje neuron spiking is also present in mouse models of SCA2 [26], SCA3 [27] and SCA6 [28], and is associated with motor onset, suggesting a shared pathogenic mechanism across multiple diseases. Additionally, protein kinase C and inositol triphosphate/calcium signalling have recently been implicated in polyglutamine SCA pathogenesis [29,30], further converging on alterations to intracellular ion dynamics as a disease modifier.

Dysregulated proteostasis

Increased levels of toxic, polyglutamine-expanded proteins in the SCAs have been shown to arise through altered post-transcriptional and -translational modifications or perturbed autophagy.

For example, whilst ATXN1's regulation via its 3' untranslated region (UTR) has been extensively studied, recent findings implicate a role for its unusually long 5' UTR. This region, which undergoes alternative splicing, possesses up to 12 out-of-frame upstream AUGs (uAUGs) that impair translational efficiency [31]. Splicing activity variably affects ATXN1 levels, and is altered in human SCA1 cerebellar tissue, with the impact on SCA1 pathology currently being examined. Additionally, an miRNA (miR760) binds a conserved site within this region, leading to RNA degradation and translational inhibition [32]. AAV-mediated overexpression of miR760 in the cerebellum of *Atxn1*$^{154Q/2Q}$ mice reduced ATXN1 levels and ameliorated motor phenotypes, demonstrating its effects are disease-mitigating.

ATXN1 levels can also be regulated post-translationally. Phosphorylation of serine 776 (S776) abnormally stabilises the protein and is crucial for SCA1 pathogenesis. Protein kinase A (PKA) was shown to regulate this in ATXN1 (82Q) mice, whereby a 30% reduction in PKA diminished S776 phosphorylation, decreased ATXN1 levels and delayed ataxic onset [33]. PAK1, another kinase, was also identified as a key modulator of ATXN1 levels by a forward genetic screen in *Drosophila* [34]. siRNA-mediated knockdown of PAK1 reduced ATXN1 levels in mammalian cells independently of S776 phosphorylation and inhibition of this pathway alongside S776 phosphorylation demonstrated a stepwise decrease in ATXN1 levels [34].

However, therapeutics such as kinase inhibitors would reduce phosphorylation of both wild-type (WT) and mutant ATXN1. In $Atxn1^{154Q/2Q}$ mice, preventing phosphorylation of the mutant allele by CRISPR-mediated site-directed mutagenesis of residue 776 (S776A) reduced ATXN1 protein expression in disease-affected regions, improved motor phenotype and extended lifespan [35]. Conversely, when homozygous S776A mutations were introduced, a reduced rescue effect was observed, suggesting that WT ATXN1 possesses neuroprotective properties.

Altered autophagy also plays a role in SCA pathogenesis: for example, polyglutamine-expanded ATXN2 results in abnormal autophagy, leading to increased STAU1 abundance in the cerebellum of ATXN2 (127Q) and BAC (72Q) mice and in SCA2 patient cells [36]. STAU1, an RNA-binding protein, colocalises with ATXN2 in stress-granule-like aggregates, sequestering SCA2-related transcripts [36]. This in turn promotes apoptosis through activation of the autophagy-inhibiting PERK-CHOP arm of the unfolded protein response (UPR) in SCA2 fibroblasts, creating a pathogenic feed-forward mechanism [37]. Moreover, STAU1 overabundance has been associated with hyperactive mTOR signalling - a master regulator of autophagy - in patient fibroblasts in HD, SCA2 and several other neurodegenerative diseases, which is normalised by silencing STAU1 in SCA2 cell models [38].

Spinal and bulbar muscular atrophy

Spinal and bulbar muscular atrophy (SBMA) is a recessive, X-linked disorder, with its causative CAG repeat expansion occurring in the androgen receptor (AR) gene (Table 1). SBMA usually only affects males, as the mutant protein binds to its natural ligand testosterone to trigger disease onset [39]. Similar to other polyglutamine diseases, transcriptional dysregulation and perturbed proteostasis contributes to neuronal dysfunction.

AR encodes a ligand-dependent transcription factor, with polyglutamine length correlating with transcription rate - recently suggested to be due to increased helicity [40]. Transcriptional dysregulation can occur through AR-mediated histone modifications, with epigenetic dysregulation of metabolic genes contributing to reduced mitochondrial ATP production in SBMA patient iPSC-derived motor neurons (iMNs) [41]. Transcriptomic analysis of cultured embryonic motor neurons from AR (100Q) mice implicated biological pathways including p53 and Wnt signaling, mitochondrial depolarisation and DNA repair [42]. The authors showed a key dysregulated transcript, *Chmp7*, was altered in the spinal cord and muscle of these mice *in vivo* prior to symptom onset, and in SBMA patient iMN precursors, suggesting it may play a causal role in SBMA. *Chmp7* encodes a component of the ESCRT-III complex, involved in autophagic flux and the endosome-lysosome system. Indeed, stimulation of autophagy via trehalose administration increases clearance of mutant AR and reverses aggregation in SBMA patient iMNs [43].

Antisense oligonucleotides (ASO)

Therapeutics for polyglutamine diseases have recently converged on the potential of antisense oligonucleotides (ASO), degrading the cognate RNA of the disease-causing gene prior to translation (Figure 3). A recent phase 1-2a clinical trial by Ionis Pharmaceuticals carried out intrathecal administration of a pan-*HTT*-lowering ASO (HTT_{RX}, later termed Tominersen by Roche) at 4-week intervals, which showed a dose-dependent reduction in mutant *HTT* in cerebrospinal fluid (CSF) with no serious side-effects [44]. Unfortunately, the phase 3 Generation HD1 study was later halted in March 2021 [45], although this was not due to any new safety concerns. Further work is needed to understand why the trial was halted and whether this is due to ongoing safety issues, lack of efficacy, or both and the possible mechanistic reasons for this. Learnings from this large phase 3 trial will be important for future ASO trials in HD and the other polyglutamine diseases. Alternatively, ASOs targeting HD-modifiers in the DNA repair pathway (e.g. *MSH3)* could alter disease pathogenesis by repressing somatic expansion and are currently in development for HD and SCAs. Preclinical ASO studies have also shown promise in SCA1 [46], SCA2 [47,48], SCA3 [27,49-51] and SCA7 [52]. For instance, intracerebroventricular injection of an *ATXN2* ASO in ATXN2 (127Q) or BAC (72Q) mice reduced cerebellar *ATXN2* transcript and protein, rescued motor deficits and almost completely restored normal Purkinje cell firing frequency [48]. Collectively, these studies confirm the tolerability of ASOs short-term in humans, with several phenotypic benefits seen in preclinical models.

Conclusion

Emerging data from the family of polyglutamine diseases have uncovered key pathogenic mechanisms, such as the role of DNA repair in somatic expansion in HD and dysregulation of ion channels in the SCAs. These offer new avenues for therapeutics, which may prove as promising as the potential use of ASOs.

Declaration of interest:

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Figure Legends and Table Captions

Figure 1: Role of MMR genes and FAN1 in somatic expansion. (1) Strand slippage within long segments of CAG repeat tract forms extrahelical extrusions ('loop-outs'), which are recognised directly by the MutSβ complex (MSH2-MSH3) **(2)** MutSβ recruits a MutL complex (MutLα; MLH1-PMS2 or MutLγ; MLH1-MLH3), activating its endonuclease function. MutLβ (MLH1-PMS1) has also been implicated in the process (not shown), although since lacking an endonuclease function its role is less clear. FAN1 stabilises the repeat through a currently unknown mechanism. It is hypothesised that either FAN1 sequesters MutL complexes, blocks access of MutSβ at the extrusion or directly repairs the extrusion itself. **(3)** The nicked strand is excised and resynthesized by a DNA polymerase. **(4)** DNA ligase 1 (LIG1) seals the DNA to complete repair, with the tract now containing more repeat units than in **(1)**. Figure created using BioRender.com.

Figure 2: Proposed mechanism of incomplete splicing at the *HTT* locus. Reduced rates of transcription by Polymerase-II (Pol-II) in the presence of longer CAG repeats leads to increased rates of incomplete splicing. The kinetics of transcription likely provide poly-adenylation (polyA) factors more time to recognise cryptic polyA sites in intron 1, causing a truncated exon 1 fragment to be transcribed. Full-length HTT can also undergo proteolytic cleavage to form N-terminal fragments. Exon 1, and potentially other N-terminal fragments, start the aggregation process and are responsible for downstream toxicity and pathological phenotypes. Figure created using BioRender.com.

Figure 3: Pathogenic mechanisms in polyglutamine diseases. DNA damage and erroneous repair of the repeat tract leads to somatic expansion. The expanded repeat tract is transcribed and translated into toxic polyglutamine-expanded protein. In HD, full-length HTT protein is produced, as well as toxic N-terminal fragments, which arise through incomplete splicing and proteolysis. These toxic proteins can enter the nucleus, where they form aggregates and cause dysregulated transcription. In the SCAs, this includes dysregulation of ion channel transcripts leading to altered membrane excitability in Purkinje neurons. Cytoplasmic aggregates also form, exacerbated by disease-related impairment of proteostasis, disrupting other downstream cellular process. **Alternative splicing also appears to occur in SCAs; however, the pathological importance of the splice variants is less defined.* Figure created using BioRender.com.

Table 1: Summary table of polyglutamine diseases. Table describing features of polyglutamine diseases including disease loci; normal and disease repeat length ranges at these loci; whether somatic expansion is observed and associated neuropathology and clinical phenotype. *(CAT = cytosine-adenine-thionine repeat interruption*).