Developing an antisense oligonucleotide treatment for Spinocerebellar Ataxia Type 3

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General introduction

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POLYGLUTAMINE DISORDERS

The polyglutamine disorders are a group of 9 hereditary neurodegenerative disorders in which symptoms generally present around midlife. The disorders are caused by a similar type of genetic change, namely an abnormal expansion of a CAG repeat in the coding region of a gene [1]. The CAG repeat is translated into an expanded polyglutamine (polyQ) stretch in the protein and these disorders were hence termed polyQ disorders. The CAG expansion underlying the polyQ disorders occur in 9 otherwise unrelated genes, and the repeat lengths that result in disease vary between the different polyQ disorders (Table 1). There is however, in all cases, a clear correlation between the length of the CAG repeat and the age of onset of a polyQ disorder [2]. The involved proteins in which polyQ expansion is present are mostly ubiquitously expressed in all cells, but clinical features such as age of onset and most severely affected brain regions differ between the polyQ disorders. The research in this thesis is focused primarily on spinocerebellar ataxia type 3.

Table 1. Polyglutamine disorders (adapted from Riley and Orr [3])

Disease	Phenotype	Gene locus	Protein	protein mass (kDa)	Wild-type allele repeat length	Mutant allele repeat length
SBMA	Proximal muscle atrophy	Xq11-12	Androgen receptor	99.2	6–39	40–63
HD	Psychiatric, cognitive, motor abnormalities	4p16.3	Huntingtin	347.6	6–34	36–121
SCA1	Ataxia	6p22-23	Ataxin-1	86.9	8–44	39–83
SCA2	Ataxia	12q23-24	Ataxin-2	140.3	13–33	32–77
SCA3/MJD	Ataxia	14q24-31	Ataxin-3	41.8	12–40	54–89
SCA6	Ataxia	19p3	CACNA1A	282.4	4–18	19–33
SCA7	Ataxia, retinal degeneration	3p12-21	Ataxin-7	95.5	4–35	37–306
SCA17	Ataxia	2q13	TATA-BP	37.7	29–42	47–55
DRPLA	Epilepsy, ataxia, dementia	12q	Atrophin-1	125.4	6–36	49–84

DRPLA: Dentatorubral-pallidoluysian atrophy, HD: Huntington's disease, SCA: spinocerebellar ataxia, SBMA: Spinal and bulbar muscular atrophy.

The 9 polyQ disorders are all caused by a polyglutamine repeat expansion in proteins that are expressed ubiquitously throughout the brain. Yet, the most severely affected brain regions and symptoms vary substantially between the disorders.

1 SPINOCEREBELLAR ATAXIA TYPE 3

Spinocerebellar ataxia type 3 (SCA3), or Machado–Joseph disease (MJD)[4], is the most common spinocerebellar ataxia [5, 6] and the second most common polyglutamine (polyQ) disease after Huntington's disease (HD) [7]. Similar to the other polyQ disorders, SCA3 is inherited in an autosomal dominant fashion [8], is neurodegenerative and ultimately fatal. There are currently only therapeutic strategies to alleviate the symptoms, but not to counteract disease progression [9]. SCA3 is clinically heterogeneous, with the main feature being progressive ataxia that can affect balance, gait and speech. Other frequently described symptoms include pyramidal signs, progressive external ophthalmoplegia, dysarthria, dysphagia, rigidity, distal muscle atrophies and double vision [8, 10-12]. These symptoms usually start around midlife, with the exact age of onset being variable. Neuropathological studies have detected widespread neuronal loss in the cerebellum, thalamus, midbrain, pons, medulla oblongata and spinal cord of SCA3 patients, as reviewed by Riess et al [13]. Also, peripheral neuropathy with axonal and demyelinating characteristics were observed in 55% of SCA3 patients, though the contribution hereof to presented symptoms in SCA3 is not clear [14].

SCA3 is caused by an expanded stretch of CAG triplets in the coding region of the *ATXN3* gene on chromosome 14q32.1, encoding the ataxin-3 protein[15]. Healthy individuals have up to 40 CAG repeats, whilst affected individuals have between 54 and 89 glutamine repeats. A repeat range from 40 to 54 is associated with incomplete penetrance of the disease[15-17]. SCA3 patients with two mutant alleles show a more severe disease phenotype than those with a single mutant allele [18]. Also, there is a clear correlation between CAG repeat size and age of onset, though CAG repeat length only accounts for approximately 50% of the total variability in age of onset [19]. The expanded CAG repeat leads to formation of an expanded polyQ tract in the C-terminal region of the ataxin-3 protein, leading to toxic gain of function of the protein and formation of characteristic neuronal aggregates [20]. The neurotoxic properties of these aggregates are still under debate since the number of aggregates does not correlate to the level of neurodegeneration or the *ATXN3* CAG repeat length [21]. Involvement of proteolytic cleavage of the mutant ataxin-3 protein, liberating shorter protein fragments containing the expanded polyQ fragment, appears an important pathway to cellular toxicity and aggregate formation [22]. Observations in Drosophila models for polyQ disorders also hint towards possible involvement of the expanded CAG repeat itself inducing toxicity at the RNA level [23]. Despite being a monogenetic disease, SCA3 pathogenesis has proven to be complex. Over the last decade extensive studies in cell and animal have led to the identification of several cellular processes potentially involved in SCA3 pathology.

Nonetheless, much remains to be elucidated regarding the toxicity resulting from mutant ataxin-3 RNA and protein, and a more comprehensive understanding of the many cellular processes involved would be of great benefit for the development of therapeutic strategies.

ATAXIN-3: A DEUBIQUITINATING ENZYME

Two years following the discovery of the *ATXN3* gene, the ataxin-3 protein was detected, and was found to be expressed throughout the brain [24]. Today it is known that ataxin-3 is in fact expressed throughout the entire body, and 20 isoforms have been described which are all potentially protein-coding [25]. Of these, 2 isoforms have been extensively investigated, with the isoform most prominently expressed in brain being encoded by 11 exons (RefSeqNM_004993) and consisting of 361 amino acids (Fig.1) [26-28]. The ataxin-3 protein has a molecular weight of approximately 42 kDa, which increases depending on the size of the C-terminal polyQ repeat. Ataxin-3 is a deubiquitinating enzyme, with a total of 3 ubiquitin interacting motifs (UIMs) and with a catalytic Josephin domain located at the N-terminus[29, 30]. The UIMs can bind and position the ubiquitin chains in such a manner that the catalytic Josephin domain is then able to cleave these chains [30]. In this manner, ataxin-3 can either rescue proteins from degradation or stimulate breakdown by the removal of inhibitory poly-ubiquitin chains and by the regeneration of free and reusable ubiquitin[30, 31]. Besides protein degradation, the ubiquitin-proteasomal pathway is involved in various cellular processes such as endocytosis, transcriptional regulation and antigen presentation.

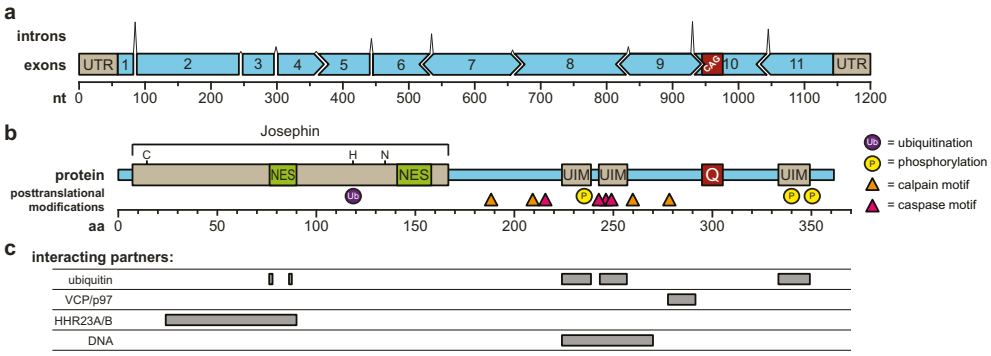

Figure 1. Schematic representation of *ATXN3* gene and protein with interacting partners (adapted from Evers *et al* [32]). a. The ATXN3 gene (Ensembl transcript ID: ENST00000558190.5) consists of 11 exons with the start codon in exon 1 and the CAG repeat in exon 10. The shape of the boxes depict the reading frame, nt: nucleotides. Based on CAG repeat of 10. The height of the introns are relative to their length. **b.** The ataxin-3 protein consists of 361 amino acids (aa), with an N-terminal Josephin domain that contains crucial amino acids for its isopeptidase activity [cysteine 14 (C), histidine 119 (H), and asparagine 134 (N)] and two nuclear export signals (NES). The C-terminal part contains three ubiquitin interacting motifs (UIMs) and the polyglutamine (polyQ) repeat. Specific amino acids known to undergo posttranslational modifications are indicated with circles. Preferential cleavage sites for calpain and caspases potentially generating toxic protein fragments are depicted with triangles. **c.** Binding domains of the main interacting partners: ubiquitin, VCP/p97 valosin-containing protein, hHR23A and hHR23B human homologues of yeast protein RAD23, and DNA binding.

Amino acid cysteine 14, histidine 119, and asparagine 134 of the Josephin domain of ataxin-3 are essential for its isopeptidase function and are highly conserved between Josephin and other ubiquitin C-terminal hydrolases and ubiquitin-specific proteases [33, 34]. The UIMs mediate selective binding to ubiquitin chains and restrict the types of chains that can be cleaved by the Josephin domain. Ataxin-3 is known to recognise poly-ubiquitin chains of four or more ubiquitins [31, 35] and binds the poly-ubiquitin linkages lysine 48, lysine 63 and mixed linkage ubiquitin chains, with preference for lysine 63-tagged ubiquitins [30, 36]. Editing and removal of poly-ubiquitin chains as well as recycling of ubiquitin is critical for cellular homeostasis. Polyubiquitin chains linked through lysines 6, 11, 27, 29, 33 and 48 target proteins for proteasomal degradation. In contrast, lysine 63 or linear polyubiquitin chains have non-proteolytic functions such as activation of kinases and autophagy, where it is proposed to be involved in the biogenesis of protein inclusions [37].

Ataxin-3 protein interactions

Ataxin-3 has been found to interact with the valosin-containing protein (VCP/p97) [38, 39] through a motif close to the polyQ repeat (Fig. 1) [40]. VCP/p97 has numerous functions, one of which is the regulation of misfolded protein degradation in a process named endoplasmic reticulum -associated degradation (ERAD)[38, 41]. The ataxin-3-VCP/p97 complex is involved in assisting targeted proteins to the proteasome [42]. Ataxin-3 is also known to interact with the human homologues of yeast protein RAD23, hHR23A and hHR23B. hHR23A and hHR23B are involved in DNA repair pathways as well as the delivery of ubiquitinated substrates to the proteasome for degradation[39]. The binding site of hHR23B to ataxin-3 is located in the second ubiquitin binding site of the Josephin domain, and in concordance, hHR23B was shown to compete with ubiquitin binding [34]. Cell stress resulted in altered interactions with both VCP/p97 and HR23B, which were found mainly in the cytoplasm, although no effect on protein degradation was reported [43]. Another association between ataxin-3 and the DNA damage repair pathway stems from the interaction between ataxin-3 and polynucleotide kinase 3′-phosphatase (PNKP). PNKP is an DNA end-processing enzyme, and it was found that wild-type ataxin-3 stimulates PNKP activity [44]. In addition, ataxin-3 is recruited to DNA double-strand breaks, and through its deubiquitinating activity was shown to regulate the chromatin dwell time of mediator of DNA damage checkpoint protein 1 (MDC1), which in turn recruits DNA repair proteins [45].

Ataxin-3 and transcriptional regulation

Besides the clear role of ataxin-3 in protein degradation, ataxin-3 has been shown to regulate transcription. Ataxin-3 is, for instance, able to repress matrix metalloproteinase-2 (MMP-2) transcription, and increased nuclear localisation of ataxin-3 through phosphorylation enhances this transcriptional repression [46]. Ataxin-3 can also regulate PTEN transcription, in turn influencing PI3K pathway activity [47]. In cells overexpressing wild-type ataxin-3, transcriptional repression of cell surface- and ECM-associated genes was observed [48]. These effects on transcription by ataxin-3 may occur through interactions with transcriptional regulators.

Indeed, ataxin-3 is known to interact with p300, p300/CBP-associated factor (PCAF)[49], histone deacetylases (HDAC) 3 and 6, nuclear receptor co-repressor (NCoR1) [48]. However, ataxin-3 can also directly bind to target DNA sequences in chromatin regions of MMP-2, after which transcription is repressed through recruitment of HDAC3 [50].Whether this direct DNA binding by ataxin-3 is an important pathway of gene regulation is currently still an open question.

Is ataxin-3 an essential protein?

Whether ataxin-3 is an essential protein for normal cellular function remains uncertain. On one hand ataxin-3 appears dispensable, as knockout of *ATXN3* in a *C. elegans* model did not negatively affect lifespan [51] and conferred a resistance to stress [52]. Downregulation of ataxin-3 in the striatum of rats using shRNA did not result in overt signs of toxicity [53]. In line with this, ataxin-3 knock-out mice did not present problems with viability or fertility [54, 55]. However, closer examination of the molecular phenotype of ataxin-3 knockout models has revealed subtle changes, particularly with regards to ubiquitination, that are important to consider. Firstly, depletion of ataxin-3 using siRNA in cultured non-neuronal human and mouse cells resulted in accumulation of ubiquitinated material in the cytoplasm, cytoskeletal disorganisation, loss of cell adhesion and increased cell death [56]. Additionally, increased levels of protein ubiquitination were observed in tissues of an ataxin-3 knockout mouse, and a subtle increase in anxiety of the mice was reported [54]. More recently, the role of ataxin-3 in the DNA damage response has been established, with ataxin-3 depletion compromising double-strand DNA break repair [45]. Taken together, it can be concluded that loss of ataxin-3 is tolerated in mice, but several subtle alterations in cellular homeostasis indicate that ataxin-3 may not be completely dispensable for cellular functioning in the long term.

PATHOGENIC MECHANISMS OF MUTANT ATAXIN-3

Wild-type ataxin-3 in the normal population has a polyQ stretch of between 13 and 44 [13]. Following mutational expansion to over 52 repeats, the ataxin-3 protein becomes toxic to brain cells. No correlation between ataxin-3 mRNA expression levels and the extent of neurodegeneration was found for the brain regions affected in SCA3 [57]. It hence appears that there is no difference in expression levels of mutant ataxin-3 to explain the regional differences in brain pathology and cytotoxicity in a straightforward manner. Rather, specific brain regions are more sensitive to the toxic effects of ataxin-3. It is currently unclear what factors are involved in conveying this specific toxicity, but several hypotheses regarding mutant ataxin-3 pathogenesis have been proposed and will be discussed below.

> Despite being ubiquitously expressed and well conserved among species, it is still unclear whether ataxin-3 is an essential protein for cellular functioning.

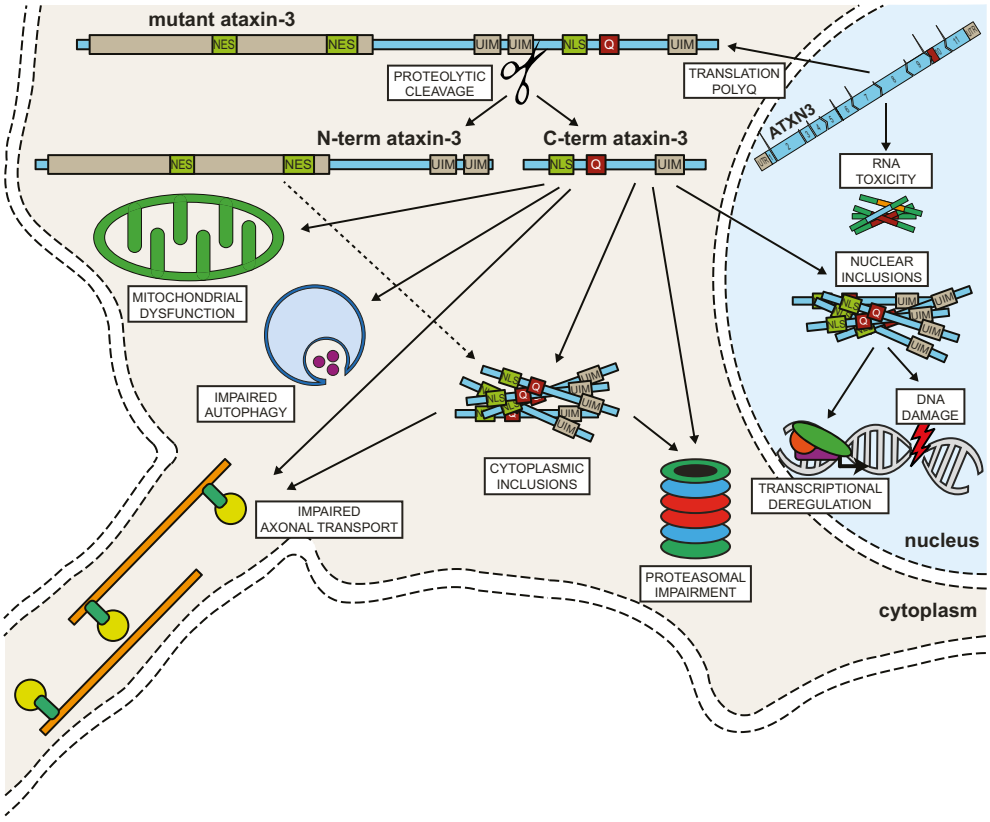

Figure 2. Cellular mechanisms of mutant ataxin-3 toxicity (adapted from [32]). In SCA3 the CAG repeat of the *ATXN3* gene is translated into a polyQ expansion in the mutant ataxin-3 protein. The expanded polyQ repeat causes misfolding of the protein. Proteolytic cleavage of ataxin-3 can generate C-terminal protein fragments containing the polyQ repeat. Full length and cleaved forms of ataxin-3 can form soluble monomers, oligomers or large insoluble aggregates, both in the nucleus and in the cytoplasm that cause toxicity. Other cellular disturbances in SCA3 pathogenesis include transcriptional deregulation, impaired autophagy, mitochondrial dysfunction, proteasomal impairment, compromised axonal transport and DNA damage. Finally, there is also evidence implicating RNA toxicity of the mutant *ATXN3* transcript in disease pathogenesis.

Mutant ataxin-3 aggregation

Similar to the other polyQ disorders, intracellular aggregates in neurons of brain material from SCA3 patients were observed as a hallmark of disease. Aggregates were observed in the substantia nigra, globus pallidus, dorsal medulla and dentate nucleus [20]. Using overexpression systems in cell- and animal models, mutant ataxin-3 was indeed found to accumulate in intracellular aggregates [58, 59].There is a clear correlation between the length of the polyQ stretch in ataxin-3 and its propensity to form aggregates [60, 61]. In particular, the nuclear accumulation of ataxin-3 appears to be important to induce toxicity, as targeting ataxin-3 to the nucleus worsens the SCA3 phenotype in mice [62]. Ubiquitinated ataxin-3 inclusions can be found in several brain regions

of SCA3 patients [20, 27]. During disease progression, SCA3 mice show a decrease in the level of soluble mutant atxin-3 in the cerebellum, whilst both nuclear aggregate formation and the ataxic phenotype progress [63]. Ataxin-3 aggregates are not exclusively found in the cell nucleus, however. Axonal ubiquitinated aggregates have been identified in SCA3 brain tissue fiber tracts [64] and cytoplasmic inclusions were also observed in several affected brain regions [65].The ataxin-3 inclusions in the cytoplasm have been proposed to interfere with axonal transport. However, there appears to be no direct correlation between the number of aggregates and severity of neurodegeneration [21, 64]. Additionally, it is currently not clear whether the observed ataxin-3 aggregates consist of full-length ataxin-3 protein, or are initiated by shorter polyQ containing ataxin-3 fragments.

The hallmark neuronal aggregates of SCA3 are closely associated with proteolytic cleavage of mutant ataxin-3.

Proteolytic cleavage and induction of toxicity

Proteolytic cleavage of proteins is a continuous process in a normally functioning cell, and serves many purposes from degradation of proteins to post-translational processing [66]. However, in the case of mutated proteins, proteolytic processing may contribute to disease. In the case of SCA3, research has shown that proteolytic cleavage of mutant ataxin-3 can generate shorter protein fragments containing the polyQ stretch. These protein fragments are more toxic to cells than the full length ataxin-3 protein, and in addition are more prone to aggregate [22, 67]. It is therefore thought that the proteolytic cleavage, which can generate short C-terminal ataxin-3 fragments containing the polyQ stretch, may be a driving force in SCA3 pathogenesis. The two main families of enzymes that have been investigated in this regard for ataxin-3 are the caspase and calpain families of proteases. Caspases cleave at defined motifs containing an aspartate residue, and at least 3 such sites located in ataxin-3 are in a position capable of generating a short polyQ containing fragment [68]. However, caspase cleavage of ataxin-3 occurs less efficiently than for huntingtin and atrophin-1[69] and inhibition of caspase cleavage did not reduce aggregate formation in SCA3 neuronal cell culture experiments [68].

Cleavage of ataxin-3 by the calpain-2 enzyme occurs throughout the protein, but a key cleavage site that is well investigated is the one just upstream of the polyQ repeat, at amino acid 260 [70, 71] (Fig.1). Cleavage at this site generates C-terminal ataxin-3 fragments that are highly prone to aggregation [70]. Inhibition of calpains in SCA3 mice results in a reduction of ataxin-3 proteolysis, nuclear localisation, aggregation and was successful in reducing toxicity [71, 72]. Conversely, stimulation of calpain activity *in vivo* worsened the ataxic phenotype of mice [70]. There is also evidence that ataxin-3 with longer polyQ stretches is more prone to calpain cleavage than ataxin-3 with shorter polyQ stretches, potentially exacerbating the formation of the toxic fragments [70]. It appears that during disease progression in mice,

soluble levels of mutant ataxin-3 in the brain decrease, possibly as a result of calpain cleavage, whilst the aggregate burden increases [63]. It has been suggested that calpains are involved in the neuronal specificity of SCA3 pathology, as excitation-mediated calcium influx in neuronal cells activated calpain-2 *in vitro*, and lead to ataxin-3 cleavage and aggregation [73]. A recent study, however, was unable to reproduce this excitation related ataxin-3 aggregation in similar neuronal cells [74], and further research is thus required to comprehensively establish ataxin-3 cleavage induced neuronal pathology.

Impairment of protein degradation

Ataxin-3 is involved in the proteasomal protein degradation pathway and this pathway is indeed found affected in SCA3 as mutant ataxin-3 appears to interfere with degradation of substrates [43, 75]. The mechanism behind this, however, appears to be more so the result of toxic gain of mutant ataxin-3 function rather than loss of function. For instance, a reduction in the level of protein deubiquitination was reported in a cell model expressing mutant ataxin-3, despite mutant ataxin-3 showing similar ubiquitin chain proteolysis as wild type ataxin-3 [30]. Mutant ataxin-3 was also shown to bind to VCP more efficiently [38, 43, 76]. For this reason, it is more likely that both mutant and wildtype ataxin-3 get trapped in the ubiquitin-rich aggregates together with components of the proteasomal machinery [20, 77]. The interaction with VCP is important, as expression of a N-terminal ataxin-3 fragment lacking the VCP binding site resulted in an impaired unfolded protein response and ER stress, without apparent changes in levels of ERAD components when tested in a mouse model [78].

Besides ERAD dysregulation, the process of autophagy also was shown impaired in SCA3 as observed in other neurodegenerative disorders[79]. Ataxin-3 aggregates in SCA3 were shown to contain components of autophagy machinery, such as beclin-1 [80]. Indeed, autophagy stimulation through beclin-1 overexpression was shown to alleviate disease pathogenesis in a SCA3 rat model [80], in parallel to previous research for HD [81]. Recent evidence shows that the polyQ repeat is required for the interaction between beclin-1 and ataxin-3, which is important to maintain adequate beclin-1 protein levels and thus normal initiation of autophagy. The polyQ expansion competes for this interaction, thereby contributing to impairment of autophagy [82]. Together, current research suggest SCA3 pathology to at least partly result from loss of function from ERAD and autophagy machinery, together culminating in impaired protein degradation and cellular stress.

SCA3 pathology is predominantly the result of gain of toxicity of mutant ataxin-3, rather than loss of ataxin-3 protein functioning.

Mitochondrial dysfunction

It has been suggested that increasing oxidative stress and inability to protect against free radicals with age could lead to mitochondrial dysfunction and cell damage in polyQ disorders [83-86]. For SCA3, a cell model overexpressing mutant ataxin-3 with 78 glutamines indeed showed reduced antioxidant enzyme levels, increased mitochondrial DNA damage, and reduced energy supply, which indicates impaired mitochondrial function [86]. In SCA3 mice, mitochondrial DNA damage was seen in affected brain regions [87], and evidence of moderate compromised mitochondrial complex II was found [88]. Finally, in brain tissue of SCA3 patients, downregulation of superoxide dismutase was detected, suggesting diminished antioxidant enzyme function. As damaged mitochondria will not be able to scavenge free radicals and prevent cell energy impairment as effectively, this process may therefore further increase oxidative stress in the cell. Oxidative stress is then able to modulate vital cellular functions, potentially resulting in activation of apoptosis or excitotoxicity, two of the main causes of neuronal death [89].

Transcriptional deregulation

Since ataxin-3 displays DNA-binding properties and interacts with transcriptional regulators, transcriptional deregulation has been suggested to play a central role in the SCA3 pathogenesis [3]. In SCA3 and other polyQ disorders, transcription factors are sequestered into nuclear aggregates, resulting in deregulation of their function as transcriptional co-repressor or activator [90, 91]. Transcription of genes involved in inflammatory processes, cell signalling and cell surface-associated proteins were found to be altered in SCA3 cell and mouse models [48, 92, 93]. Likewise, some corresponding proteins like MMP-2, amyloid β-protein and interleukins were found to be significantly increased in SCA3 patient brain material [92]. A second mode of transcriptional deregulation arises from impairment of ataxin-3 function. Wild-type ataxin-3 can repress transcription through recruitment of histone deacetylase 3 and nuclear receptor corepressor, resulting in histone deacetylation. However, mutant ataxin-3 results in reduced histone deacetylation in this context, allowing for aberrant transcription to take place [50].

RNA toxicity and repeat-associated non-ATG translation

Until recently, it was believed that polyQ disorders are solely the result of gain of toxic protein function and, to a lesser extent, loss of wild-type protein function. However, increasing evidence suggests involvement of the expanded CAG repeat at the RNA level in polyQ pathogenesis. This may occur through various mechanisms, including alternative splicing, bidirectional transcription, involvement of the RNA interference pathway, as well as repeat-associated non-ATG (RAN) initiated translation [32]. Evidence from toxicity of the CAG repeat itself stems from the observed neural dysfunction in *Drosophila melanogaster* models where the repeat was positioned in the 3′ untranslated region (UTR). Interspersing the repeat with CAA codons resulted in only a mild phenotype [23]. The pathways underlying RNA toxicity could be sequestration of proteins to the hairpin structure of the CAG repeat, like for

Table 2. Potential toxicity pathways involved in SCA3 pathogenesis

Pathway	Established in	contribution to pathology*	References
Nuclear ataxin-3 inclusions/ aggregates	cell and mouse models, patient brain material	++++	20, 27, 63, 73
Proteolytic ataxin-3 cleavage/ toxic protein fragments	cell culture, animal models	+++	22, 68, 70, 94
Proteasomal impairment	cell models, patient brain material	++	77, 95
Autophagy impairment	cell and mouse models	+++	80, 82
Axonal transport impairment	patient brain material	++	64
Transcriptional deregulation	cell and animal models	+++	48, 93, 96
RNA toxicity	Drosophila	+	23
RAN translation	indirect; evidence in HD	unknown	97

RAN = Repeat associated non-ATG translation, HD = Huntington disease *This scale is an arbitrary measure based on current evidence in literature and the interpretation of the author.

instance the transcription factor muscleblind-like 1 (MBNL1) [98], resulting in misregulation of splicing [99]. Additionally, MID1 protein is recruited to expanded CAG repeat containing RNA, which in turn results in aberrant translation of proteins from the CAG mRNAs [100, 101]. However, the actual contribution of this direct RNA toxicity may be limited in SCA3, since other research suggests that CAG repeats in the UTR are only toxic when considerably exceeding the repeat length normally found in SCA3 patients [98, 102].

For SCA3 and other polyQ disorders, reading frame shifts and subsequent translation of homopolymeric repeats such as polyalanine (polyA) have been established [103, 104]. The polyA repeat is associated with increased toxicity over the polyQ repeat, and may therefore be a substantial contributor to toxicity associated with the expanded CAG repeat [105]. Of note, expansion proteins from all three reading frames can be produced without an AUG start codon, in a process termed RAN translation [97]. Frameshifting and RAN translation are distinct translational steps, but are both dependent on the repeat sequence length [106]. RAN translation is usually associated with sequences that are considered to be non-coding, but may nonetheless be involved in polyQ disorder toxicity as well.

DNA damage

Ataxin-3 has recently been discovered to interact with, or modulate activity of, several DNA damage response associated proteins, such as HHR23A, HHR23B[39], MDC1[45], polynucleotide kinase 3′-phosphatase (PNKP)[44, 107], and checkpoint kinase 1 (Chk1) [108]. Interestingly, a large increase in DNA damage has been demonstrated in cells, mouse brain and human brain material of SCA3 [107], suggesting DNA damage may be involved in SCA3 pathogenesis. Indeed, defects in the DNA repair have been linked to several other neurological disorders [109]. In the case of SCA3, it was shown that wild-type ataxin-3 stimulates PNKP activity, whereas mutant ataxin-3 inhibits this activity [44]. The deactivation of PNKP by mutant ataxin-3 in

fact appears to be involved in inducing DNA damage and subsequent cell death underlying neurodegeneration [107], suggesting a direct pathologic link. It is currently unclear whether the interfering effect of mutant ataxin-3 on PNKP functioning is the result of PNKP inhibition through inclusions in aggregates or a more direct inhibition due to binding of mutant ataxin-3 [110]. Additionally, it will be of importance to determine whether the deubiquitinating activity of ataxin-3 is involved in the PKNP modulation, such as observed for the checkpoint mediator protein MDC1[45]. Mutant ataxin-3 is able to deubiquitinate and stabilize Chk1 protein at a comparable capacity as wild-type ataxin-3, arguing against involvement of altered enzymatic activity of mutant ataxin-3 as a initiator of DNA damage in this context [108].

Collectively, there is abundant evidence that ataxin-3 is involved in the DNA repair pathway, but it remains to be elucidated how mutant ataxin-3 relates to the increased DNA damage observed in SCA3, and to what extent this contributes to pathology.

THERAPIES

Currently no therapies are available to delay SCA3 disease progression and patients are only treated symptomatically. Nonetheless, several interesting new compounds and RNA targeting therapeutics are currently in preclinical development. Firstly, compounds resulting in increased clearance of mutant ataxin-3 are being investigated. Increasing intracellular protein degradation by various compounds have shown to be capable of improving the phenotype of SCA3 mice [111,112], but strong modulation of autophagy may cause neurotoxicity [113] and these compounds thus have to be implemented with great care. Additionally, several chemical chaperones capable of reducing ataxin-3 aggregation have been tested with success in cell culture experiments[114], and trehalose [115] is currently in phase II clinical trial for the treatment of SCA3. A screening of FDA-approved drugs has recently identified the serotonin reuptake inhibitor as a drug capable of improving the phenotype in *c.elegans* and SCA3 mice [116]. However, only the autophagy inhibitor lithium has been tested in SCA3 patients so far, but no beneficial effect on disease progression could be determined [117]. Various other compounds and strategies have been tested in SCA3 mouse models. Firstly, preventing SCA3 disease progression through modulation of calcium homeostasis has been investigated using Dantrolene [118] and caffeine [119,120] with promising results that warrant further investigation of these compounds. Additionally, the HDAC inhibitor sodium butyrate was capable of inducing beneficial effects on the phenotype of SCA3 mice, possibly by ameliorating transcriptional repression [121]. Thirdly, inhibition of ataxin-3 cleavage by treatment with the calpain inhibitor BDA-410 successfully prevented motor deficits in SCA3 mice [94]. Caloric restriction was found to reduce motor deficits in SCA3 mouse models, through rescue of sirtuin 1 (SIRT1) levels [122].

Recently there has been increased interest in stem cell transplantation as treatment strategy for neurodegenerative disorders. Transplantation of cerebellar neural stem cells in SCA3 mice led to differentiation of the cells into neurons and supportive cells, and alleviated motor behaviour impairment and neuropathy [123]. An exciting aspect of stem cell transplantation is that they can potentially induce cell replacement to compensate for the neuronal loss in SCA3.

Indeed, transplanted embryonic neurons were shown capable of integrating in pre-existing brain circuits in mice[124]. However, clinical success of stem cell use in for instance Alzheimer and Parkinson's disease have been modest thus far [125, 126], and some concerns regarding safety have been risen [127]. Due to the monogenetic nature, SCA3 is also an ideal candidate for therapies directly targeting the *ATXN3* transcript. Downregulation of ataxin-3 using shRNA and siRNA has shown success in mouse models [128-130]. Additionally, antisense oligonucleotides can be used to down regulate[131, 132] or modify ataxin-3 through exon skipping [133]. The use of antisense oligonucleotide therapies in neurodegenerative disorders is extensively reviewed in **Chapter 2.**

Scope of the thesis

In this thesis, antisense oligonucleotides (AONs) are investigated as a potential treatment for SCA3.

Chapter 2 describes the current state of development of AONs and their use as therapy for neurodegenerative disorders. In **Chapter 3,** an AON based strategy to reduce formation of toxic ataxin-3 cleavage fragments in cell models is described. **Chapter 4** describes pathogenic mechanisms and biomarkers in a SCA3 mouse model using a multi-omics approach. In **Chapter 5,** a novel AON based therapy to remove the toxic polyQ repeat from ataxin-3 in the SCA3 mouse model is investigated. **Chapter 6** provides an overview of potential side effects and toxicity related to 2'OMe AONs after intracerebroventricular injection in mice. **Chapter 7** discusses the main findings of the thesis, and how these relate to the current state of SCA3 research and AON based therapies.

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