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General discussion  
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## 6.1. Introduction

In this thesis I have discussed various AON-mediated therapies to reduce polyQ-induced toxicity, particularly in HD and SCA3. These expanded polyQ proteins are known to undergo proteolytic processing and this results in polyQ-containing protein fragments that are considered to be the main toxic entities in polyQ disorders. By targeting the polyQ-encoding transcripts, translation of mutant polyQ protein is reduced or the polyQ protein is modified. This is achieved by targeting the CAG repeat directly (chapter 3), removing the motifs that are implicated in the formation of polyQ fragments (chapter 4), or by removal of the CAG repeat-encoding exon (chapter 5).

Recently however, it has also been suggested that the polyQ pathogenesis is not only caused by polyQ protein toxicity. The significance of these novel findings will be assessed together with implication for the AON-mediated treatments proposed in this thesis. Subsequently, I will discuss the prospect of applying similar AON-mediated therapies to other polyQ disorders. Finally, using existing knowledge from preclinical studies with AONs in rodents and clinical trials with disease-modifying drugs, I will discuss what is required for the enrolment of future clinical trials of genetic therapies in polyQ diseased patients.

## 6.2. Main findings

In this thesis I propose various novel genetic therapies for polyQ disorders, aiming at reducing and/or modifying polyQ disease-causing proteins. While much research has been done on the underlying polyQ disease mechanisms, knowledge on mRNA and protein regulation and expression levels are limited. Chapter 2 describes differences in HTT RNA and htt protein expression in adult-onset HD with less mutant HTT mRNA, but equal wild-type and mutant htt protein levels. Juvenile HD subjects did show less mutant htt protein expression compared to wild-type, indicating subtle differences in htt protein expression between adult-onset and juvenile HD.

In chapter 3, CUG triplet-repeat AONs are used to reduce mutant polyQ transcript and protein levels in several polyQ disorders. A slight reduction in wild-type CAG transcript levels was observed as well, showing that the (CUG)<sub>7</sub> AON is not completely specific for the mutant allele. However, several other non-expanded CAG-containing transcripts investigated were not affected by (CUG)<sub>7</sub> AON treatment, implying the preference of the (CUG)<sub>7</sub> AON for the expanded transcript. Furthermore, chapter 2 describes that the basal levels of mutant HTT mRNA and protein are equal or lower than wild-type, providing feasibility for AONs such as the (CUG)<sub>7</sub> AON described in chapter 3, that are not completely specific for the mutant HTT allele.

Next to specifically targeting the expanded CAG repeat transcripts to reduce the translation of mutant polyQ protein, chapter 4 reports on removing motifs that are implicated in the formation of toxic polyQ fragments. Preventing the formation of a 586 amino acid N-terminal htt fragment, implicated in HD toxicity, is achieved by AONs that induce skipping of exon 12 in HTT pre-mRNA. This resulted in a shorter protein lacking the caspase-6 cleavage motif with subsequently reduced 586 amino acid N-terminal htt fragments. This proof of concept shows a completely novel approach to reduce mutant htt toxicity not by reducing its expression levels, but by modifying the protein.

A more direct genetic approach to reduce polyQ-induced toxicity is shown in chapter 5, where the toxic polyQ repeat is removed from the ataxin-3 protein through AON-mediated skipping of the CAG repeat-enclosing ATXN3 exon 10. A modified ataxin-3 protein is formed that lacks the toxic polyQ repeat, but may well maintain many important wild-type functions of the protein.

In chapter 4 and 5 the feasibility of the AONs is shown *in vivo* by ICV injections of the murine AONs into control mice. Above described results suggest that both targeting the CAG repeat directly, or AON-mediated exon skipping may be suitable therapeutic approaches to reduce polyQ-induced toxicity in HD, SCA3 and other polyQ disorders.

## 6.3. Recent developments

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. Recent findings have shown that the molecular pathogenesis of these disorders is more complex. More and more observations suggest that next to polyQ protein toxicity, RNA toxicity and bidirectional transcription are also involved in the polyQ disease pathogenesis. These potential novel toxic mechanisms have to be taken into account when designing an AON-mediated disease-modifying treatment.

### CAG repeat-induced RNA toxicity

Recently, in patient-derived juvenile HD fibroblasts expanded CAG repeat-induced RNA foci were shown (DE MEZER *et al.*, 2011), suggesting a RNA toxicity component in HD. Furthermore, overexpression of untranslated repeats with 200 CAGs showed clear nuclear RNA foci, progressive neural dysfunction, reduced breeding efficiency, and premature death in various transgenes (LI *et al.*, 2008; HSU *et al.*, 2011; WANG *et al.*, 2011). Although the exact mechanism of this expanded CAG repeat-induced toxicity remains elusive, there is some evidence that RNA toxicity may result from stable CAG hairpin structures which can sequester RNA binding proteins in nuclear foci, resulting in misregulation of alternative splicing (LI *et al.*, 2008; MYKOWSKA *et al.*, 2011; WANG *et al.*, 2011). Next to CAG repeat-induced deregulation of splicing, short CAG repeat-containing RNAs of around 21 nucleotides, originating from mutant CAG repeat-containing RNAs were found to induce cell death (BANEZ-CORONEL *et al.*, 2012). The toxicity was CAG repeat dependent, since toxicity was blocked by AONs against the CAG repeat sequence, similar to the (CUG)<sub>7</sub> AON described in chapter 3 (BANEZ-CORONEL *et al.*, 2012).

The size of the CAG repeat is probably critical for the contribution of mRNA aggregation from the expanded CAG repeat because overexpression of untranslated repeats with 83 and 93 CAGs did not appear to result in a phenotype (MCLEOD *et al.*, 2005; WANG *et al.*, 2011). Likewise, in chapter 2 the levels of wild-type and mutant HTT mRNA and htt protein did not differ in adult-onset HD fibroblasts, suggesting that there is no mutant CAG repeat-induced mRNA aggregation in HD fibroblasts. To note, the repeat sizes of the adult-onset HD patients described in chapter 2 are shorter compared to the juvenile HD fibroblasts (with 68 and 151 CAGs repeats) in which RNA foci were observed (de Mezer *et al.*, 2011), and therefore CAG repeat-induced RNA toxicity could still play a role in patients with extremely expanded CAG repeat lengths.

In chapter 5 a novel protein modification approach is proposed to reduce mutant ataxin-3 toxicity by removing the toxic polyQ repeat from the ataxin-3 protein. In case of CAG repeat-induced RNA toxicity, this AON-mediated exon skipping is still applicable, since the toxic CAG repeat is removed from the mRNA. In the same way the RNA toxicity component in polyQ disorders will be prevented by the (CUG)<sub>7</sub> AON described in chapter 3, through direct binding of the (CUG)<sub>7</sub> AON to the expanded CAG repeat. The potential RNA toxicity is not targeted by

the AON-mediated htt protein modification described in chapter 4. This potential therapeutic approach prevents the formation of toxic N-terminal htt fragments and leaves the hypothetical toxic RNA entities intact. However, removal of the 586 caspase-6 site from the full-length mutant htt protein was shown to alleviate the phenotype in transgenic HD mice (GRAHAM *et al.*, 2006; POULADI *et al.*, 2009), supporting the significance of this particular htt protein fragment. Therefore the role of RNA toxicity in the disease pathology remains to be assessed.

### **Bidirectional transcription in polyQ disorders**

For many genes in the genome transcription occurs from both DNA strands (KATAYAMA *et al.*, 2005; HE *et al.*, 2008). Several studies have shown that bidirectional transcription occurs from triplet repeat disorder genes (CHO *et al.*, 2005; MOSELEY *et al.*, 2006; WILBURN *et al.*, 2011). Although bidirectional transcriptional tags have been identified for all nine polyQ disease-causing loci (HE *et al.*, 2008), only for HD and SCA7 antisense transcripts have been studied in more detail (CHUNG *et al.*, 2011; SOPHER *et al.*, 2011). The SCA7 antisense noncoding transcript 1 (SCAANT1) and HTT antisense (HTTAS) transcripts are thought to be involved in downregulation of the corresponding sense *ATNX7* and *HTT* gene expression (CHUNG *et al.*, 2011; SOPHER *et al.*, 2011). In total 3 HTTAS versions have been identified, of which one, HTTAS\_v1 contained the CTG repeat whereas two other, HTTAS\_v2.1 (CHUNG *et al.*, 2011) and HTTAS\_v2.2 (chapter 2), did not. It was hypothesized that HTTAS\_v1 was downregulated in HD patients since HTTAS\_v1 with an expanded CTG repeat was not detected in HD brains (CHUNG *et al.*, 2011). However, in chapter 2 we did show HTTAS\_v1 expression in human-derived fibroblasts homozygous for the CAG repeat expansion, suggesting that there is bidirectional transcription from the expanded CAG repeat, questioning the role of HTTAS in HTT regulation.

Altogether, these novel findings suggest that in some triplet repeat disorders bidirectional transcription could potentially play a role in the disease pathology. On the other hand, for only two out of nine polyQ disorders antisense transcripts have been characterized thus far, questioning the significance of bidirectional transcription in deregulation of sense transcripts and disease manifestation.

### **Other potential toxic entities in polyQ disorders**

A major line of thinking with regard to polyQ disorder pathogenesis termed the 'toxic fragment hypothesis' concerns the proteolytic cleavage of polyQ expanded protein. This proteolytic cleavage is thought to lead to generation of short cytotoxic and aggregation-prone fragments containing the expanded polyQ repeat. Most research on the formation of toxic entities has been performed on HD, for which various rodent models expressing full-length or N-terminal mutant htt fragments have been generated (HENG *et al.*, 2008).

Recently, it was shown in various knock-in and transgenic HD mouse models that alternative splicing of HTT pre-mRNA results in a short HTT transcript with a stop codon in the beginning of intron 1 and a polyA tail 700 nucleotides into intron 1. This HTT exon 1-intron 1 transcript was shown to be translated into a short polyQ-containing htt protein of 216 amino acids (with 150Qs) (SATHASIVAM *et al.*, 2013). Interestingly, this aberrantly spliced transcript occurred in a

repeat-dependent manner, probably by altered binding of splicing factors to the expanded CAG repeat (SATHASIVAM *et al.*, 2013). While this alternative splicing of HTT pre-mRNA is interesting, it is difficult to assess the importance of this finding. Although the authors showed the aberrantly spliced transcript in patient-derived fibroblasts, they could only detect this HTT exon 1-intron transcript in two out of four *post-mortem* HD brain tissues. Neither did they show protein data from human brain samples, suggesting low abundance of this short N-terminal polyQ fragment in HD brain tissue. Still, these results show that not only posttranslational modification, but also gene expression alteration can generate short toxic N-terminal htt fragments. If this would be the main or sole pathogenic mechanism, this would mean that the removal of the 586 caspase-6 site from the full-length mutant htt protein as proposed in chapter 4 would not be therapeutically beneficial. On the other hand, the (CUG)<sub>7</sub> AON described in chapter 3 would still prevent the formation of these short polyQ-containing htt protein fragments.

Other toxic entities resulting from reading frame shifting have been suggested to be involved in the polyQ disease pathogenesis. Next to expanded polyQ repeats, also polyA stretches occur in cells derived from HD and SCA3 patients (GASPAR *et al.*, 2000; DAVIES AND RUBINSZTEIN, 2006). These polyA stretches in the full-length ataxin-3 protein were toxic when overexpressed in *D. melanogaster* and neuronal cell models (STOCHMANSKI *et al.*, 2012). Next to reading frame shifting, repeat associated non-ATG translation was recently also proposed as a novel class of protein toxicity, in which coding RNA transcripts with mutant CAG repeats are translated in the absence of an ATG start codon (ZU *et al.*, 2011). This repeat associated non-ATG translation was found in all three possible CAG repeat reading frames, resulting in the translation of proteins with polyQ, polyA, and polyserine (polyS) repeats (ZU *et al.*, 2011). However, these non-ATG translated htt and ataxin-3 proteins were only shown in artificial overexpression systems with low expression levels and their contribution to the polyQ disease pathology is as yet unclear.

To conclude, although most evidence points towards a toxic gain of polyQ protein fragments, novel toxic entities have been described that are potentially involved in the pathogenesis of polyQ disorders. These toxic proteins could be the result of aberrant splicing, proteolytic cleavage, reading frame shifting, or repeat associated non-ATG translation. On transcriptional level bidirectional transcription and CAG repeat-induced RNA toxicity could also be involved. Although extensive research is necessary to assess the relevance of these novel toxic entities, in many cases AON-mediated protein lowering approaches would still have an effect as they interfere with mRNA levels and compositions.



## 6.4. Future directions

There are several neurodegenerative disorders where AON-mediated therapies moved from preclinical to clinical testing during the course of this PhD research. All completed clinical trials on AON-mediated therapies into the CSF reported thus far have been successful and no major adverse events were reported (RIGO *et al.*, 2012; MILLER *et al.*, 2013). Although recent preclinical results using non-allele-specific, SNP-specific and CAG repeat-targeting oligonucleotides look promising (GRAHAM *et al.*, 2006; ALVES *et al.*, 2008; HU *et al.*, 2009b; KORDASIEWICZ *et al.*, 2012; OSTERGAARD *et al.*, 2013), no trials of genetic therapies in polyQ patients have been done thus far.

### Modulating splicing in polyQ disorders

In chapter 3 an allele-specific silencing is achieved based on the common denominator of all polyQ patients, being their expanded CAG repeat. A reduction of the mutant transcript is shown in fibroblasts derived from patients with DRPLA, HD, SCA1, and SCA3. Unfortunately for the other 5 polyQ disorders the allele-specific effect of the (CUG)<sub>7</sub> AON could not be investigated, because no fibroblasts were available. Still, based on the mutant CAG repeat sizes of SBMA, SCA2, SCA7 and SCA17, it is expected that these can also be targeted by the (CUG)<sub>7</sub> AON. For SCA6 this could be more complicated since the pathogenic repeat size starts at already 19 CAGs.

Next to targeting the mutant CAG repeat directly, chapter 5 describes removal of the CAG repeat-containing exon 10 from ATXN3 pre-mRNA in SCA3. Recently, it was also shown that a (CUG)<sub>7</sub> single-stranded silencing RNA (ss-siRNA), comparable to the (CUG)<sub>7</sub> AON described in chapter 3, resulted in exon skipping of ataxin-3 exon 10 (LIU *et al.*, 2013). The (CUG)<sub>7</sub> ss-siRNA was found to mask exon 10 from the splicing machinery, resulting in the activation of a downstream stop codon and subsequently the formation of a shorter, C-terminus-lacking ataxin-3 protein. CAG repeat-containing exon skipping could hypothetically also be used for other polyQ disorders, except for HD, SBMA, SCA2 and SCA6, where the CAG repeat is located in the first or last exon of the transcript. In SCA7 the CAG repeat is located in the third exon of ATXN7, which is the first coding exon. In this case therefore, the activation of an alternative start 3' of the CAG repeat codon would be necessary for the translation of a polyQ lacking ataxin-7 protein. In SCA1 the CAG repeat is also located in the first coding exon, however, the ATXN1 transcript only consists of 2 coding exons, making exon skipping unsuitable. As explained in chapter 4 and 5, it could be necessary to use more AONs to remove additional exons to preserve the reading frame. To remove the CAG repeat in DRPLA and SCA17 this would mean skipping of two exons to induce translation of a modified polyQ-lacking atrophin-1 and TBP.

Chapter 4 describes a proof of concept for AON-mediated exon skipping to remove proteolytic cleavage motifs from the htt protein. Proteolytically processed polyQ fragments are implicated in toxicity of HD and other polyQ disorders (SHAO AND DIAMOND, 2007). Removal of these coding regions that code for the proteolytic cleavage site would ideally reduce the formation of toxic fragments. For SCA3 this would mean removal of ATXN3 exon 8 and 9 to

subsequently remove calpain and caspase cleavage sites from the ataxin-3 protein (WELLINGTON *et al.*, 1998; BERKE *et al.*, 2004; HUBENER *et al.*, 2012). For DRPLA, it is preferred that the caspase-3 cleavage site near the N-terminus of the protein is removed from the protein. This is encoded by exon 5 of the *ATN1* gene (WELLINGTON *et al.*, 1998; ELLERBY *et al.*, 1999A). Skipping of exon 5, and exon 6 to maintain the reading frame, would result in the removal of both the proteolytic cleavage encoding region as well as the CAG repeat. The known caspase-3 cleavage motif in the AR is encoded by the first exon (ELLERBY *et al.*, 1999B) and therefore AON-mediated skipping of this caspase-3 motif-encoding exon is not an option as treatment for SBMA. For the remaining polyQ disorders proteolytic processing has been implicated in disease pathogenesis, but the exact proteases and motifs are thus far unknown and therefore no AON-mediated therapy to remove proteolytic cleavage motifs can be designed for those disorders.

### **Towards clinical trials of genetic therapies for polyQ disorders**

Some disease-modifying drugs were successful in small studies to prevent or even slow down the progression of polyQ disorders. But none of them were shown to be effective in larger, randomised, double-blind, placebo-controlled trials. For example, drugs aiming at correcting mitochondrial dysfunction, such as coenzyme Q10, ethyl-eicosapentaenoic acid, and antihistamine latrepirdine, were shown not to improve cognition or global function in HD patients in phase III placebo-controlled trials (HUNTINGTON STUDY GROUP, 2001; HUNTINGTON STUDY GROUP TREND-HD INVESTIGATORS, 2008; HORIZON *et al.*, 2013). Likewise, the dopamine neurotransmission-stabilizing molecule pridopidine, which was promising in phase II, did not result in improved motor score in HD patients in a phase III clinical trial (de Yebenes *et al.*, 2011). A phase II/III clinical trial with lithium carbonate was recently carried out in SCA3 patients. The mechanism of lithium is thought to rely on upregulation of autophagy, though anti-apoptotic effects have also been implicated (JIA *et al.*, 2013). Due to the limited group size, this did not show a significant effect on disease progression as determined by the neurological examination score for spinocerebellar ataxia (SAUTE *et al.*, 2013).

For potential genetic therapies it would be best to start treatment before onset of clinical symptoms, before irreversible brain atrophy has occurred. Indeed, in symptomatic transgenic HD mice, AON treatment did not lead to a significant phenotypical improvement, whereas treatment of younger mice did show disease reversal (KORDASIEWICZ *et al.*, 2012). Yet after disease onset there seems to be a window of opportunity for AON treatments. In a conditional knockout model of SCA1 it was shown that removal of mutant ataxin-1 in 6 week old mice with mild motor deficits and some aggregation in Purkinje cells resulted in complete disease reversal (ZU *et al.*, 2004). Likewise, knockdown of mutant ataxin-1 in more severe 12 week old transgenic SCA1 mice with clear signs of ataxia and Purkinje cell atrophy a partial recovery was seen (ZU *et al.*, 2004), suggesting (partial) disease reversal in later stages.

The full penetrance of the polyQ mutation in combination with the availability of predictive genetic testing at the age of 18 provides the opportunity to start treatment in presymptomatic polyQ patients. The disease stage prior to onset of (typically motor) symptoms is variably called the presymptomatic, premanifest, preclinical or prodromal stage. The stage in which

subtle symptoms can be identified upon refined comparisons is called the prodromal stage. To assess the therapeutic benefit of disease-modifying compounds in polyQ patients who do not yet manifest signs of illness, there is a need for sensitive and stable clinical endpoints, such as subtle cognitive or motor improvement, brain imaging, or disease-specific biomarkers. As example, for HD the unified HD rating scale (UHDRS) is thus far used in clinical trials to show improvements on disease phenotype after disease-modifying drugs (HUNTINGTON STUDY GROUP, 1996), whereas for ataxias, the scale for the assessment and rating of ataxia (SARA) clinical scale has been developed as clinical measurement for trials (SCHMITZ-HUBSCH *et al.*, 2006). However, available rating scales can only be reliably employed after onset of clinical symptoms and are therefore inadequate to assess therapeutic potency in presymptomatic polyQ patients. Likewise, both scales require a large number of patients to show a possible significant effect of treatment, which is not always feasible for the rare polyQ disorders.

Recently, much effort has been put into characterizing the polyQ disease stage before onset of symptoms. For HD, two extensive longitudinal observational studies are ongoing (TABRIZI *et al.*, 2013; BIGLAN *et al.*, 2013). Half-term reports showed that various motor and cognitive task scores were significantly decreased over 3 year period in presymptomatic HD individuals (TABRIZI *et al.*, 2013). Combining these individual measurements into a multidimensional diagnosis may result in a more sensitive diagnosis of presymptomatic HD (BIGLAN *et al.*, 2013). For the SCAs 1, 2, 3, and 6 a prospective observational study has also been enrolled to define presymptomatic disease stage. The first baseline data from the RISCA showed mild coordination deficits in SCA1 and SCA2 patients respectively 10 to 20 years before age of onset (JACOBI *et al.*, 2013). Follow-up studies of this cohort will hopefully gain more biological and clinical characteristics for SCA patients.

In summary, polyQ pathology can be measured long before the onset of clinical symptoms and preferably disease-modifying drugs such as AON-mediated therapeutics need to be administered in the presymptomatic stage to prevent polyQ-induced toxicity and with best prospect of reversal of disease.

## 6.5. In conclusion

This thesis proposes various novel genetic therapies for polyQ disorders, aiming at reducing and/or modifying polyQ disease-causing proteins. Since AONs are never 100% effective, it is likely that a combination of lowering mutant polyQ protein levels and modifying the remaining mutant polyQ protein will be the most optimal therapy for polyQ disorders. Future experiments in small rodents are required to evaluate whether AON-mediated treatment improves the polyQ induced phenotype. Furthermore, it will also be necessary to assess (1) whether the novel modified normal proteins retain their function and localization, and (2) whether the novel modified mutant proteins are less toxic and less aggregation-prone. Also the best route of administration to the brain, optimal dosage, and treatment regime needs to be determined.

The ultimate aim should of course be to cure patients, but a more realistic short-term objective would be to delay the age of onset or reduce the progression and thereby increase the quality of life for both polyQ patients and their families. Although extensive *in vitro* and *in vivo* research is required to rule out toxic off-target effects of the various AONs and the resulting modified proteins, these preclinical AON-mediated treatments look promising. Hopefully this thesis will help in the quest to develop treatments for individuals with one of the polyQ disorders.

