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General introduction: Genetic therapies for polyglutamine disorders

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1.1. Introduction

More than two decades ago for the first time an expansion of a repeated microsatellite sequence was discovered to be the cause of a disease. A CGG triplet repeat expansion in the gene responsible for fragile X syndrome was found (Verkerk *et al.*, 1991). In the same year the mutation in fragile X syndrome was identified, a CAG trinucleotide repeat expansion in the *AR* gene was found to be the cause of a X-linked progressive neurodegenerative disorder called spinal and bulbar muscular atrophy (SBMA), or Kennedy disease (La Spada *et al.*, 1991). The CAG trinucleotide repeat expansion in the *AR* gene results in a mutant androgen receptor (AR) with an expanded polyglutamine (polyQ) tract (La Spada *et al.*, 1991). Next to SBMA, eight other neurodegenerative disorders have since been identified resulting from an expanded polyQ protein (**Table 1**). These disorders are Huntington disease (HD), the spinocerebellar ataxias (SCAs) 1, 2, 3, 6, 7, and 17), and dentatorubro-pallidoluysian atrophy (DRPLA) (**Table 1**).

This chapter will first introduce a group of inherited disorders caused by a triplet repeat expansion and provide a short overview of the main characteristics of polyQ disorders (**paragraph 1.2**). The underlying clinical and molecular genetics of the most prevalent and best studied polyQ disorders, HD and SCA3, will be extensively reviewed in **paragraph 1.3 and 1.4**. While I will provide a short overview of the disease-causing polyQ proteins and the role of known disease mechanisms of the other polyQ disorders; DRPLA, SBMA, and SCAs 1, 2, 6, 7 and 17, in **paragraph 1.5**. Next, opportunities for protein lowering approaches of polyQ disorders will be discussed (**paragraph 1.6**). What can we learn from other neurodegenerative disorders where genetic therapies are in development or already used as therapy (**paragraph 1.7**). The challenges in delivery and cellular uptake of genetic therapies for neurodegenerative disorders to the brain and specifically neurons will be discussed in **paragraph 1.8.** Finally, the scope and outline of this thesis "developing genetic therapies for polyglutamine disorders" will be defined.

1.2. Triplet repeat expansion disorders

Since the early nineties, 14 inherited triplet repeat expansion disorders have been identified. Over the years other repeat expansions, such as tetra, penta and dodecanucleotide repeats, have also been linked to human diseases (Mirkin, 2007). In this thesis I will only focus on neurological and neuromuscular disorders caused by triplet repeat expansions. These triplet repeat disorders are categorised according to their disease mechanism, being (1) functional cellular impairment due to loss of function of the gene containing the repeat, (2) cellular impairment due to production of RNA containing an expanded CUG tract, or (3) functional cellular impairment due to production of a protein containing an expanded polyQ. Here, I will provide a short overview of all three categories and successively I will focus on polyQ disorders.

Loss of protein function

As described previously, in 1991 the Human Genome Project led to the discovery of the gene responsible for fragile X syndrome (Verkerk *et al.*, 1991). Fragile X syndrome is characterized by mental retardation, macroorchidism, and distinct facial features (Jacquemont *et al.*, 2007). The CGG repeat is located in the 5′ untranslated region (UTR) of the *FMR1* gene (Kremer *et al.*, 1991; Verkerk *et al.*, 1991). A trinucleotide repeat expansion of over 200 CGGs results in decreased FMR1 expression due to hypermethylation at the promoter, decreased fragile X mental retardation protein (FMRP) levels and loss of function (Pieretti *et al.*, 1991; Meijer *et al.*, 1994). The CGG repeat in fragile X syndrome is considered to be highly unstable once it exceeds a certain threshold length (Richards and Sutherland, 1992). This phenomenon where the triplet repeat size increases upon the next generation, causing symptoms at an earlier age, is called anticipation. The full mutation alleles are derived from meiotically unstable maternal premutation alleles, with 55 to 200 CGG repeats (Fu *et al.*, 1991). Patients with 45 to 54 repeats do not transmit directly to the full mutation, even though these intermediate alleles are slightly unstable, particularly when maternally transmitted (Zhong *et al.*, 1996).

Gain of RNA toxicity

A decade after the discovery of the CGG triplet repeat expansion responsible for fragile X syndrome, it was found that carriers with premutation alleles developed a late age of onset neurodegenerative disorder called fragile X-associated tremor/ataxia syndrome (FXTAS) (Hagerman *et al.*, 2001; Jacquemont *et al.*, 2003). Although not fully penetrant, especially males with premutation alleles containing of more than 70 CGG repeats develop FXTAS with intention tremor and cerebellar ataxia (Jacquemont *et al.*, 2006). Remarkably, FXTAS patients had close to normal FMRP protein levels, ruling out the loss of protein function as shown in fragile X syndrome (Kenneson *et al.*, 2001). Peripheral blood leucocytes derived from FXTAS patients did show elevated FMRP mRNA levels in (Kenneson *et al.*, 2001). Likewise, *post-mortem* brain tissue showed intranuclear mRNA inclusions (RNA foci) containing the expanded CGG-repeat (Tassone *et al.*, 2004), suggesting RNA-mediated neurodegeneration.

Originally, the idea for gain of toxic function at RNA level came from myotonic dystrophy type 1 (DM1) (Davis *et al.*, 1997). DM1 is a member of CTG expansion disorders that derive from triplet repeat expansions located in non-coding regions of the corresponding genes. Other CTG repeat expansion disorders are HD-like 2 (HDL2), SCA8 and SCA12. DM1 is caused by a CTG expansion in the 3′ UTR of the *dystrophia myotonica-protein kinase* (*DMPK*) gene (Brook *et al.*, 1992; Fu *et al.*, 1992; Mahadevan *et al.*, 1992). This unstable CTG triplet repeat expansion results in the most common form of adult muscular atrophy. Like other triplet repeat expansions, DM1 shows genetic anticipation with an earlier onset and more severe phenotype after transmission from one generation to the next (Howeler *et al.*, 1989). Patients with DM1 were shown to have RNA foci with sequestration of the muscleblind-like 1 (MBNL1) splicing factor in muscle nuclei (Miller *et al.*, 2000; Fardaei *et al.*, 2001). The CUG repeat mRNA can form stable hairpin structures which can sequester RNA binding proteins, such as MBNL1. This binding of MBNL1 to double stranded CUG RNA is believed to result in depleted MBNL1 function and consequently misregulation of alternative splicing, resulting in cellular toxicity (Wheeler and Thornton, 2007).

Gain of toxic polyQ protein function

Since the discovery that an expanded CAG repeat in the *AR* gene and subsequent translation of a mutant polyQ-repeat containing androgen receptor result in SBMA, eight other neurodegenerative polyQ disorders have been identified (**Table 1**). All polyQ disorders are caused by a CAG triplet repeat expansion in exons of different genes and are the result of a gain of toxic polyQ protein function. These disorders are HD, SCAs 1, 2, 3, 6, 7, and 17, and DRPLA (**Table 1**). PolyQ disorders can be subdivided based on their main clinical feature: SBMA is mainly characterized by motor weaknesses, HD by chorea and the other 7 polyQ disorders by ataxia.

It is known that the prevalence of each polyQ disorder significantly varies per country and ethnicity. The prevalence of the polyQ SCAs was estimated to be about 3 per 100,000 individuals in the Netherlands (van de Warrenburg *et al.*, 2005) and for DRPLA 0.1 per 100,000 individuals in Japan (Hirayama *et al.*, 1994). Worldwide HD (3-5 per 100,000), SBMA (1-2 per 100,000), and SCA3 (0.5-1 per 100,000) are the most prevalent polyQ disorders (Schols *et al.*, 2004; Banno *et al.*, 2012; Pringsheim *et al.*, 2012).

Although the mutations occur in very different genes, polyQ disorders have a lot in common (Fischbeck, 2001). They all result in progressive neurodegeneration with psychiatric, cognitive and motor symptoms. Except for SBMA, they are all autosomal dominant and disease onset is around midlife. For all disorders the CAG repeat length correlates with the age of onset, which means that the longer the CAG repeat, the earlier the disease manifestation (Doyu *et al.*, 1992; Ikeuchi *et al.*, 1995; Lund *et al.*, 2001; van de Warrenburg *et al.*, 2005; Roos, 2010). All polyQ disorders have a CAG repeat threshold, meaning that carriers with a CAG repeat above this threshold will certainly develop the disorder (**Table 1**). PolyQ diseases also have genetic anticipation, mainly upon paternal transmission, which means that the next generation will likely inherit a longer CAG repeat, resulting in a more severe disease with an earlier age of

Table 1: Overview of polyQ disorders and CAG repeat location and number of causative genes.

disease		gene		CAG repeat	repeat number	
symbol	full name	symbol	full name	location	wt	mut
HD	Huntington disease	*HTT*	huntingtin	exon 1	< 29	≥ 40
SCA3/ MJD	spinocerebellar ataxia type 3/ Machado-Joseph disease	*ATXN3*	ataxin 3	exon 10	< 40	≥ 52
SBMA	spinal and bulbar muscular atrophy/ Kennedy disease	*AR*	androgen receptor	exon 1	< 31	≥ 40
SCA1	spinal and bulbar muscular atrophy/	*ATXN1*	ataxin 1	exon 8	< 36	≥ 39
SCA2	Kennedy disease	*ATXN2*	ataxin 2	exon 1	< 31	≥ 32
SCA6	spinocerebellar ataxia type 6	*CACNA1A*	calcium channel, voltage-dependent, P/Q type, alpha 1A subunit	exon 47	< 18	≥ 19
SCA7	spinocerebellar ataxia type 7	*ATXN7*	ataxin 7	exon 3	< 35	≥ 38
SCA17	spinocerebellar ataxia type 17	*TBP*	TATA box-binding protein	exon 3	< 42	≥ 50
DRPLA	dentatorubral-pallidoluysian atrophy	*ATN1*	atrophin 1	exon 5	< 35	≥ 49

Abbreviations: wt: normal repeat size, mut: full penetrant repeat size

onset (Rub *et al.*, 2013).

As described above, the polymorphic polyQ tract consists of CAG repeats. However, polyQ is also encoded by CAA codons and usually a combination of both CAG and CAA triplets encodes the polyQ tract. In polyQ disease-causing proteins the polyQ tracts are commonly composed of long uninterrupted CAG triplets. The polyQ tracts encoded by mixtures of CAG and CAA codons seems to be less prone to expansion and more stable upon transmission to the next generation (Frontali *et al.*, 1999). In accordance, interruption of the CAG repeat by CAA (silent) or CAT (missense) mutations results is altered aggregation properties and delay the onset age (Menon *et al.*, 2013).

Except for SCA6, polyQ disease-causing proteins are ubiquitously expressed throughout the body. Apart from the expanded polyQ repeat, mutant polyQ proteins have no homologous sequences or functional domains, assuming that the expanded polyQ repeat is causative for the observed pathogenesis (Zoghbi and Orr, 2000). It has been suggested that in polyQ disorders increasing oxidative stress and inability to protect against free radicals with age could lead to mitochondrial dysfunction and cell damage (Kim *et al.*, 2003; Goswami *et al.*, 2006; Miyata *et al.*, 2008; Ajayi *et al.*, 2012).

A prominent pathological hallmark of these diseases is the accumulation of aggregated polyQ proteins in the brain (Davies *et al.*, 1997). However, the exact role of protein aggregation in disease pathogenesis is controversial and whether the aggregates are neurotoxic or neuroprotective is still under debate (Klement *et al.*, 1998; Saudou *et al.*, 1998; Yamada *et al.*, 2006). Probably the smaller soluble polyQ species generated by proteolytic cleavage during the aggregation process are the toxic entities and the large SDS-insoluble aggregates may likely be less harmful end products of the upstream toxic event (Shao and Diamond, 2007). Misfolding of the expanded polyQ proteins probably results in proteolytic cleavage and altered interactions, resulting in neurodegeneration and neuronal loss (Shao and Diamond, 2007).

1.3. Huntington disease

HD is an autosomal, dominantly inherited neurodegenerative disorder. HD is rare, but more common in Western countries. The prevalence of HD in America is approximately 5 in 100,000 (Shoulson and Young, 2011) and in Europe, the prevalence of HD may be even higher with estimates in England and Wales as high as 12 in 100,000 individuals (Rawlins, 2010).

Post-mortem studies show that there is a 10-20 percent weight reduction in HD brains (Vonsattel *et al.*, 1985). Neurodegeneration occurs throughout the forebrain and specifically affects GABAergic medium spiny neurons of the striatum (Levesque *et al.*, 2003). Severe cell loss in the striatal complex, the caudate nucleus and putamen, results in striatal atrophy (Halliday *et al.*, 1998). This causes an enlargement of the lateral ventricles. The medium spiny projection neurons, containing enkephalin, are more susceptible to degeneration than substance P containing projection neurons while interneurons seem to be spared (Walker, 2007). With disease progression, degeneration expands throughout the brain and other structures become affected (Tabrizi *et al.*, 2009). Cortical atrophy is characterized by thinning of the cerebral cortex and the underlying white matter. Neuronal loss is abundant in cortical layers III, V and VI (Rosas *et al.*, 2008) but is also prominent in the *Cornu Ammonis* (CA1) region of the hippocampus, with a reduction of about 9 percent (Rosas *et al.*, 2003).

Disease onset usually occurs around midlife and is clinically characterized by a combination of symptoms: cognitive impairments, movement abnormalities, and emotional disturbances (Roos, 2010). Motor symptoms of HD include chorea and occasionally bradykinesia and dystonia (Tabrizi *et al.*, 2009). Choreic movements, recognized as involuntary and unwanted movements, start in the distal extremities. During the course of HD these movements become more profound and eventually all muscles of the body are affected. These symptoms can initially appear as lack of concentration or nervousness and unsteady gait (Kremer *et al.*, 1992). Psychiatric symptoms often precede the onset of motor symptoms. Irritability is commonly one of the first signs and occurs throughout the course of the disease. Other psychiatric symptoms involve anxiety, obsessive and compulsive behaviour while apathy and psychosis can appear in advanced stages. However, the most frequent psychiatric symptom is depression (Reedeker *et al.*, 2012). Like psychiatric symptoms, cognitive symptoms can be present prior to the onset of the motor symptoms. The cognitive symptoms comprise mainly of impairment in executive functions, including abstract thinking, problem solving, and attention (Paulsen and Long, 2014). Furthermore, the ability to learn new skills is affected (Paulsen *et al.*, 2001). Altogether these symptoms dramatically impede social and professional functioning. Eventually patients are incapable to adequately perform daily activities finally leading to progressive disability, requiring full-time care, followed by death (Simpson, 2007). Death generally occurs 15 to 20 years post diagnosis due to complications such as pneumonia, falls, dysphagia, heart disease or suicide (Roos *et al.*, 1993).

The disease is caused by a CAG trinucleotide repeat expansion in the first exon of the *HTT* gene. The *HTT* gene was the first autosomal disease locus to be mapped by genetic linkage

analysis in 1983 (Gusella *et al.*, 1983) on the short arm of chromosome 4 (4p16.3). The huntingtin protein (htt) was found to be ubiquitously expressed throughout the body, with highest expression in testis and brain (Strong *et al.*, 1993), however, cells in the brain are specifically vulnerable to the toxic function of mutant htt. The CAG repeat expansion in the *HTT* gene results in an expanded polyQ repeat in the htt protein (The Huntington's Disease Collaborative Research Group, 1993). When the number of CAG repeats exceeds 39, the gene encodes a mutated form of the htt protein that is prone to aggregation. Alleles ranging from 36 to 39 repeats, lead to reduced penetrance of the disease or to a very late onset (Kremer *et al.*, 1992; McNeil *et al.*, 1997; Losekoot *et al.*, 2013) and both sexes are affected with the same frequency (Walker, 2007). Repeat numbers exceeding 55-60 result in clinical manifestation of the disease before the age of 20, known as juvenile HD (Andresen *et al.*, 2007). Intergenerational CAG repeat changes are extremely rare on normal chromosomes but on expanded chromosomes changes in CAG repeat size take place in approximately 70 percent of meioses and expansion is more likely via the paternal line (Kremer *et al.*, 1995).

There is a strong inverse correlation between repeat numbers and the age of onset of the disease. The repeat length accounts for approximately 70 percent of the variance in age of onset (Roos, 2010). The relationship between repeat size is and rate of progression and duration of the disease is still under debate (Rosenblatt *et al.*, 2012). Neuropathological changes, such as atrophy and the number of aggregates found in the brain are clearly correlated with the CAG repeat number.

For patients, only symptomatic treatment is available and a treatment to slow down the progression or delay the onset of the disease remains elusive.

Huntingtin protein

When the *HTT* gene was discovered in 1993, the htt protein had an unknown function. Since then, enormous research efforts have revealed many functions of the wild-type protein (discussed in the present paragraph) and many toxic gain of functions of the mutant protein (discussed in the next paragraph) (**Figure 1**).

Wild-type htt is mainly localized in the cytoplasm, although a small proportion is present in the nucleus (Hoogeveen *et al.*, 1993; de Rooij *et al.*, 1996; Kegel *et al.*, 2002). The protein is known to be associated with microtubules, the plasma membrane, Golgi complex, the endoplasmic reticulum (ER), and mitochondria. Furthermore htt is associated with vesicular structures, such as clathrin-coated and non-coated vesicles, autophagic vesicles, endosomal compartments or caveolae (Kegel *et al.*, 2005; Atwal *et al.*, 2007; Rockabrand *et al.*, 2007; Strehlow *et al.*, 2007; Caviston *et al.*, 2011).

Three of the first 17 amino acids at the amino terminus of htt are lysines, which are targets for post translational modifications that regulate htt half-life and are proposed to be involved in targeting htt to various intracellular membrane-associated organelles (Kalchman *et al.*, 1996; Steffan *et al.*, 2004; Kegel *et al.*, 2005; Atwal *et al.*, 2007; Rockabrand *et al.*, 2007). The polyQ repeat starts at the 18th amino acid and is thought to form a polar zipper structure, important for the interaction between different polyQ-containing transcription factors (Perutz *et al.*, 1994; Harjes

and Wanker, 2003). The polyQ stretch is followed by a polymorphic polyproline repeat, which is thought to be involved in keeping the protein soluble (Steffan *et al.*, 2004). Additionally, three main HEAT (htt, elongation factor 3, protein phosphatase 2A, and the yeast PI3-kinase TOR1) repeat motifs are present which are known to form superhelical structures and are involved in protein-protein interactions (Takano and Gusella, 2002; Li *et al.*, 2006). Htt is palmitoylated at the cysteine residue 214 by htt interacting protein (Hip) 14, which is thought to be involved in htt trafficking (Huang *et al.*, 2004). Htt has various proteolytic cleavage motifs, with a hotspot between amino acid 500 and 600 that are recognized by various proteases, such as caspases 1, 3, 6, 7 and 8 and calpain (Gafni and Ellerby, 2002; Wellington *et al.*, 2002; Kim *et al.*, 2006). The significance of wild-type htt cleavage is not completely clear, but the N-terminal proteolytic cleavage products tend to be trafficked across the nuclear membrane (Warby *et al.*, 2008). In the case of mutant htt, the accumulation of N-terminal proteolytic cleavage products in the nucleus has major impact on the pathogenesis (see below).

Mutant huntingtin: gain of toxic function

Although countless toxic gain of functions of the mutant htt have been proposed in the last two decades, the exact order of pathogenic events in HD, as well as interactions between mutant htt and other cellular proteins, are still poorly understood. Mutant htt is known to undergo conformational changes and interferes with various cellular processes, such as cellular trafficking, inhibition of chaperones, proteasomes, and autophagy, causing accumulation of htt and other abnormally folded proteins (**Figure 1**).

The characteristic protein aggregates are located throughout the brain and can already be found before the onset of the first symptoms (Weiss *et al.*, 2008). The rate of aggregate formation is correlated to the length of the polyQ repeat (Legleiter *et al.*, 2010), although there is growing evidence suggesting that these aggregates are not good indicators for disease onset and progression (Wanker, 2000; van Roon-Mom *et al.*, 2006). Whether accumulation of these aggregates is neurotoxic or neuroprotective is still under debate but increasing evidence suggests that soluble shorter mutant htt, and fragments thereof, are the main toxic components (Davies *et al.*, 1997; Saudou *et al.*, 1998; Arrasate *et al.*, 2004; Sathasivam *et al.*, 2013). Mutant htt is more subject to increased caspase activity and proteolytic cleavage of mutant htt results in the formation of small toxic N-terminal mutant htt fragments (**Figure 1**) (Cooper *et al.*, 1998). In HD brains, more htt protein fragments are found within the striatum compared to control brains, as well as upregulation of caspases, suggesting that cleavage may be disease specific (Mende-Mueller *et al.*, 2001; Graham *et al.*, 2010). Furthermore, strong evidence was presented for an important role of htt protein fragments in the pathogenesis of HD, as a HD mouse model containing capase-6-resistant expanded htt did not show neuronal dysfunction in contrast to the same mouse with caspase-6-sensitve expanded htt (Graham *et al.*, 2006). The only difference between these mice was the presence or absence of a 586 amino acid caspase-6 cleaved htt fragment containing the expanded polyQ repeat.

Although many genes and proteins have been identified to be involved in the HD pathogenesis, there is not one main cellular process affected in HD. Below I will review the

best studied cellular processes known to be involved in HD pathology.

Transcriptional deregulation

Like other polyQ disorders, altered gene expression is a prominent molecular hallmark of HD. As described above, mutant htt is proteolytically cleaved and N-terminal fragments are abundant in the nucleus, where they form aggregates (Cooper *et al.*, 1998). Various transcription factors have been found to co-localize with htt aggregates, such as TATA box-binding protein (TBP), cAMP response element-binding protein binding protein (CBP) and p53 (Steffan *et al.*, 2000; van Roon-Mom *et al.*, 2002). These co-aggregated proteins can no longer assert their normal function and could thereby contribute to transcriptional deregulation (Nucifora, Jr. *et al.*, 2001). A large set of genes involved in cellular processes affected have been found to be differentially expressed in various cellular and animal models of HD (Cha, 2007) and HD patient-derived *post-mortem* brain (Hodges *et al.*, 2006). In HD, mutant htt binds less efficient to the RE1 silencing transcription factor (REST), causing transcriptional repression of various genes, including the brain-derived neurotrophic factor (BDNF) (Zuccato *et al.*, 2001; Zuccato *et al.*, 2007). BDNF is vital for neuronal survival and is involved in synaptic plasticity processes (Huang and Reichardt, 2001). Next to reduced gene transcription, mutant htt also disrupts vesicular transport and release of BDNF, possibly leading to excitotoxicity (Gauthier *et al.*, 2004).

Impaired protein degradation

Protein aggregates in HD patient-derived brain material shows a clear co-localization of htt and ubiquitin (Difiglia *et al.*, 1997), suggesting an involvement of the ubiquitin-proteasomal protein degradation in the disease. Mutant htt is misfolded, resulting in an aggregation-prone conformation (Rousseau *et al.*, 2009). Misfolded, aggregation-prone proteins are generally cleared either by the ubiquitin-proteasome system (UPS) (short-lived proteins) or through the autophagy-lysosome pathway (long-lived cytoplasmic proteins and protein complexes) (Rubinsztein, 2006). Aggregated htt protein and long stretches of Qs are known to impair the UPS *in vitro* and in *post-mortem* brain tissue (Bence *et al.*, 2001; Venkatraman *et al.*, 2004; Diaz-Hernandez *et al.*, 2006; Raspe *et al.*, 2009; Park *et al.*, 2013b), resulting in an inefficient degradation of mutant htt. The UPS is also involved in ER-associated protein degradation (ERAD). In an overexpressing cell system mutant htt was found to sequester various ERAD proteins, thereby inhibiting their function (Duennwald and Lindquist, 2008), which can result in ER stress-induced autophagy. To note, the involvement of the UPS in processing of expanded polyQ repeats has been the subject of controversy. Overexpressed N-terminal polyQ fragments were found to be entirely degraded by cellular proteasomes (Juenemann *et al.*, 2013). Also the entrapment of components of the UPS in aggregates could not be validated in HD mouse models (Bett *et al.*, 2009; Maynard *et al.*, 2009). Still, UPS activity is known to decrease with age and this reduced UPS activity is associated with increasing N-terminal expanded polyQ fragments aggregates in an HD knock-in mouse model (Zhou *et al.*, 2003), suggesting an involvement of UPS impairment in the HD pathogenesis.

In HD, two types of autophagy are affected, being macroautophagy and chaperone-mediated

autophagy (Cortes and La Spada, 2014). By macroautophagy cytosolic materials are sequestered in double membrane vesicles called autophagosomes. Although in HD cells autophagosomes are formed correctly and fused with lysosomes, its cargo recognition is disrupted by mutant htt, leading to empty autophagosomes (Martinez-Vicente *et al.*, 2010). Thus, it seems that the in HD reduced macroautophagy is not caused by comprised autophagosomes formation, but due to impaired toxic substrate removal. Blockage of macroautophagy results in upregulation of chaperone-mediated autophagy (Kaushik *et al.*, 2008). Chaperones usually assist target substrates, including phosphorylated (mutant) htt, directly to the lysosome (Thompson *et al.*, 2009; Qi *et al.*, 2012). In HD, this chaperone-mediated autophagy is impaired, probably by reduced phosphorylation of mutant htt (Thompson *et al.*, 2009), or by binding of mutant htt to chaperone proteins (Qi *et al.*, 2012), resulting in a reduced clearance of mutant htt. The reduced macroautophagy seen in HD is perhaps initially compensated by chaperone-mediated autophagy, but this overcompensation decreases with age, resulting in impaired clearance of toxic entities, increased oxidative damage and eventually neuronal cell death (Cortes and La Spada, 2014).

Mitochondrial dysfunction

N-terminal mutant htt fragments were found to be associated with the surface of mitochondria in transgenic and knock-in HD mice (Panov *et al.*, 2002; Orr *et al.*, 2008). The accumulation of mutant htt on mitochondria increases with age and correlates with disease progression. Soluble mutant htt impairs microtubule-based transport of proteins that are involved in the transport of mitochondria, which could lead to decreased ATP supply in nerve terminals (Orr *et al.*, 2008). Mutant htt is also suggested to be involved in mitochondrial energy metabolism defects. Metabolic energy defects could be the result of mutant htt's capability to induce mitochondrial permeability transition pore opening (Choo *et al.*, 2004). This leads to low mitochondrial membrane potential and high glutamate transmission, resulting in overactive glutamate receptors (excitotoxicity) (Choo *et al.*, 2004). Abnormal mitochondrial respiratory chain function seen in HD leads to reduced ATP levels and subsequent partially depolarized membrane (Milakovic and Johnson, 2005). This voltage change leads to chronic calcium influx and activation of proteases, causing more reactive oxygen species production and eventually oxidative stress. Nevertheless, this respiratory chain impairment is probably not caused by mutant htt directly but as late secondary event of autophagy pathway impairment and transcriptional deregulation (Oliveira, 2010).

Impaired axonal transport

In HD, axonal transport of mitochondria is impaired (Chang *et al.*, 2006). Next to transport of mitochondria, also transport of other organelles, such as BDNF-containing organelles and vesicles that store neurotransmitters and other peptides, exists in synapses (Gauthier *et al.*, 2004; Li *et al.*, 2009). In *C. elegans* and *D. melanogaster* HD models, mutant htt overexpression resulted in axonal aggregate formation and subsequently impaired axonal trafficking of synaptic vesicles and mitochondria (Parker *et al.*, 2001; Gunawardena *et al.*, 2003; Sinadinos *et al.*,

2009). This impaired transport of vesicles is confirmed by the finding that in early stage HD patients synaptic vesicle proteins show an altered subcellular location (Modregger *et al.*, 2002). Finally, various proteins involved in exocytosis are known to have decreased expression levels in HD patients. Proteins involved in docking and fusion of vesicles show reduced transcript expression, suggesting a defect in the neurotransmitter release machinery in HD patients (Smith *et al.*, 2007).

To conclude, many cellular processes have been identified that are impaired in HD, making it difficult to pinpoint offhand which processes are crucial for the disease pathology. Nevertheless, the toxic N-terminal polyQ protein fragments are thought to be crucial in the pathogenesis of HD. Why certain neuronal populations are more vulnerable to polyQ-containing peptides than others, remains elusive.

Loss of wild-type htt function

As described above, the main cause of HD is a gain of toxic mutant htt function. Mutant htt can bind and sequester wild-type htt into aggregates, potentially causing loss of wild-type htt function (Kazantsev *et al.*, 1999; Busch *et al.*, 2003). Since wild-type htt has anti-apoptotic properties and is important for cell survival in adult brain, loss of wild-type htt function could also be involved. Knock-out of the homologous *htt* mouse gene was found to be early embryonic lethal (Duyao *et al.*, 1995; Nasir *et al.*, 1995; Zeitlin *et al.*, 1995) and htt inactivation in the forebrain and testis of adult mice was shown to result in progressive neurodegeneration, sterility and reduced lifespan (Dragatsis *et al.*, 2000). Removal of endogenous htt in a *D. melanogaster* HD model was found to exacerbate the neurodegenerative phenotype (Zhang *et al.*, 2009). Htt is reported to be involved in BDNF-mediated neurotrophic support (Zuccato *et al.*, 2001) and act as protector of brain cells from apoptotic stimuli (Rigamonti *et al.*, 2000). These neuronal survival pathways are compromised due to mutant htt, once more supporting the view that loss of wild-type htt function is also involved in the disease pathogenesis.

Chapter 1

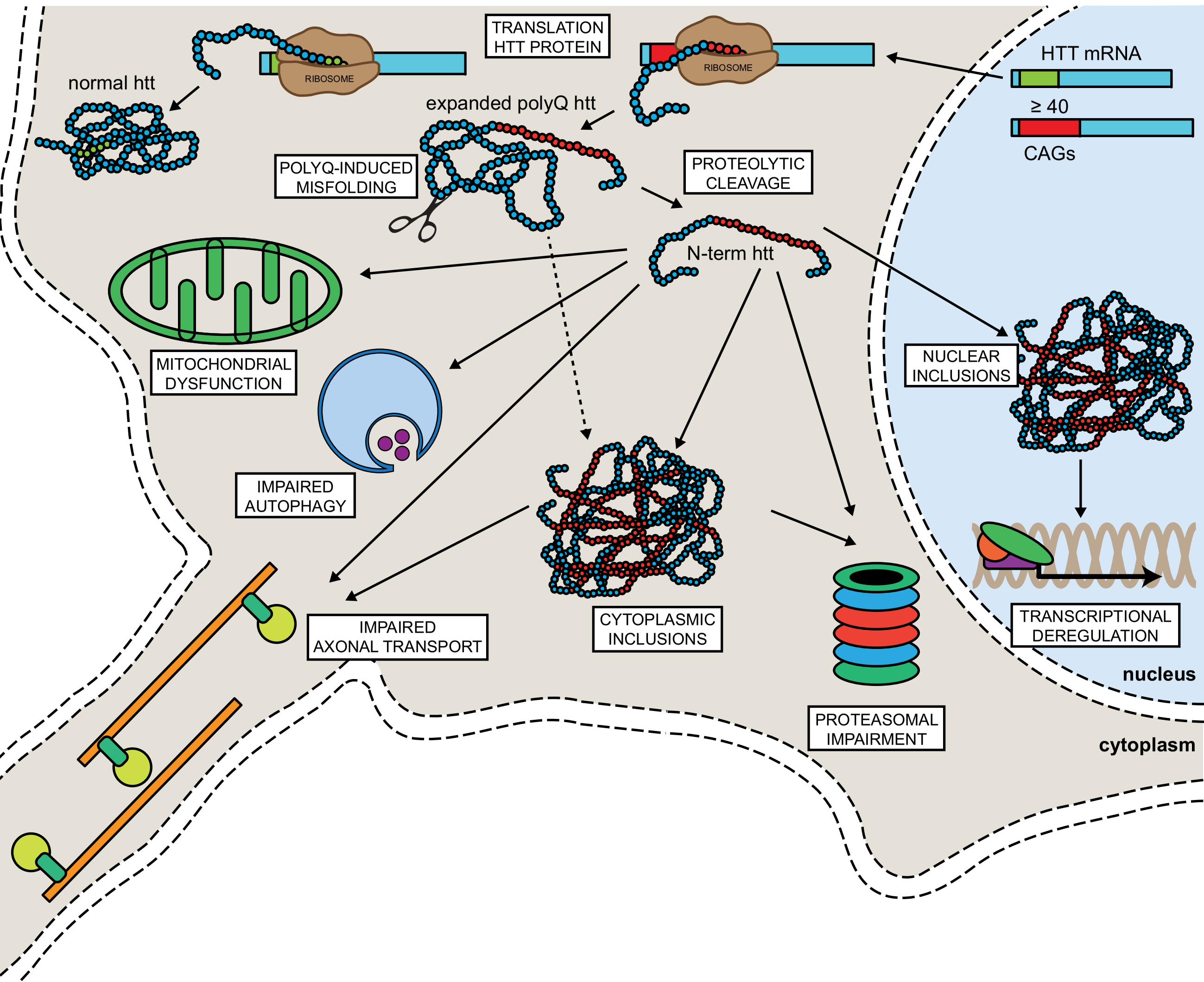

Figure 1. Schematic representation of cellular pathogenesis in Huntington disease (HD). The *HTT* gene is transcribed into pre-mRNA containing 67 exons which is spliced into mature mRNA. The expanded CAG repeat is located in the first exon and the transcript is translated into a mutant polyglutamine (polyQ) repeat-containing huntingtin (htt) protein. This polyQ repeat triggers conformational changes, resulting in abnormally folded mutant htt. Mutant htt is proteolytically cleaved, giving rise to N-terminal fragments that are aggregation-prone. Full-length and cleaved forms of htt form soluble monomers, oligomers or large insoluble aggregates, both in the nucleus and in the cytoplasm that cause toxicity. Other cellular disturbances resulting from mutant htt presence involved in HD pathogenesis include: transcriptional deregulation, impaired autophagy, mitochondrial dysfunction, proteasomal impairment, and compromised axonal transport.

1.4. Spinocerebellar ataxia type 3

SCA3, or Machado-Joseph disease (MJD) (Haberhausen *et al.*, 1995), is the most common spinocerebellar ataxia (Ranum *et al.*, 1995; Silveira *et al.*, 1996) and the second most common polyQ disease after HD (Pringsheim *et al.*, 2012). Similar to the other polyQ disorders is SCA3 inherited in an autosomal dominant fashion (Coutinho and Andrade, 1978), neurodegeneration is progressive and is ultimately fatal. Current therapeutic strategies are only able to provide symptomatic relief (Bauer and Nukina, 2009). SCA3 is clinically heterogeneous, but the main feature is progressive ataxia, affecting balance, gait and speech. Other frequently described symptoms include pyramidal signs, progressive external ophthalmoplegia, dysarthria, dysphagia, rigidity, distal muscle atrophies and double vision (Coutinho and Andrade, 1978; Rosenberg, 1992; Soong *et al.*, 1997; Teive *et al.*, 2012). 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.*, (Riess *et al.*, 2008).

SCA3 is caused by an expanded stretch of CAG triplets in the penultimate exon of the *ATXN3* gene on chromosome 14q32.1, encoding the ataxin-3 protein (Kawaguchi *et al.*, 1994). Healthy individuals have up to 44 CAG repeats, whilst affected individuals have between 52 and 86 glutamine repeats. A repeat range from 45 to 51 is associated with incomplete penetrance of the disease (Kawaguchi *et al.*, 1994; Durr *et al.*, 1996; Padiath *et al.*, 2005). SCA3 patients with two mutant alleles show a more severe disease phenotype than those with a single mutant allele (Carvalho *et al.*, 2008). 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 (Maciel *et al.*, 1995). The expanded CAG repeat leads to formation of an expanded polyQ tract in the C-terminal region of the ataxin-3 protein, leading to a toxic gain of function of the protein and formation of characteristic neuronal aggregates (Paulson *et al.*, 1997b). As is the case in HD, the neurotoxic properties of these aggregates are still under debate since the number of aggregates does not mirror the level of neurodegeneration or *ATXN3* CAG repeat length (Rub *et al.*, 2006).

Extensive studies in cell and animal models over the last decade 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 protein

The ataxin-3 protein has a molecular weight of approximately 42 kDa, depending on the isoform and size of the polyQ repeat. The CAG repeat, located in the penultimate exon, is translated into a polyQ repeat located at the C-terminus of the protein. In blood, 56 splice variants of ATXN3 have been identified, of which 20 could potentially be translated into a functional ataxin-3 protein (Bettencourt *et al.*, 2010). Of these 20 isoforms, only two isoforms, which differ in their C-terminal tail, have been studied extensively thus far. The isoform of ATXN3 most commonly expressed in brain consists of 11 exons, and is translated into an ataxin-3 protein consisting of 361 amino acids (Schmidt *et al.*, 1998; Trottier *et al.*, 1998; Harris *et al.*, 2010), based on a polyQ repeat length of 13 [Ensembl transcript ID ENST00000393287] (**Figure 2**).

Ataxin-3 is found throughout the cell and is able to translocate from the cytoplasm to the nucleus and back (Paulson *et al.*, 1997a; Schmidt *et al.*, 1998; Tait *et al.*, 1998; Trottier *et al.*, 1998; Pozzi *et al.*, 2008). Different regions of the ataxin-3 protein influence its subcellular localisation. It is not yet known if ataxin-3 plays a more important role in the nucleus or the cytoplasm, but enzymatically active ataxin-3 has been shown to localise to the nucleus more frequently compared to its inactive form (Todi *et al.*, 2007). In silico analysis predicted a nuclear localisation signal (NLS) in the proximity of the polyQ repeat at amino acid 273 to 286 (**Figure 2**) (Tait *et al.*, 1998; Albrecht *et al.*, 2004; Antony *et al.*, 2009; Macedo-Ribeiro *et al.*, 2009). This NLS showed a weak nuclear import activity *in vitro* (Antony *et al.*, 2009). However, mutating or deleting the proposed core NLS sequence from amino acid 282 to 285 had no effect on subcellular distribution, thus questioning the importance of the ataxin-3 NLS in its cellular localisation (Mueller *et al.*, 2009; Breuer *et al.*, 2010).

Another region that has been implicated in regulating ataxin-3 cellular localisation lies in the first 27 amino acids. Ataxin-3 lacking these first 27 amino acids could not be found in the nucleus, though the responsible mechanism involved is still unknown (Pozzi *et al.*, 2008). Furthermore, ataxin-3 contains six potential nuclear export signals (NES) (Albrecht *et al.*, 2004; Antony *et al.*, 2009; Macedo-Ribeiro *et al.*, 2009), of which two (amino acid 77 to 99 and 141 to 158) (**Figure 2**), showed significant nuclear export activity (Antony *et al.*, 2009; Macedo-Ribeiro *et al.*, 2009).

The N-terminus of ataxin-3 contains a large Josephin domain (**Figure 2**) that is known to have a low isopeptidase activity (Winborn *et al.*, 2008), implicating a role for ataxin-3 in the UPS pathway (Burnett *et al.*, 2003). The Josephin domain, together with the ubiquitin interacting motifs (UIM), 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(Burnett *et al.*, 2003; Winborn *et al.*, 2008; do Carmo *et al.*, 2010). The UPS pathway is involved in various cellular processes, such as protein degradation, endocytosis, transcriptional regulation and antigen presentation. Ubiquitination is a cascade of processes involving activating enzyme E1, transfer to ubiquitin conjugating enzymes E2 and association with ubiquitin ligases E3, resulting in addition of ubiquitins via isopeptide linkages to lysines

in the targeted protein (Fang and Weissman, 2004). Ubiquitins can bind individually, or as a poly-ubiquitin chain. 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 (Lim and Lim, 2011). Amino acid cysteine 14, histidine 119, and asparagine 134 of the Josephin domain (**Figure 2**) 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 (Mao *et al.*, 2005; Nicastro *et al.*, 2005). 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 (Burnett *et al.*, 2003; Berke *et al.*, 2005) and binds the poly-ubiquitin linkages at lysine 48, lysine 63 and mixed linkage ubiquitin chains, with preference for lysine 63-tagged ubiquitins (Fang and Weissman, 2004; Winborn *et al.*, 2008). Especially the first and second UIMs are very important for binding of poly-ubiquitin chains, since mutations of leucine 229 and 249 resulted in almost abolished binding to ubiquitins (Burnett *et al.*, 2003).

Ataxin-3 has been found to bind the valosin-containing protein (VCP/p97) (**Figure 2**) (Wang *et al.*, 2000; Zhong and Pittman, 2006). VCP/p97 has numerous functions, of which one is the regulation of ERAD (Zhong and Pittman, 2006; Liu and Ye, 2012). A potential VCP/p97 binding domain has been mapped to an arginine/lysine-rich motif just prior to the polyQ repeat (Boeddrich *et al.*, 2006). The ataxin-3-VCP/p97 complex is involved in assisting targeted proteins to the proteasome (Wang *et al.*, 2006). Ataxin-3 is also known to interact with the human homologues of yeast protein RAD23, hHR23A and hHR23B (**Figure 2**) (Wang *et al.*, 2000). hHR23A and hHR23B are involved in DNA repair pathways as well as the delivery of ubiquitinated substrates to the proteasome for degradation (Wang *et al.*, 2000). 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 (Nicastro *et al.*, 2005). 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 (Laco *et al.*, 2012a).

Besides the clear role of ataxin-3 in protein degradation, ataxin-3 has been shown to be capable of regulating the transcriptional process. Ataxin-3 is, for instance, able to repress matrix metalloproteinase-2 (MMP-2) transcription, and improved nuclear localisation of ataxin-3 through phosphorylation enhances this transcriptional repression (Mueller *et al.*, 2009). Transcriptional regulation by ataxin-3 might arise through different mechanisms, since ataxin-3 is known to interact with numerous transcriptional regulators such as TBP-associated factor 4 (TAF4) (Shimohata *et al.*, 2000), CBP (McCampbell *et al.*, 2000; Chai *et al.*, 2002; Li *et al.*, 2002), p300 (Li *et al.*, 2002), p300/CBP-associated factor (PCAF) (Li *et al.*, 2002), nuclear receptor co-repressor (NCoR1), histone deacetylase (HDAC) 3 and 6 (Burnett, 2005; Evert *et al.*, 2006), forkhead box O (FOXO) transcription factor FOXO4 (Araujo *et al.*, 2011), and RAD23 (Wang *et al.*, 2000). Also, direct binding of ataxin-3 to DNA can likely take place through a leucine zipper motif located at amino acid 223 to 270 (**Figure 2**) (Evert *et al.*, 2006). This basic leucine zipper

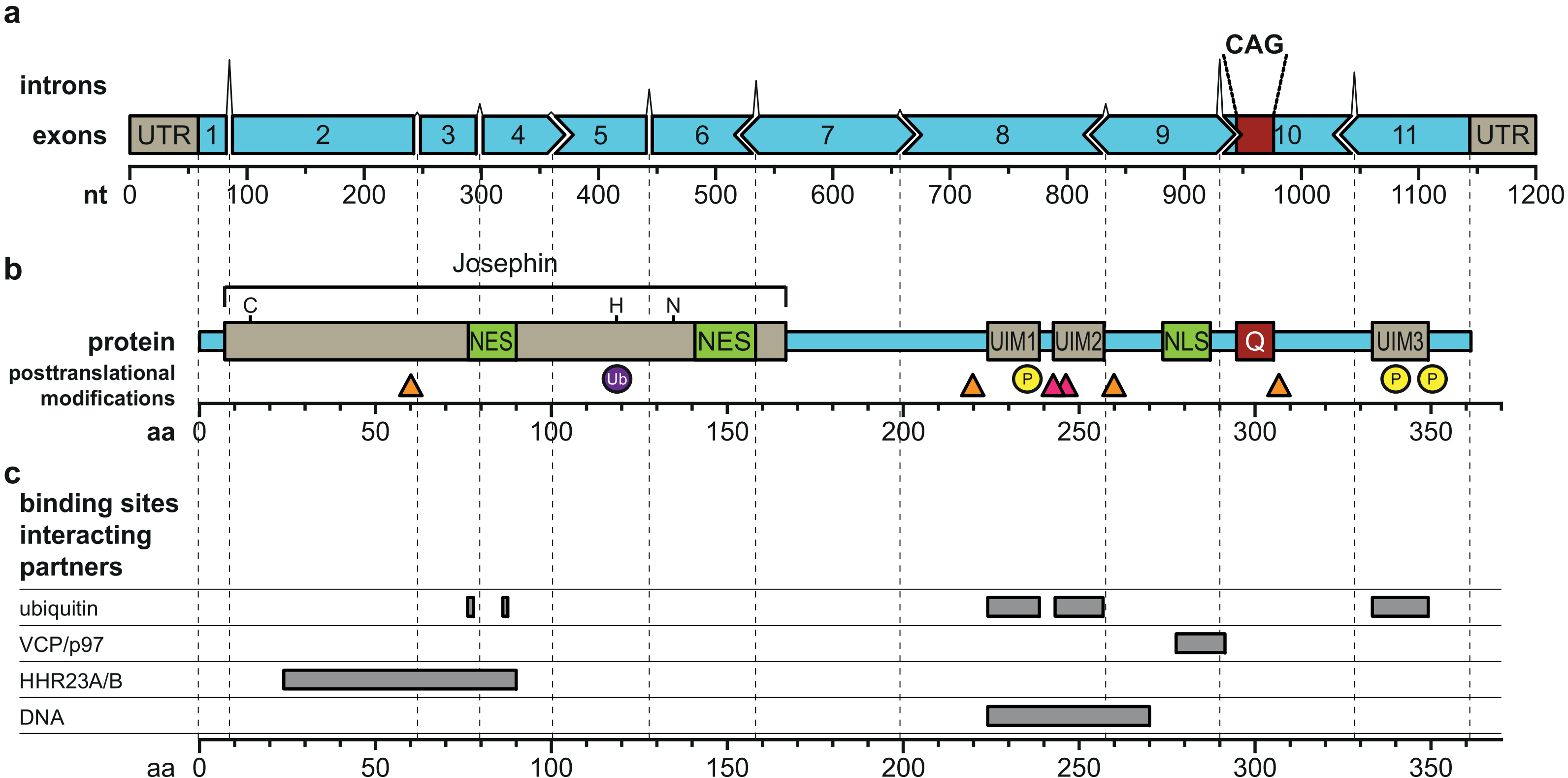

Figure 2. Schematic representation of the *ATXN3* gene, exon-intron structure and protein product showing protein functional domains, posttranslational modifications and binding domains of the main interacting partners. (a) The *ATXN3* gene (Ensembl ENST00000393287 transcript ID) consists of 11 exons 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. The height of the introns are relative to their size. **(b)** The ataxin-3 protein consists of 361 amino acids (aa) with a Josephin domain in the N-terminal part 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 (UIM 1 to 3), a nuclear localisation signal (NLS) and the polyQ repeat. Specific amino acids known to undergo posttranslational modifications are indicated as follows: Yellow circles, phosphorylation **(P)**; purple eclipse, ubiquitination (Ub); orange triangle, calpain cleavage site; pink triangle, caspase cleavage motif. **(c)** Binding domains of the main interacting partners: ubiquitin; VCP/p97, valosin-containing protein; hHR23A and hHR23B, human homologs of yeast protein RAD23; and DNA.

motif was previously shown to bind to the GAGGAA consensus sequence in DNA (Landschulz *et al.*, 1988).

In summary, ataxin-3 is a well-established deubiquitinating enzyme, directly regulating the UPS machinery. Next to the proteasomal degradation, ataxin-3 is also implicated to be involved in regulation of misfolded ER protein degradation and might also directly interact with important transcriptional regulators and components of the DNA repair pathway.

Mutant ataxin-3 gain of toxic function

In SCA3, the expanded polyQ stretch in the C-terminus of ataxin-3 most likely leads to conformational changes of the protein, in turn resulting in altered binding properties, loss of protein function, altered subcellular localisation, aggregation, and perhaps altered proteolytic cleavage (Jana and Nukina, 2004). Although in the past decade there has been extensive research into the SCA3 disease mechanisms, it is still not well understood how the ataxin-3 polyQ expansion results in the observed pathology. In brain, the *ATXN3* gene expression levels were not found to be higher in the predominantly affected brain regions, suggesting that ATXN3 gene expression levels do not directly correlate with the selective neurodegeneration seen in SCA3 patients (Schmitt *et al.*, 1997). Therefore, other alterations induced by the mutant ataxin-3 protein are most likely important in SCA3 pathogenesis as well.

One of the first observations made in SCA3 patient derived brain material were the intracellular aggregates in neurons of the ventral pons and less frequently in the substantia nigra, globus pallidus, dorsal medulla, and dentate nucleus (Paulson *et al.*, 1997b), a feature that was reproduced in cell and animal models overexpressing mutant ataxin-3 (Ikeda *et al.*, 1996; Evert *et al.*, 1999; Schmidt *et al.*, 2002). Mutant ataxin-3 is known to accumulate in the cell nucleus, a property that is required for *in vivo* toxicity (Paulson *et al.*, 1997b; Schmidt *et al.*, 1998; Bichelmeier *et al.*, 2007). In line with this, transgenic SCA3 mice show a decrease of soluble mutant ataxin-3 protein in the cerebellum during disease progression, whilst aggregate formation increases and the disease phenotype progresses (Nguyen *et al.*, 2013). The nuclear environment has been suggested to promote the formation of nuclear aggregates, and additional proteins, such as TBP and CBP, were found to be recruited to the aggregates in human brain (van Roon-Mom *et al.*, 2005) and SCA3 animal models (Perez *et al.*, 1998). Indeed, reducing nuclear localisation of mutant ataxin-3 led to a reduction in nuclear inclusions (Fei *et al.*, 2007; Mueller *et al.*, 2009). The intranuclear aggregates only arise when ataxin-3 contains the expanded polyQ tract (Paulson *et al.*, 1997b).

In SCA3, proteolytic cleavage of mutant ataxin-3 is thought to lead to generation of cytotoxic and aggregation prone shorter soluble fragments containing the expanded polyQ toxic entity (Berke *et al.*, 2004; Haacke *et al.*, 2006; Takahashi *et al.*, 2008; Koch *et al.*, 2011). In a mouse model, ataxin-3 derived cleavage fragments were shown to contain expanded polyQ-containing protein fragments C-terminal of amino acid 221 (Goti *et al.*, 2004). Interestingly, in the two SCA3 brains tested, the ataxin-3 C-terminal fragments were enriched in disease-relevant brain structures, such as the cerebellum and substantia nigra, compared to an unaffected brain region or control brain material (Goti *et al.*, 2004). In subsequent studies, several caspase and

calpain proteolytic enzymes were identified that could be responsible for the generation of the potentially toxic ataxin-3 fragments. These mutant ataxin-3 fragments are highly susceptible to aggregation (Hubener *et al.*, 2012), and capable of inducing both aggregation and toxicity to a larger extent than full length mutant ataxin-3 (Ikeda *et al.*, 1996; Teixeira-Castro *et al.*, 2011).

In the past decade there has been extensive research into the SCA3 disease mechanisms, and various cellular processes, which I will review below, were found to be altered in SCA3.

Impaired protein degradation

Though ubiquitin chain proteolytic activity does not appear to vary between wild-type and mutant ataxin-3 (Winborn *et al.*, 2008), a widespread reduction of protein deubiquitination was reported in a mutant ataxin-3 cell model (Winborn *et al.*, 2008). This potential loss of deubiquitination function in SCA3 might in part be explained by trapping of ataxin-3 and various other components of the proteasomal machinery in the large ubiquitin-rich aggregates (Paulson *et al.*, 1997b; Chai *et al.*, 1999). Mutant ataxin-3 binds the ERAD-mediated protein degradation component VCP/p97 more efficiently than wild-type ataxin-3, possibly because of conformational changes (Hirabayashi *et al.*, 2001; Zhong and Pittman, 2006; Laco *et al.*, 2012a). In spite of this more efficient binding, mutant ataxin-3 seems to interfere with the degradation of target substrates (Doss-Pepe *et al.*, 2003; Laco *et al.*, 2012a). Additionally, N-terminal ataxin-3 fragments of 259 amino acids lacking the VCP/p97 binding domain were found to result in ER stress and impaired ER mediated unfolded protein response when expressed in a mouse model, though ERAD component levels appeared unchanged (Hubener *et al.*, 2011).

Not only ER degradation is altered in SCA3 but also autophagy, in which the degradation of cellular components through the lysosomal machinery, is impaired (**Figure 3**). Aggregates of mutant ataxin-3 were found to contain molecular components involved in autophagy. For instance, beclin-1, a protein crucial in the autophagy pathway was found to be trapped in protein aggregates in SCA3 brains (Nascimento-Ferreira *et al.*, 2011). In a rat model overexpressing mutant ataxin-3, increased beclin-1 expression resulted in clearance of the mutant protein (Nascimento-Ferreira *et al.*, 2011). This observation is in accordance with impairments in autophagy seen in other neurodegenerative disorders (Wong and Cuervo, 2010), and the fact that stimulation of autophagy was found to alleviate symptoms *in vivo* (Ravikumar *et al.*, 2004).

These observations suggest that the SCA3 pathology may partly be the result of loss of function of ERAD machinery as well as compromised autophagy, together resulting in impaired protein degradation, accumulation of ubiquitinated proteins, and cellular stress.

Mitochondrial dysfunction

A cell model overexpressing mutant ataxin-3 with 78 CAGs showed reduced antioxidant enzyme levels, increased mitochondrial DNA damage, and reduced energy supply, which indicates impaired mitochondrial function (Yu *et al.*, 2009). Recently, mitochondrial DNA damage was also seen in SCA3 transgenic mice expressing full length ataxin-3 with 98 to 104 glutamines (Kazachkova *et al.*, 2013). In the disease affected pontine nuclei of these transgenic SCA3 mice less mitochondrial DNA copies were seen, as compared to the unaffected

hippocampus (Kazachkova *et al.*, 2013). Additionally, less mitochondrial DNA copy numbers were observed in the mutant cells and SCA3 patient samples, implying mitochondrial DNA damage due to oxidative stress (Yu *et al.*, 2009). In line with this, the antioxidant enzyme superoxide dismutase was found downregulated in pontine brain tissue of SCA3 patients (Araujo *et al.*, 2011), suggesting diminished antioxidant enzyme function.

Additionally, mitochondrial dysfunction was verified by the finding that the mitochondrial respiratory chain complex II activity was somewhat compromised in SCA3 (Laco *et al.*, 2012b). 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 interfere with vital cellular functions, potentially resulting in activation of apoptosis or excitotoxicity, two of the main causes of neuronal death (Emerit *et al.*, 2004).

Above described findings indicate that, like other polyQ disorders, defects in the cellular defence mechanism against oxidative stress could play a role in the pathogenesis of SCA3.

Transcriptional deregulation

Since ataxin-3 has DNA-binding properties and interacts with transcriptional regulators, transcriptional deregulation has been suggested to play a central role in the SCA3 pathogenesis (Riley and Orr, 2006). In SCA3 and other polyQ disorders, transcription factors, together with polyQ proteins, are sequestered into nuclear aggregates, resulting in deregulation of their function as transcriptional co-repressor or activator (Perez *et al.*, 1998; van Roon-Mom *et al.*, 2005). 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, suggesting transcriptional deregulation in SCA3 (Evert *et al.*, 2001; Evert *et al.*, 2003; Chou *et al.*, 2008). Likewise, some corresponding proteins like MMP-2, amyloid-β precursor protein (APP) and interleukins were found to be significantly increased in SCA3 patient brain material (Evert *et al.*, 2001). However, thus far no gene expression studies have been performed on SCA3 patient material to replicate above described findings.

Ataxin-3 was shown to inhibit histone acetylase activity. When mutated, this inhibition of histone acetylase is impaired, and increased acetylation of total histone H3 was indeed observed in mutant ataxin-3 overexpressing cells and human SCA3 brain material, resulting in an increase of transcription in SCA3 cells (Evert *et al.*, 2006). This transcriptional upregulation was supported by the discovery that in cells overexpressing mutant ataxin-3, MMP-2 was found upregulated (Evert *et al.*, 2003).

Although in SCA3 changes in gene expression have not been as extensively studied as the impaired protein degradation, the discovery of altered transcription of many genes suggests a role of transcriptional deregulation in SCA3 pathogenesis.

Loss of wild-type ataxin-3 function

Although the ataxin-3 protein has been well studied, it is still uncertain to what extent ataxin-3 is an essential protein for normal cellular functioning. In support of an essential role for ataxin-3, depletion of ataxin-3 using small-interference RNA (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 (Rodrigues *et al.*, 2010). Likewise, knock-out of ataxin-3 was found to result in lower levels of stress-induced chaperone proteins in mouse brain, proposing an significant role for ataxin-3 in cellular homeostasis (Reina *et al.*, 2012). Other evidence however suggests that ataxin-3 is not necessary for normal cellular functioning. First, ataxin-3 knock-out in *C. elegans* did not alter the lifespan (Rodrigues *et al.*, 2007), and remarkably resulted in resistance to stress (Rodrigues *et al.*, 2011). In mice, local knock-down of endogenous ataxin-3 in the striatum for 2 months did not show any toxicity (Alves *et al.*, 2010). Likewise, ataxin-3 knock-out mice, loss of ataxin-3 did not affect viability or fertility (Schmitt *et al.*, 2007; Boy *et al.*, 2009; Switonski *et al.*, 2011). However, these mice did show a mild behavioural phenotype with increased anxiety, as well as increased levels of ubiquitinated proteins, particularly in cells that are known to express high levels of ataxin-3 in wild-type mice (Schmitt *et al.*, 2007). Furthermore, ataxin-3 has also been proposed to serve as a neuroprotectant, since in flies expressing mutant polyQ proteins overexpression of ataxin-3 was found to alleviate neurodegeneration (Warrick *et al.*, 2005). In contrast, double transgenic mice, co-expressing mutant and wild-type ataxin-3, did not show any phenotypic improvement as compared to single transgenic SCA3 mice, suggesting that ataxin-3 may not act as neuroprotectant (Hubener and Riess, 2010)

Whilst absence of ataxin-3 thus does not appear to be directly detrimental to cellular vitality in most studies, the subtle phenotypes observed in rodent ataxin-3 knock-out models and the fact that ataxin-3 contains several well conserved regions amongst different species (Albrecht *et al.*, 2003) indicate that the protein may not be completely dispensable.

1.5. Clinical and molecular genetics of other polyQ disorders

Spinal and bulbar muscular atrophy

SBMA is X-linked and females typically exhibit a reduced pathology (Banno *et al.*, 2012). Next to lower motor weakness, males affected by SBMA suffer from muscle cramps, gynecomastia with abdominal obesity, and progressive loss of libido (Katsuno *et al.*, 2012). There is marked degeneration of anterior horn cells, bulbar neurons, and dorsal root ganglion cells (Orr and Zoghbi, 2007). Patients with 40 or more CAGs in the first exon of the *AR* gene will develop SBMA (La Spada *et al.*, 1991).

The AR protein is a well characterized nuclear hormone with the polyQ repeat located in its N-terminal transactivation domain. Next to this transactivation domain, the AR contains a DNA binding domain and an androgen binding domain. Binding of android to the AR in the cytoplasm results in translocation into the nucleus, where it dimerizes and subsequently stimulates transcription of androgen responsive genes. AR is essential for male foetus development, male sexual characteristics and spermatogenesis maintenance (Brinkmann, 2011). The main pathological mechanisms leading to SBMA are altered protein-protein interactions and transcriptional deregulation. Intranuclear aggregates have been found to sequester AR binding partners (Beitel *et al.*, 2013). Other known polyQ gain of function mechanisms like mitochondrial deregulation, autophagy, and impaired transport were also suggested to be involved in SBMA (Beitel *et al.*, 2013).

Spinocerebellar ataxia type 1

SCA1 is clinically characterized by dysphagia, oculomotor disturbance, pyramidal and extrapyramidal disease signs, sensory deficits as well as mild cognitive decline (Sasaki *et al.*, 1996). SCA1 is caused by a repeat expansion of 39 or more CAGs in the first coding exon (exon 8) of the *ATNX1* gene, resulting in severe cerebellar and brain stem atrophy (Orr *et al.*, 1993). In healthy individuals, when the repeat is longer than 21 CAGs, it is interrupted by one to three histidine-encoding CAT triplets. Loss of one of these CAT codons results in an uninterrupted CAG repeat which is instable upon transmission (Menon *et al.*, 2013). There is a clear inverse correlation between the pure CAG repeat number and the age of onset (Menon *et al.*, 2013). Patients with 70 or more CAG repeats will develop a juvenile form of SCA1 (Donato *et al.*, 2012).

The function of the ataxin-1 protein is largely unknown, although various domains and phosphorylation sites have been identified that are involved in protein-protein interactions, cellular localisation and stability (Chen *et al.*, 2003; La Spada and Taylor, 2010). Ataxin-1 is able to translocate from the cytoplasm to the nucleus where it can interact with various transcription factors (Orr, 2012). Mutant ataxin-1 can still translocate to the nucleus, but transport back to the cytoplasm is impaired (Irwin *et al.*, 2005).

Spinocerebellar ataxia type 2

Compared to SCA1, patients with SCA2 show slower eye movements and more pronounced hyporeflexia and tremor (Giunti *et al.*, 1998). SCA2 is characterized by olivopontocerebellar, spinal and cortical atrophy (Geschwind *et al.*, 1997a). SCA2 is caused by a CAG repeat expansion in the first exon of the *ATXN2* gene which is translated into an expanded polyQ containing ataxin-2 protein (Imbert *et al.*, 1996). The CAG repeat is interrupted by one or two CAA triplets and loss of one of the CAA triplets makes the repeat very unstable upon transmission to the next generation. SCA2 is an unique polyQ disorder as it does not show reduced penetrance. Patients with 32 or more CAGs will develop SCA2, whereas individuals with 31 do not (Magana *et al.*, 2013).

Though no reduced penetrance has been reported for SCA2, an expansion between 27 to 33 CAGs in ATXN2 has been associated with sporadic and familial amyotrophic lateral sclerosis (ALS). In these cases, an altered interaction between ataxin-2 and the ALS related TAR DNA-binding protein (TARDBP) is thought to result in cytoplasmic aggregations in neurons derived from ALS patients (Elden *et al.*, 2010). The ataxin-2 protein is thought to be involved in transcriptional regulation via its interaction with mRNA metabolism complexes (Orr, 2012).

Spinocerebellar ataxia type 6

SCA6 is a slow progressing pure cerebellar ataxia with mainly cerebellar atrophy mild peripheral neuropathy (Schols *et al.*, 1998). Patients show pronounced oculomotor disturbance and problems with the vestibulo-ocular reflex (Yabe *et al.*, 2003). SCA6 is the only non-fatal polyQ disease, probably because the brain stem is not affected (Zhuchenko *et al.*, 1997; Geschwind *et al.*, 1997b). The CAG repeat located in the 3'UTR of the *CACNA1A* gene on chromosome 19p13 was found when mapping the gene responsible for familial hemiplegic migraine (FHM) and episodic ataxia type-2 (EA-2) (Ophoff *et al.*, 1996). SCA6 is the result of an expansion of 20 till 33 CAG repeats, usually located in the 3'UTR of the *CACNA1A* gene (Riess *et al.*, 1997). However, in SCA6 alternative splicing leads to an alpha 1A subunit of the voltage-dependent P/Q type calcium channel ($Ca_v2.1$) isoform containing the toxic polyQ repeat at its C-terminus. This alternative splicing disrupts the reading frame at the 3' end of the CACNA1A transcript due to a GGCAG pentamer read-through at the intron 46 and exon 47 boundary (Tsunemi *et al.*, 2008).

The pathogenic polyQ expansion in the encoded C-terminus of $Ca_v2.1$ is by far the smallest of all polyQ disorders and within the range of normal repeats in other polyQ disease-causing proteins. $Ca_v2.1$ is highly expressed in the cerebellar cortex and is expressed in only a few neuronal specific cell types. The pathology of SCA6 is thought to be a straightforward channelopathy, although SCA6 knock-in mice, which displayed aggregation of mutant $Ca_v2.1$, did not show an electrophysiological phenotype, suggesting that the polyQ repeat itself does not affect the intrinsic electrophysiological properties of the channels (Watase *et al.*, 2008). Proteolytic processing is implicated in disease pathogenesis, because C-terminal fragments and cytoplasmic protein aggregates have been reported in SCA6 *post-mortem* brain tissue (Ishikawa *et al.*, 1999; Kubodera *et al.*, 2003).

Spinocerebellar ataxia type 7

Next to ataxia and dysarthria, the clinical presentation of SCA7 consists of oculomotor disturbance, pyramidal disease signs, and visual loss due to pigmentary retinopathy (Enevoldson *et al.*, 1994). This clinical presentation resulting from the olivopontocerebellar atrophy is highly variable and depends on the length of the CAG repeat in exon 3 of the *ATXN7* gene (David *et al.*, 1997). Individuals with 36 or more CAG repeats will certainly develop the disease around midlife and in the case of more than 100 repeats the disease manifests itself in infancy or early childhood (van de Warrenburg *et al.*, 2001). Intermediate alleles of 28 to 35 CAG repeats are meiotically unstable and were shown to result in pathological repeat lengths after paternal transmission (Stevanin *et al.*, 1998).

The ataxin-7 protein is part of various transcription regulating complexes, where it functions as transcriptional repressor via histone acetylation (Strom *et al.*, 2005). The pathogenic role of expanded ataxin-7 is still not exactly known and expanded ataxin-7 overexpression systems showed conflicting results on its histone acetylation activation (Martin, 2012). Although its gain of function mechanism is thus far unidentified, proteolytic cleavage and entrapment of truncated and full-length ataxin-7 in nuclear aggregates are part of the disease process (Holmberg *et al.*, 1998; Garden *et al.*, 2002).

Spinocerebellar ataxia type 17

SCA17 was the last identified polyQ disorder. In SCA17 patients the repeat size correlates with clinical characteristics; patients with 43 to 50 repeats display a reduced penetrance and show an HD-like phenotype (Rolfs *et al.*, 2003), patients with 50 to 60 Qs show intellectual disability, dystonia and pyramidal signs, whereas patients with over 60 Qs have early childhood onset with rapid progression (Cloud and Wilmot, 2012). The expanded CAG repeat, interspersed by CAAs, is located in exon 3 of the *TBP* gene (Nakamura *et al.*, 2001). Because the CAG repeat is interrupted by CAA codons, the expanded repeat is quite stable upon inheritance, and anticipation is rare in SCA17.

The expanded mixed repeat encodes a polyQ expansion in the transcription initiation factor TBP. TBP has the longest pure glutamine stretch in the healthy human proteome and can contain up to 42 Qs. An homozygous expanded polyQ repeat in TBP does not impair normal function during development, ruling out loss of function in SCA17 (Zuhlke *et al.*, 2003; Toyoshima *et al.*, 2004). Although transcriptional deregulation has been suggested, the exact mechanism by which expanded TBP cause neurotoxicity is not yet known (Friedman *et al.*, 2008).

Dentatorubro-pallidoluysian atrophy

DRPLA is characterized by progressive ataxia, dementia, choreoathetosis, myoclonus, and epilepsy (Naito and Oyanagi, 1982). It is caused by an expanded CAG repeat in exon 5 of the *atrophin-1* (*ATN1*) gene, resulting in degeneration of the dentatorubral and pallidoluysian systems of the central nervous system (CNS) (Naito and Oyanagi, 1982). Patients with a repeat of 49 or more will develop symptoms (Nagafuchi *et al.*, 1994). Patients with a repeat number of 65

or more will develop myoclonic epilepsy during childhood, whilst infantile onset with severe atrophy was reported in patients with more than 90 CAGs (Shimojo *et al.*, 2001).

Although the exact function of atrophin-1 is still unknown, it was suggested to act as transcriptional co-repressor (Zhang *et al.*, 2002). Mutant atrophin-1 is prone to proteolytic cleavage and N-terminal fragments accumulate in nuclei of neurons (Nucifora, Jr. *et al.*, 2001). This transcriptional deregulation is thus far the only pathogenic mechanism proposed to contribute to the neurodegeneration in DRPLA.

1.6. Protein lowering approaches for polyQ disorders

Most therapeutic strategies for polyQ diseases under investigation are targeting one of the many altered cellular processes caused by toxic mutant polyQ protein. To date, several clinical trials with small patient numbers have been carried out for polyQ disorders as symptomatic treatment to reduce depression, chorea, parkinsonian phenotype, restless leg syndrome, and sleepiness (Ogawa, 2004; Bettencourt and Lima, 2011; Scott, 2011). Clearly, there is demand for polyQ disorder therapies directed at preventing or slowing the progression of neurodegeneration. Targeting a single cellular process might be inadequate to be clinically beneficial. A more effective approach would be to reduce the expression of the mutant gene and thereby inhibiting all downstream toxic effects. Since polyQ disorders are monogenic and are the result of a gain of toxic polyQ protein, reducing the expression of the CAG repeat expansion containing gene should ideally halt the disease progression. However, for most of the polyQ disorders, the exact function and importance of the polyQ-containing protein is not fully understood, and therefore specific lowering of the mutant polyQ protein levels, leaving wild-type levels unchanged, would be favoured over a generic downregulation (Miller *et al.*, 2003; Rodriguez-Lebron and Paulson, 2006). For instance, TBP haploinsufficiency was already shown to cause cognitive deficits in mice, indicating that complete knockdown of TBP cannot be used as potential therapy for SCA17 (Rooms *et al.*, 2006).

For SCA2 and SCA17, no reports are available that use oligonucleotide-mediated modulating therapeutics as potential treatment, although if there would be therapies existing for these disorders, rodent models that nicely mimic phenotypic characteristics, are available to test these protein lowering strategies (Kelp *et al.*, 2013; Magana *et al.*, 2013). For the remaining polyQ disorders, there have been reports on protein lowering strategies, which will be shortly outlined below. I will first in short describe the basic principles of oligonucleotide-mediated therapies. Next I will focus on studies showing non-allele-specific lowering of total polyQ protein levels (**Figure 3a and b**), followed by different approaches for allele-specific lowering of mutant polyQ proteins (**Figure 3c**), than oligonucleotide-mediated modulating therapeutics targeting the common denominator of polyQ disorders, being the expanded CAG repeat (**Figure 3d**), and finally I will discuss lowering polyQ protein levels by targeting its binding partner.

Basic principles of antisense oligonucleotide-mediated therapies

Oligonucleotide-mediated therapies are widely used to manipulate the expression of specific disease-causing genes, as well as modulating splicing to rescue protein expression. Nowadays, there are many types of nucleic acid molecules that can interfere at the RNA level, such as double stranded RNAs (siRNAs, short hairpin RNAs (shRNA), and microRNAs (miRNA or miR)) (Maxwell, 2009) or single stranded RNAs (single-stranded silencing RNA (ss-siRNA)) (Yu *et al.*, 2012), that promote selective degradation of homologous cellular mRNAs through the RNA-induced silencing complex. In this thesis I will mainly focus on antisense oligonucleotides (AON), which are small pieces of modified RNA or DNA that can hybridize to RNA. They can

generate different effects depending on the AON chemistry and target site (**Figure 3 and 4**).

Initially, AONs were used to induce gene knockdown (Dias and Stein 2002). This can be achieved through RNase H, a ubiquitous enzyme that cleaves RNA:RNA or RNA:DNA hybrids. The AONs used for this application are generally modified with a phosphorothioate (PS) backbone, which increases AON stability and enhances uptake of the AON across cell membranes. AONs modified further containing DNA molecules with 2'sugar moieties at the wings (DNA gapmers) will, upon binding to target mRNA, recruit RNase H to this RNA:DNA heteroduplex, resulting in cleavage and degradation of the target by nucleases (Wu *et al.*, 2004) (**Figure 3a**).

Suppressing RNA translation into protein is also achieved by AONs targeting the translation start site by sterically blocking the binding of RNA binding protein complexes, such as ribosomal subunits (Kole *et al.*, 2012) (**Figure 3b**). Here, AONs can be modified further to render them RNase H resistant by addition of a methyl (Me) or methoxyethyl (MOE) group to the 2'O sugar ribose, which is the target cleavage site of the RNase H enzyme. The 2' sugar PS AONs targeted at pre-mRNA splicing elements can also block the access of proteins involved in the splicing machinery, causing exon skipping or inclusion (**Figure 4**).

Alternatively, nucleotides have been modified even further, e.g. using phosphorodiamidate morpholino oligomers (PMO), peptide nucleic acids (PNA) or locked nucleic acids (LNA). PMOs have been widely used for developmental studies in zebrafish embryos (Nasevicius and Ekker 2000; Nasevicius *et al.*, 2000). Multiple RNase H-dependent AONs are in clinical trials including one against high-grade glioma in phase IIb (trabedersen (Bogdahn *et al.*, 2011), and one has even been registered as a drug for cytomegalovirus induced retinitis (vitravene) (Marwick 1998).

Lowering total polyQ protein levels

For HD, various synthetic oligonucleotides with different modifications and backbones have been used in rodents to lower htt expression (Sah and Aronin, 2011). A partial reduction of 25 to 35% of both normal and mutant htt by using shRNAs was well-tolerated in wild-type rats up to 9 months without signs of toxicity or striatal degeneration (Drouet *et al.*, 2009). Non-allele-specific reduction of htt transcripts up to 75% by using shRNAs (McBride *et al.*, 2008; Boudreau *et al.*, 2009; Grondin *et al.*, 2012) and chimeric MOE PS AONs (**Figure 3b**) (Kordasiewicz *et al.*, 2012) was found to be well tolerated in HD rodents and non-human primates. Intracerebroventricular (ICV) infusion of MOE PS AONs in transgenic HD mice for two weeks targeting both the human HTT transgene and endogenous murine htt resulted in reduced toxicity, extended survival, and improved motor performance up to 3 months post treatment (Kordasiewicz *et al.*, 2012). Interestingly, the observed phenotypic improvement was comparable to the mice who were treated with exclusively the human HTT-specific AON, suggesting that the therapeutic reversal is caused by total lowering of htt protein levels (Kordasiewicz *et al.*, 2012). Since htt lowering strategies will be most beneficial for patients when administered over many years, the long-term safety needs to be assessed.

Reducing AR protein expression using siRNAs was shown to reduce truncated mutant AR-induced toxicity in mutant AR overexpressing *D. melanogaster* and human cell models of SBMA (Caplen *et al.*, 2002). However, no follow-up studies have been published since showing

an *in vivo* effect of reduced AR protein expression levels.

In a transgenic mouse model of SCA1, it also has been shown that ataxin-1 is an essential protein for cellular functioning (Burright *et al.*, 1995). Depletion of endogenous ataxin-1 resulted in learning deficits and decreased hippocampal paired-pulse facilitation. This suggests that complete knockdown of ataxin-1 is not favourable (Matilla *et al.*, 1998). The first attempt to use oligonucleotides as treatment for SCA1 was performed in a SCA1 transgenic mouse model, where injection of lentiviral shRNAs into the cerebral ventricles resulted in reduced mutant ataxin-1 protein expression, causing improved neuropathology and motor coordination (Xia *et al.*, 2004). Silencing of mutant ataxin-1 *in vivo* was also achieved with miRNAs (Keiser *et al.*, 2013). However, these shRNAs or miRNAs are uniquely attacking the transgenic human 82 CAGs-containing *ATXN1* gene, whereas the endogenous ataxin-1 is left untouched, limiting its value as a model for the human intervention where all the ataxin-1 protein will probably be affected.

As potential gene silencing treatment for SCA3, non-allele-specific downregulation of all ataxin-3 transcripts has been tested in both wild-type and SCA3 rats (Alves *et al.*, 2010). Striatal knock-down of endogenous ataxin-3 by injection of lentiviruses encoding shRNAs into the brain of wild-type rats did not show any toxicity (Alves *et al.*, 2010). Interestingly, in SCA3 rats, this non-allele-specific silencing of ataxin-3 in the striatum for a 2 month period resulted in locally reduced neuropathology (Alves *et al.*, 2010).

Lowering specifically mutant polyQ protein

Suppression of human mutant htt by 50% to 80%, for 4 months in transgenic rodent models of HD (expressing one human mutant htt and two wild-type murine htt transcripts) was found to improve motor and neuropathology abnormalities and prolonged longevity in HD mice (Harper *et al.*, 2005; Wang *et al.*, 2005). These studies showed that allele-specific lowering mutant htt without reducing wild-type htt levels, result in an improved pathology. Various studies have shown that a pronounced decrease of mutant htt levels with only minor reduction in wild-type htt is feasible using allele-specific oligonucleotides (Krol *et al.*, 2007; van Bilsen *et al.*, 2008; Hu *et al.*, 2009b; Lombardi *et al.*, 2009; Pfister *et al.*, 2009; Carroll *et al.*, 2011). One way to design a molecule that can distinguish between the wild-type and polyQ disease-causing allele is to target a single nucleotide polymorphism (SNP) that is unique to the mutant transcript (Miller *et al.*, 2003). SNPs are DNA sequences in which a single nucleotide is different between the two alleles of a gene. The first study showing allele-specific silencing in HD using SNP-specific siRNAs was obtained in human cells overexpressing an additional copy of HTT containing the targeted SNP (Schwarz *et al.*, 2006). The first proof of principle of endogenous mutant htt silencing using this approach was shown in fibroblasts derived from HD patients (van Bilsen *et al.*, 2008). Next to siRNAs, SNP-targeting RNase H-dependent AONs (**Figure 3c**) were shown to allele-specifically reduce mutant htt expression in patient-derived cells and a humanized HD mouse model (Carroll *et al.*, 2011; Ostergaard *et al.*, 2013). Subsequent genotyping revealed a group of 22 SNPs that are highly associated with mutant htt alleles in a European HD cohort (Warby *et al.*, 2009). Since then, various groups have shown that the vast majority of the HD

patient population could be treated in this way using 5 (75% of HD patients) or 7 (85% of the HD patients) different siRNAs (Lombardi *et al.*, 2009; Pfister *et al.*, 2009). The most promising SNP is located in exon 67 of the *HTT* gene. This SNP is strongly associated with the mutant allele while 48% of the total Western HD population was heterozygous at this site (Pfister *et al.*, 2009). Although the allele specificity obtained from above described SNP targeting siRNAs are very promising, there are some limitations. The diversity of SNPs within patient populations would make it necessary to develop multiple oligonucleotides. Furthermore, for HD patients that do not exhibit heterozygosity at any of the most frequent SNPs this approach is not applicable.

A cre-recombinase conditional knock-out SCA1 mouse model proved that removal of mutant ataxin-1 at an early stage of the disease results in clearance of nuclear inclusions and reversal of disease symptoms (Zu *et al.*, 2004). To date, there is only one report on allele-specific silencing of mutant ataxin-1 by targeting a heterozygous SNP. Using SNP-specific siRNAs in SCA1 patient-derived fibroblasts, only a moderate reduction of the mutant ataxin-1 transcript was achieved, whereas the normal ataxin-1 allele was also somewhat reduced (Fiszer *et al.*, 2012).

Whilst absence of ataxin-3 does not appear to be directly detrimental to cellular vitality in most studies, subtle phenotypes were observed in rodent ataxin-3 knock-out models (Schmitt *et al.*, 2007; Boy *et al.*, 2009; Switonski *et al.*, 2011). The fact that ataxin-3 contains several well conserved regions amongst different species (Albrecht *et al.*, 2003), together with its important function in protein degradation, transcription and possibly DNA repair, suggests that a strategy which reduces mutant ataxin-3 protein toxicity, whilst maintaining wild-type ataxin-3 protein levels, would be a preferable approach for therapeutic application in SCA3. Allele-specific silencing was achieved using shRNAs directed against a SNP unique to the mutant ataxin-3 transcript (Alves *et al.*, 2008). This targeted SNP at the 3′ end of the *ATXN3* gene was found to be present in over 70% of SCA3 patients (Gaspar *et al.*, 2001). The SNP-specific shRNA was able to specifically silence mutant ataxin-3 and was found to be neuroprotective in SCA3 mouse and rat models (Alves *et al.*, 2008; Nobrega *et al.*, 2013), thus showing good promise of allele-specific reduction for clinical implementation in SCA3 patients.

In the case of SCA6, complete removal of the $Ca_v2.1$ protein would probably result in cerebellar dysfunction due to Purkinje cell loss (Saito *et al.*, 2009). However, mice with one functional *CACNA1A* allele are phenotypically normal (Fletcher *et al.*, 2001). Therefore, an allele-specific, or even better, a specific reduction of the $Ca_v2.1$ isoform containing the toxic polyQ repeat at its C-terminus would be preferred. This splice isoform-specific knockdown of polyQ-containing $Ca_v2.1$ was performed *in vitro* in transiently transfected human cells using siRNAs, as well as in human neuronal cells using miRNAs targeting a specific sequence encoding the polyQ-containing C-terminus of $Ca_v2.1$ (Tsou *et al.*, 2011).

Recently, conditional knockdown of mutant ataxin-7, one month after onset of motor symptoms, resulted in reversal of aggregation and alleviated some behavioural deficits in a tamoxifen-inducible SCA7 transgenic mouse model (Furrer *et al.*, 2013). They concluded that a 50% downregulation of the mutant ataxin-7 protein expression already would show

major impact on the SCA7 phenotype. On the other hand, complete removal of the ataxin-7 homolog in *D. melanogaster* was shown to be lethal at the larval stage (Mohan *et al.*, 2014). Furthermore, knockdown of ataxin-7 in *D. melanogaster* resulted in reduced deubiquitinase activity, pronounced neurodegeneration, reduced locomotion, and decreased life span (Mohan *et al.*, 2014), suggesting that complete removal of ataxin-7 is not a proper strategy for SCA7. Thus far only one potential treatment for SCA7 showed to some extent an allele-specific reduction of mutant ataxin-7. Using shRNAs, a reduction of mutant ataxin-7 was achieved by targeting an expanded CAG repeat-linked SNP located in the last coding exon of ATXN7 (Scholefield *et al.*, 2009; Scholefield *et al.*, 2014). In a heterozygous wild-type and mutant ataxin-7 overexpressing cell model it was found that using this SNP-specific shRNA a 97% and 26% reduction of respectively mutant and wild-type ataxin-7 was achieved, which ameliorated the SCA7 phenotype (Scholefield *et al.*, 2009). However, in patient-derived fibroblasts this allele-specific reduction of mutant ataxin-7 transcript levels was less pronounced and was only found at very low doses of SNP-specific shRNAs (Scholefield *et al.*, 2014), questioning the allele-specificity of this particular small RNA, especially at higher dosage or of more realistic (less favourable) agent : target ratios.

PolyQ expansion-specific protein lowering

Another approach to achieve allele-specific silencing targets the common denominator of all polyQ disorders; their expanded CAG repeat (**Figure 3d**). Here selective silencing is either based on the hypothesis that there are structural differences between wild-type and mutant HTT mRNA, or based on the larger number of CAGs in the expanded repeat and subsequent more binding possibilities of CAG-targeting oligonucleotides. The first proof for allele discrimination by targeting the CAG repeat was achieved in HD human fibroblasts using an siRNA with 7 consecutive CUG nucleotides (Krol *et al.*, 2007). Further studies with CAG repeat targeting siRNAs showed a low selectivity for the mutant allele, making siRNAs unsuitable for CAG repeat-directed allele-specific silencing (Hu *et al.*, 2009b). Other chemical modifications and oligomers show much higher specificity for expanded CAG repeat transcripts. Single stranded PNAs, LNAs and 2OMePS AONs targeting CAG repeats (**Figure 3d**) have been used to specifically reduce expanded HTT transcripts *in vitro* in patient-derived fibroblasts (Hu *et al.*, 2009b; chapter 3). Other endogenous CAG repeat containing transcripts with important cellular functions were unaffected by the tested CUG oligonucleotides (Hu *et al.*, 2009b; chapter 3). To note, PNA selectivity was less pronounced in CAG repeat lengths (40 to 45 CAGs) that occur most frequently in the HD patient population. The allele-specific reduction with 2OMePS AONs and LNAs with 7-mer CUG repeats was more pronounced in the average HD CAG repeat length, making these 2OMePS AONs and LNAs more suitable for polyQ expansion-specific protein lowering.

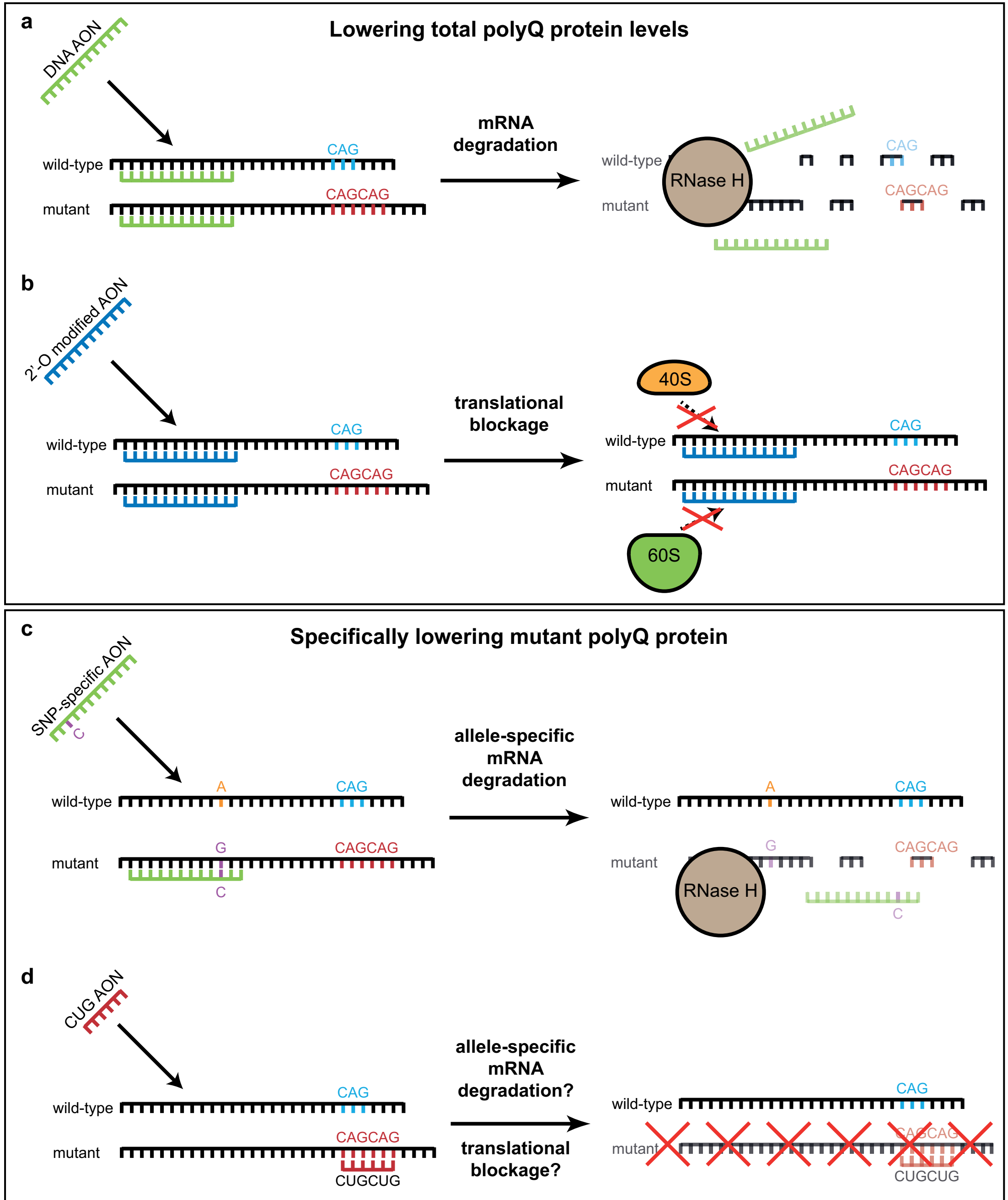

Figure 3. AON-mediated therapeutic approaches for lowering polyQ protein levels. Two different polyQ protein lowering strategies used for polyQ disorders are: 1) Lowering total polyQ protein levels by **(a)** using 2'-O-modified-phosphorothioate (PS) AONs blocking translation from both transcripts or **(b)** using (chimeric 2'-O-modified-PS) DNA AONs resulting in a RNA:DNA hybrid, which activates RNase H. RNase H will cleave the mRNA and prevents the translation into a protein. 2) Specifically lowering mutant polyQ protein by **(c)** targeting a unique heterozygous SNP linked to the mutant transcript and subsequently RNase H-induced cleavage of the mutant mRNA or **(d)** targeting the expanded CAG repeat by using CUG triplet AONs complementary to the CAG repeat, resulting in polyQ expansion-specific protein lowering.

Lowering polyQ protein levels by targeting its modulating partner

Next to targeting the (mutant) transcript directly, targeting binding partners to lower polyQ protein levels have been recently proposed as potential therapeutic intervention for various polyQ disorders. For SBMA, a possible approach to lower AR protein levels that does not directly target the AR, but targets a binding partner of AR, acts through miRNAs. Exploration of miRNA expression differences in the spinal cords of mice expressing full length wild-type AR and mutant AR resulted in the identification of upregulated miR-196a (Miyazaki *et al.*, 2012). This miR-196a regulated CUGBP Elav-like family member 2 (CELF2). CELF2 recognizes the AR exon 1-internal CUGCUGCUG sequence and by binding increases AR mRNA stability (Miyazaki *et al.*, 2012). Lentiviral miR-196a injection in the hind limb skeletal muscle of SBMA mice resulted in reduced levels of the polyQ-specific antibody 1C2-positive aggregate formation in the spinal cord and improved motor symptoms (Miyazaki *et al.*, 2012).

For SCA1, it was recently found that multiple components of the cell-signalling RAS–MAPK–MSK1 pathway influence ataxin-1 protein expression levels (Park *et al.*, 2013a). Pharmacological inhibition of this pathway was found to decrease ataxin-1 protein levels and knockout of MSK1 rescued both behavioural and pathological phenotypes in SCA1 mice (Park *et al.*, 2013a). These results suggest that components of this pathway are potential target for oligonucleotide-mediated lowering of mutant ataxin-1 protein levels.

In summary, most research has been performed on protein lowering treatments for HD and SCA1 and SCA3. Concerning the rarer polyQ disorders, less is known about the importance of the wild-type functions of polyQ proteins and the gain of toxic pathological mechanisms of the expanded polyQ proteins. For none of the polyQ disorders, much data is available elucidating the levels of mutant and wild-type transcript and protein present in the brain. Knowledge of wild-type and mutant polyQ protein levels are required to allow researchers to better assess the impact of non-allele-specific reduction of wild-type htt protein. Knowledge on these basal levels, together with better understanding of the significance of the polyQ protein for normal cellular functioning, will eventually define which protein lowering strategy to follow; an allele-specific or general reduction.

1.7. Antisense oligonucleotides in therapy for other neurodegenerative diseases

Like polyQ disorders, many other neurodegenerative diseases originate from a mutation in a single gene, resulting in a loss- or gain of one or more toxic functions, eventually initiating disease onset. There are several neurodegenerative disorders where the use of AONs is a promising therapeutic strategy. I will show some examples where AON treatment resulted in therapeutic benefit in animal models and/or clinical trials. In neurodegenerative diseases such as polyQ disorders, multiple sclerosis (MS), ALS and Alzheimer disease (AD), the aim of AON treatment can be to reduce transcript levels of disease-causing proteins. Alternatively, the deleterious allele can be reduced or knocked-out using allele-specific approaches or the mutated element can be eliminated by modulating pre-mRNA splicing events. The latter approaches are being followed for HD (chapter 4), SCA3 (chapter 5), and spinal muscular atrophy (SMA). In SMA, altering splicing can also be used to restore the expression of a gene or increase expression of a particular isoform.

Prevent translation of mutant protein in neurodegenerative diseases

ALS is a progressive neurodegenerative disorder caused by degeneration of motor neurons in the brain and spinal cord. This eventually leads to muscle weakening, twitching, and an inability to move the arms, legs, and body (Al-Chalabi and Leigh, 2000). Only 5% of ALS cases are familial and about 20% of all familial cases result from a specific genetic defect that leads to mutation of the enzyme known as superoxide dismutase 1 (SOD1) rendering the protein toxic and prone to aggregation (Bossy-Wetzel *et al.*, 2004). The AONs that have been used in ALS were designed to lower mRNA levels of the SOD1 transcripts and were PS modified chimeric nucleotides with five MOE modifications on both the 5′ and 3′ ends and 10 deoxynucleotides in the center to support RNase H activity. Continuous ventricular infusion reduced levels of mutant SOD1 in a rodent model of ALS and significantly slowed disease progression (Smith *et al.*, 2006). A phase I study to test the safety of this AON in subjects with familial ALS with a SOD1 mutation showed no serious adverse side effects after intrathecal injection into the CSF (Miller *et al.*, 2013).

MS is an autoimmune disease of the CNS where multifocal infiltration of autoreactive T lymphocytes across the blood brain barrier (BBB) takes place. Lymphocytes in MS patients display high levels of α-4 integrin on their surface (Cannella and Raine, 1995) and this plays an important role in lymphocyte migration to sites of inflammation (Rose *et al.*, 2007). Decreasing leukocyte trafficking into various organs has been successful using monoclonal antibodies against α-4 integrin (Lobb and Hemler, 1994). In a commonly used mouse model of MS, the experimental autoimmune encephalomyelitis model, AON-induced blocking of α-4 integrin expression reduced the incidence and severity of paralytic symptoms (Myers *et al.*, 2005). The 20-mer AONs with MOE modifications and a PS backbone were designed to target a sequence just 3′ of the translation start site of the murine α-4 integrin mRNA to block its translation.

Subcutaneous daily injections reduced α-4 integrin surface expression. Although the site of actions of this particular AON is unknown, it is thought that α-4 integrin levels are reduced in peripheral lymphoid tissue and this prevents trafficking of activated mononuclear cells into brain and spinal cord (Myers *et al.*, 2005).

AD is the most common form of dementia, in which AONs are considered in yet another mode. Cleavage of amyloid β precursor protein (APP) at the β-secretase and γ-secretase site causes elevated levels of β-amyloid peptide (Aβ). This is considered a key event in the pathogenesis of AD (Van Broeck *et al.*, 2007). Point mutations near the β-secretase site in the human gene for APP lead to a dominantly inherited form of AD (Selkoe and Kopan 2003). In a transgenic mouse model of AD containing this mutated β-secretase site, translation of the APP-mRNA was blocked by AONs that bind specifically to the mutated β-secretase site. The AONs used in this study had a MOE group and capped at 5′- and 3′-ends with a PS backbone. Repeated injections into the third ventricle (once a week for 4 weeks) reduced the levels of toxic Aβ and increased the levels of soluble α-cleaved APP indicating that this could be a possible strategy to treat familial AD (Chauhan and Siegel, 2007).

Modulating pre-mRNA splicing neuromuscular diseases

Other AON applications that do not induce the lowering of transcript levels are gaining more interest. The best-known application is the manipulation of splicing. Using AONs that target splice sites or exonic/intronic inclusion signals, exons can be hidden from the splicing machinery, resulting in skipping of the target exon (**Figure 4**). This can have multiple applications, e.g. switching from one isoform to another, skipping an aberrantly introduced exon to restore the normal transcript, removing disease-causing mutations from genes, or introducing an out-of-frame deletion that results in knock down expression of a gene. The latter approach is an alternative approach to AON-induced knockout through RNAse H-dependent cleavage of RNA:DNA hybrids (Aartsma-Rus *et al.*, 2009).

The most advanced clinical application of exon skipping is the exclusion of an exon allowing the production of an internally deleted, partially functional protein. This has been extensively studied as a therapeutic approach for Duchenne muscular dystrophy (DMD). Protein restoration has been shown in patient-derived cell cultures and in animal models this led to a rescued phenotype (Aartsma-Rus *et al.*, 2006; Heemskerk *et al.*, 2009; Heemskerk *et al.*, 2010). The results in phase I and I/II clinical trials were very encouraging (Lu *et al.*, 2003; Alter *et al.*, 2006; van Deutekom *et al.*, 2007; Kinali *et al.*, 2009; Goemans *et al.*, 2011). Although the primary endpoint in a recently conducted phase III clinical trial was not reached (Flanigan *et al.*, 2014), a very clear phenotypical improvement in young children was seen (press release January 16th 2014, Prosensa Holding N.V.), indicating that exon skipping is successful in DMD patients.

Intron splicing silencers can also be targeted resulting in exon inclusion. This can be used to restore expression of a gene or inducing expression of a particular isoform. Here, the most prominent application is rescue of SMA by AON mediated stimulation of the expression of a functional homologue. SMA is an autosomal recessive neuromuscular disorder caused by dysfunction and loss of motor neurons in the anterior horn of the spinal cord and lower brain

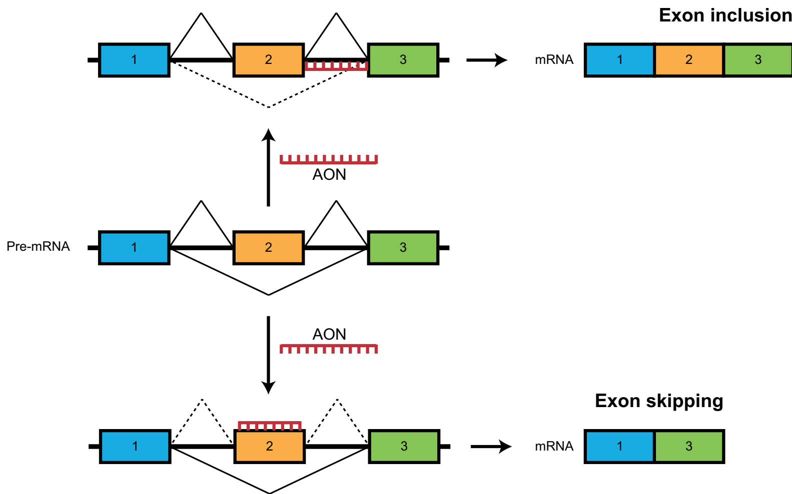

Figure 4. Schematic representation of anti-sense oligonucleotide-mediated modulation of pre-mRNA splicing. 2'OH modified RNase H-resistant or alternatively modified AONs complementary to the target pre-mRNA can result in: 1) inclusion of an exon by binding to the exonic splicing silencers (ESSs) or intronic splicing silencers (ISSs). 2) exclusion of an exon by binding to the 3' or 5' splice sites or exon-internal sequences, resulting in an in-frame transcript and translation of a shorter partly functional protein. Full lines indicate possible splicing events while dashed lines indicate non-possible events.

stem. The underlying cause of SMA is a homozygous deletion of survival motor neuron 1 (SMN1). SMN1 depletion is viable because of the presence of the almost identical *SMN2* gene. However, the majority of SMN2 mRNA transcripts lack exon 7, due to a silent mutation within this exon. This reduces the inclusion of exon 7 which results in a truncated protein and reduced expression of functional SMN protein (Lorson *et al.*, 2010). Current therapeutic strategies are aimed at modulation the splicing of SMN2 by blocking exonic splicing silencers (ESE) or intronic splicing silencers (ISS), thereby increasing exon 7 inclusion. Transfecting fibroblasts with an AON (termed ISS-N1) blocking an ISS in intron 7 of SMN2 was found to result in inclusion of SMN2 exon 7 (Singh *et al.*, 2006). Improved efficacy of the AON was achieved by incorporation of a uniform MOE chemistry and a single injection of this AONs into the cerebral ventricles in a severe mouse model of SMA showed increased exon 7 inclusion and SMN protein levels in the spinal cord resulting in increased muscle size and strength (Passini *et al.*, 2011). An increased exon 7 inclusion could also be achieved by targeting the 3' splice site region of exon 8 with 2OMe and PS backbone modified AONs (Lim and Hertel, 2001). These 2OMePS AONs were found to result in exon 7 inclusion and elevated SMN protein expression levels *in vivo* (Williams *et al.*, 2009; Hua *et al.*, 2010).

Recently, a phase I clinical trial has been completed evaluating the safety of a MOE-modified AON which aims at exon 7 inclusion and increased SMN protein levels (ClinicalTrials.gov, 2011). The so called ISIS-SMNRx was intrathecally injected in 4 increasing doses in children with SMA. In this open label safety tolerability dose-escalating study, the MOE-modified AON was well tolerated with no significant safety or tolerability findings after a single dose up to 9 mg. The intrathecal injection procedure was well tolerated and all SMA patients who participated completed the study. In the high dose treated patients, the SMN protein levels in the CSF more than doubled in the two highest dose cohorts and that those children continued to show increases in muscle function scores up to 14 months after a single injection of the MOE-

modified AON (RIGO *et al.*, 2012). Currently a phase II trial is ongoing with 6 mg or 12 mg doses of MOE-modified AON administered intrathecally on days 1, 15 and 85. The interim results reported that the MOE-modified AON was well tolerated (CLINICALTRIALS.GOV, 2012).

1.8. Drug delivery to the brain, how to cross the blood brain barrier?

One major challenge of AON-mediated therapies for neurodegenerative disorders is delivery of the AON to the brain. In this paragraph I will describe in short the BBB function and how this impairs the uptake of peripherally administered drugs. I will focus in particular on the limitations and possibilities of AON delivery to the brain and specifically neurons, and will speculate on future clinical applications.

Blood Brain Barrier

A unique feature of the brain is that it is separated from the blood by the BBB. This is a monolayer of endothelial cells forming tight junctions through the interaction of cell adhesion molecules (Palmer, 2010). Astrocytes with their processes surrounding the endothelial cells, pericytes located between the endothelial cells and astrocytes, macrophages, and the basement membrane, form the other structural components of the BBB. Endothelial cells of the BBB are characterized by only few fenestrae and pinocytic vesicles, limiting transport to and from the brain. In this respect, it should be noted that the BBB also largely separates the immune system from the brain. Despite this gate-controlling system, essential nutrients, such as glucose, are permitted to pass (Bernacki *et al.*, 2008). In neurodegenerative diseases, including HD, disruption of the BBB is common (Tomkins *et al.*, 2007; Palmer, 2010). Interestingly, in animal models, this can even lead to neurodegenerative changes itself (Tomkins *et al.*, 2007).

The BBB has been already noticed in the work of Paul Ehrlich, Nobel Prize winning bacteriologist in the late 19th century. Injected dyes stained all organs except the brain and spinal cord. However, he did not attribute this phenomenon to the presence of a barrier but to dye characteristics. His student showed later that staining of the brain was possible when the dye was injected directly into the brain (Palmer, 2010). Subsequent studies using electron microscopy allowed to directly visualize the BBB.

While essential to protect the brain, the BBB is a major challenge in CNS drug development. When a drug is administered to the body, a fraction will be bound to proteins (e.g. serum albumin, lipoprotein etc.) and a fraction will be free. The free fraction is the pharmacologically relevant fraction, since it is, in principle, available to cross the BBB (Palmer, 2010), depending on its physiochemical properties. After crossing the BBB, the drug will enter the interstitial fluid and go to the target (proteins, receptors, transporters etc.). Subsequently, the interstitial fluid drains to the CSF, which is produced at a rate of 500 ml/day in humans, while the ventricle system can house only 100-150 ml. This means that there is a continuous dehydration as well, making up for at least a threefold CSF circulation, allowing a continuous drainage of the brain's interstitial fluid.

In the process of drug discovery, the aim is to find a substance which is potent, selective and preferably bioavailable. In addition, it needs to be able to cross the BBB, and reach the target at a sufficient concentration (Alavijeh *et al.*, 2005). The following mechanisms are available

to cross the BBB. The first one is simple diffusion. Small lipophilic substances which have a hydrogen bond are more likely to pass the BBB (Gerebtzoff and Seelig, 2006). The second mechanism is via active transport mediated by transporter molecules. The most well-known is glucose with its glucose transporter 1 (GLUT1), which is the most widely expressed of the GLUT family (13 isoforms) (Guo *et al.*, 2005; Palmer, 2010). Other carriers are for instance lactate and amino acids. A well-known drug transported via this way is levodopa (Cotzias *et al.*, 1967). The third mechanism to cross the BBB is via receptor-mediation. Receptor-mediated endocytosis allows macromolecules to enter the brain, such as transferrin, insulin, leptin, and insulin-like growth factor 1 (Pardridge, 2007).

Besides systemic mechanisms to cross the BBB, there are also techniques to bypass the BBB by direct infusions into the subdural space, the brain's ventricle system, or the brain parenchyma. These infusions can be single, repeated, or continuous depending on the methodology, using either simple or sophisticated pump systems. It is possible to use one probe or more probes for infusion. Using the subdural and ventricle compartments, diffuse delivery of the drug into the brain can be achieved, while using intraparenchymal delivery, a local, but well-targeted delivery can be realized.

When a substance has successfully entered the brain, there are mechanisms preventing adequate functioning. One mechanism is active transport to remove the substance, also known as resistance. A superfamily of multidrug resistance proteins, belonging to the adenosine triphosphate (ATP)-binding cassette transporters, drives substances out by an ATP-dependent process (Palmer, 2010). One of the most abundant proteins is the P-glycoprotein. This mechanism is responsible for the failure of some anticancer drugs. Another relevant family of egress transporters is the organic anion transporting proteins.

In the field of HD, efforts are ongoing to deliver innovative drugs to the brain via the systemic route and drugs are designed to use any of the three mechanisms to cross the BBB, as explained earlier. For instance, Lee and associates described the use of a peptide nucleic acid as an antisense which was able to access endogenous transferrin transport pathways (receptor mediated endocytosis) and reach the brain in a transgenic mouse model (Lee *et al.*, 2002). However, there are also efforts to bypass the BBB, and to deliver the drug using either the ventricle system or intraparenchymally.

Cellular delivery and associated safety of oligonucleotide-mediated therapeutics

In all instances of oligonucleotide-mediated therapeutics targeting the brain, delivery is an issue. *In vivo* manipulation of gene expression with shRNA very often depends on the use of viral vectors (Di Benedetto *et al.*, 2009; Ehlert *et al.*, 2010; Kubo *et al.*, 2010), as do cre-recombinase mediated gene excision (Kolber *et al.*, 2008) or gene overexpression models (Ulusoy *et al.*, 2010; Woldbye *et al.*, 2010). However, after reaching the brain, AONs are readily taken up by neurons, and are therefore independent of viral transduction of neurons (Kordasiewicz *et al.*, 2012). The ease of delivery of the present day modified AONs seems to be linked with a lack of any major adverse side effects, making AONs suitable candidates as potential treatment for the polyQ disorders.

Associated safety of oligonucleotide-mediated therapeutics

Delivery of viral vectors has been associated with toxicity in the brain, mainly depending on viral type used. For example, adeno-associated virus (AAV) vectors have been shown to induce neurotoxicity when delivered to the CNS (Ehlert *et al.*, 2010; Jayandharan *et al.*, 2011), although serotypes may differ in that aspect (Sanchez *et al.*, 2011). Other viral types, such as retrovirus, show milder toxicity, but they are not suitable for investigation of long term effects and due to their tropism for mitotically active cells have limits in the applicability to postmitotic neurons (Kaplitt *et al.*, 1998). Lentivirus causes less inflammatory and immune response, but still share the disadvantage that pre-existing immunity to parental wild-type virus may cause an accentuated immune response. Furthermore, toxicity could also be triggered due to the lack of dosage regulation of virally-mediated shRNAs since they tend to produce an all-or-nothing effect, particularly when cre-recombinase systems are used (Kolber *et al.*, 2008; Pfeifer *et al.*, 2001).

Although AONs have a longer half-life than, for instance, siRNAs (Smith *et al.*, 2006), eventually they are degraded allowing gene expression to return to basal levels, offering the possibility to discontinue treatment (Smith *et al.*, 2006). Obviously, in instances where long-term manipulation is the goal, viral delivery may be desirable (Hua *et al.*, 2010). The lack of viral vectors makes that AON administration allows better dosage control while reducing potential toxic effects (Smith *et al.*, 2006; Heemskerk *et al.*, 2010; Hua *et al.*, 2010). For 2’-O-modified-PS AONs only very mild toxicity has been reported, which did not interfere with their desired effects after delivery in the brain via the ventricles (Liebsch *et al.*, 1999; Hua *et al.*, 2010), or in cultured neuronal cells (Muller *et al.*, 2000). Although it has been shown that PS AONs can have an immunostimulatory effect via toll-like receptors, appropriate 2’-O modifications, such as 2OMe can suppress these effects (Robbins *et al.*, 2007; Hamm *et al.*, 2010; Ma *et al.*, 2011). It is important to mention that possible toxic and immunostimulatory effects of 2OMePS AONs may also be a function of dosage, concentration, or duration of treatment (Hua *et al.*, 2010).

Cellular delivery of AONs

Single stranded AONs have a very rapid uptake within minutes to hours (Pitts *et al.*, 2009; Ma *et al.*, 2011). A typical AON used to modulate splicing is negatively charged and has a PS backbone. There are diverse chemical modifications to strengthen binding to the target mRNA and to improve pharmacokinetics by reducing nuclease-induced degradation. Most chemistries have modifications of the 2’O sugar ribose (such as 2OMePS and MOE), which is the target cleavage site of the RNase H enzyme, and thus have increased resistance to degradation. Other oligonucleotide chemistries, such as PNAs, LNAs, PMOs, are even more resistant to nuclease degradation.

Conjugating arginine-rich peptides to 2OMePS and PMOs (PPMO) have been shown to improve cellular uptake (Moulton *et al.*, 2009; Jirka *et al.*, 2014). This is not required for neurodegenerative disorders when locally administered, since 2OMePS and MOE AONs are readily taken up by neurons and translocate to the nucleus where splicing events take place

(Kordasiewicz *et al.*, 2012; Zalachoras *et al.*, 2013). Furthermore, ICV injected PMOs resulted in increased SMN protein expression in the spinal cord and total brain of severe SMA mice (Mitrpant *et al.*, 2013). Nevertheless, these "naked" AONs do not cross the BBB and therefore need to be administered into the CSF.

Interestingly, a PPMO against ataxia-telangiecatasia causing out-of-frame splicing mutations did cross the BBB after intravenous injections (Du *et al.*, 2011). The PPMOs were widely distributed throughout the brain of wild-type mice (Du *et al.*, 2011). Although remarkable, thus far no follow-up studies have been published showing an *in vivo* restoration of normal splicing and protein production in ataxia-telangiecatasia mice. Unfortunately, two PPMOs are abandoned as therapeutic agent since repetitive intravenous bolus injections of PPMOs caused lethargy and weight loss in rats (Amantana *et al.*, 2007) and tubular degeneration in the kidneys of monkeys (Moulton and Moulton, 2010).

To conclude, most *in vivo* data on splicing modulation or protein reduction in brain disorders make use of MOE PS and as described in chapter 3 to 5, 2OMePS AONs. While AONs for use in the CNS cannot be administered systemically, they have excellent entry into cells once they passed the BBB. For several chemistries, it has been shown that local injection and distribution via the CSF seem to be devoid of any major toxicity, making these oligonucleotide chemistries suitable candidates as potential treatment for the polyQ disorders.

1.9. Scope and outline of the thesis

Although polyQ disorders are caused by CAG triplet repeat expansions in different genes, they all result in gain of toxic polyQ protein function and subsequently neurodegeneration. The polyQ disorders have a monogenic cause and thus far no therapies are available to delay the age of onset or slow the disease progression. Because of the well-defined nature of the primary mutation and its direct consequence on toxic polyQ protein function, reducing the expression of the CAG repeat expansion-containing gene should in principle contribute significantly to halting the disease progression. Therefore, much effort has been put in reducing the expression of the mutant gene and thereby inhibiting all downstream toxic polyQ effects. Preclinical results during the course of this PhD research using oligonucleotide-mediated therapies for polyQ disorders, particularly HD, look promising. Despite the fact that for several neurodegenerative disorders oligonucleotide-mediated therapies moved from preclinical to clinical testing, for polyQ disorders we are not there yet.

While much research has been done on the underlying polyQ disease mechanisms, knowledge on mRNA and protein regulation and expression levels are limited. In **Chapter 2**, htt expression levels in adult-onset HD and juvenile HD patient-derived fibroblasts and *post-mortem* brain are studied. Subtle differences in htt mRNA and protein expression between adult-onset and juvenile HD are described.

By targeting the polyQ-encoding transcripts, translation of mutant polyQ protein is reduced, inhibiting all downstream toxic polyQ effects. **Chapter 3** describes the reduction of polyQ disease-causing proteins by specifically targeting expanded CAG repeat transcripts. By targeting the common denominator of all polyQ disorders, using CUG triplet-repeat AONs, mutant polyQ transcript and protein levels in several polyQ disorders were found to be reduced.

Chapter 4 describes a novel therapeutic approach for HD through removal of cleavage motifs that are implicated in the formation of toxic htt polyQ fragments. In HD, expanded polyQ htt is known to undergo proteolytic processing, which results in toxic polyQ-containing htt protein fragments. Preventing the formation of these toxic polyQ htt fragments is achieved by AONs that induce exon skipping of HTT pre-mRNA. Thus by modifying the htt protein, cleavage motifs are removed and less toxic polyQ htt fragments are formed.

Chapter 5 describes the removal of the CAG repeat-encoding exon from SCA3-causing mutant ATXN3 pre-mRNA. This AON-mediated skipping results in the removal of the toxic polyQ repeat from the ataxin-3 protein. A modified ataxin-3 protein is formed that lacks the toxic polyQ repeat, but maintains important wild-type functions of the protein.

Chapter 6 provides a general discussion of the thesis, reviewing the main findings, followed by recent developments and its implications for the genetic therapies proposed in this thesis and finally discussing future perspective.