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Ameliorating Huntington's Disease by Targeting Huntingtin mRNA

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1. Introduction

To date there are 9 known neurological diseases caused by an expanded polyglutamine (polyQ) repeat, with the most prevalent being Huntington's Disease (HD) (Cummings & Zoghbi, 2000). HD is a progressive autosomal dominant disorder. It is caused by a CAG repeat expansion in the *HTT* gene, which results in an expansion of a polyQ stretch at the N-terminal end of the huntingtin (htt) protein. This polyQ expansion plays a central role in the disease and results in the accumulation of cytoplasmic and nuclear aggregates. In this chapter we will discuss wild-type htt function and the gain of toxic function of mutant htt in HD. Currently no treatment is available to delay onset or slow disease progression. However, recently developed RNA modulating therapies have great potential to lower mutant htt levels in HD. Already promising results in animal and human studies for other neurodegenerative disorders have been obtained, from which HD research can learn.

2. Huntington's 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 & 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 with the GABAergic medium spiny neurons of the striatum as its first prominent victim, and to a lesser extent neurons in the cerebral cortex (Levesque et al., 2003). Severe cell loss in the striatal complex, the caudate nucleus and putamen results in striatal atrophy. This is accompanied by an enlargement of the lateral ventricles. The medium spiny projection neurons, containing enkephalin, are more susceptive to degeneration than substance P containing neurons while interneurons seem to be spared (Walker, 2007). With disease

progression, degeneration expands throughout the HD brain and other structures become affected (Vonsattel et al., 1985). 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 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. 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 other 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 behavior while apathy and psychosis can appear in advanced stages. However, the most frequent psychiatric symptom is depression (Craufurd et al., 2001). Like psychiatric symptoms, cognitive symptoms can be present prior to the onset of the motor symptoms. The cognitive symptoms comprise mainly impairment in executive functions, including abstract thinking, problem solving, and attention (Snowden et al., 2002). Furthermore, the ability to learn new skills is affected (Paulsen et al., 2001). Altogether these symptoms substantially 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.

The disease is caused by a CAG trinucleotide repeat expansion within the coding region 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 36 to 39 repeats, lead to an incomplete and variable penetrance of the disease or to a very late onset (McNeil et al., 1997). Repeat numbers exceeding 55-60 result in clinical manifestation of the disease before the age of 20, known as Juvenile Huntington's Disease (JHD) (Andresen et al., 2007) and both sexes are affected with the same frequency (Walker, 2007). Intergenerational CAG changes are extremely rare on normal chromosomes but on expanded chromosomes changes in CAG 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). However, no correlation with repeat size is apparent for the progression

and duration of the disease. Furthermore, neuropathological changes, such as atrophy and inclusion load 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.

2.1 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).

Wild-type htt is mainly localized in the cytoplasm, although a small proportion is present in the nucleus (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, 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; Strehlow et al., 2007; Rockabrand et al., 2007; Atwal 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 first 17 amino acids of htt have also been suggested to act as nuclear export signal (NES) by interaction with the nuclear pore protein translocated promoter region (Tpr) that then transports N-terminal htt fragments out of the nucleus (Cornett et al., 2005). The polyQ repeat starts at the 18th amino acid and is thought to form a polar zipper structure, which has been implicated in the interaction between different polyQ-containing transcription factors (Perutz et al., 1994; Harjes & Wanker, 2003). The polyQ stretch is followed by a 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 identified which are known to form superhelical structures and are involved in protein-protein interactions (Takano & 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, which are recognized by various proteases, such as caspases 1, 3, 6, 7 and 8 and calpain (Gafni & Ellerby, 2002; Wellington et al., 2002; Kim et al., 2006). In contrast to mutant htt, the significance of wild-type htt cleavage is not completely clear.

2.2 Mutant htt gain of toxic function in HD

Expanded polyQ proteins are known to undergo conformational changes, which result in the hallmark of polyQ disorders, protein aggregates. The aggregates can already be found before the onset of the first symptoms (Weiss et al., 2008). Remarkably, 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). The rate of aggregate formation is correlated to the length of the polyQ repeat (Legleiter et al., 2010). Whether accumulation of these aggregates is neurotoxic or neuroprotective is still under debate since evidence also suggests that soluble mutant htt is the main toxic component (Davies et al., 1997; Saudou et al., 1998; Arrasate et al., 2004). While the expanded polyQ repeat displays pathogenic properties it is probably not essential for normal function (Clabough & Zeitlin, 2006). Mutant htt is more disposed to proteolysis and it was shown that small N-terminal htt fragments are more toxic than full length mutant htt (Cooper et al., 1998). Proteolytic cleavage of mutant htt results in nuclear localization of toxic N-terminal mutant htt fragments. These N-terminal mutant htt fragments are important in the pathological process. Mutant htt fragments within the striatum of HD brains clearly differ from those of control brains, suggesting cleavage is disease specific (Mende-Mueller et al., 2001) and htt caspase-6 resistant HD mice did not show neuronal dysfunction (Graham et al., 2006).

Various transcription factors have been found to co-localize with htt aggregates, such as TATA box binding protein (TBP), CREB 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 HD pathology (Nucifora, Jr. et al., 2001)

Mutant htt is also suggested to act as pro-apoptotic factor triggering cell death. Htt is found to bind to the pro-apoptotic factor p53. Interestingly, p53 deficient HD mice displayed increased striatal inclusion body formation (Ryan et al., 2006). Expression of mutant htt in p53 deficient mice improved the lifespan probably by increased apoptosis initiated by mutant htt (Ryan & Scrable, 2008).

In HD the fusion machinery and axonal transport are impaired. Accumulated N-terminal fragments block the axonal machinery, resulting in transport defects (Gunawardena et al., 2003). Endocytosis is thought to be impaired since the synaptic vesicle protein PACSIN1 has an altered subcellular location in early stage HD patients (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).

N-terminal mutant htt fragments are 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 is increasing with age and correlates with disease progression. This impaired mitochondrial trafficking by N-terminal mutant htt could lead to decreased ATP supply in nerve terminals (Orr et al., 2008). Mutant htt is also suggested to be involved mitochondrial energy metabolism defects. Metabolic energy defects could be the result of mutant htt's capability to induce mitochondrial permeability transition pore opening. This leads to low mitochondrial membrane potential and high glutamate transmission, resulting in overactive glutamate NMDA receptors (excitotoxicity) (Choo et al., 2004). Abnormal mitochondrial respiratory chain function leads to reduced ATP levels and subsequent partially depolarized membrane. This voltage change leads to chronic calcium influx and activation of proteases, causing more reactive oxygen species (ROS) production. Further, increased ROS production gives rise to oxidative stress and could contribute to the vicious circle (Browne & Beal, 2006).

2.3 Loss of wild-type function in HD

As described above, the main cause of HD is a gain of toxic mutant htt function. Since various functions and post-translational modifications of htt are altered in HD, loss of wild-type htt function could also be involved. Htt expression is important for normal cellular function since knock-out of the homologous htt mouse gene was found to be early embryonic lethal (Zeitlin et al., 1995). Previous studies have shown that approximately 50% of htt protein level is required to maintain cell functionality (Dragatsis et al., 2000). Next to embryonic development, htt is also involved in regulation of apoptosis, transcription, intracellular transport and BDNF transcription (Zuccato et al., 2001; Imarisio et al., 2008).

Wild-type htt is reported to act as protector of the brain cells from apoptotic stimuli (Rigamonti et al., 2000). Reduced wild-type htt expression in transgenic HD mice resulted in worsening of the behavioural deficits and survival. In addition, no severe striatal abnormalities were visible in those HD mice, which could mean that the striatal phenotype is mainly caused by mutant htt toxicity (Zhang et al., 2003). Furthermore, overexpression of wild-type htt protected these mice against neurodegeneration. Removal of endogenous htt in a *Drosophila melanogaster (D. melanogaster)* HD model was found to exacerbate the neurodegenerative phenotype associated, suggesting that loss of normal htt function might also contribute to HD pathogenesis (Zhang et al., 2009).

3. HTT RNA

Although the main toxic component is the htt protein, recent evidence suggests that also HTT RNA could have toxic properties. There is also recent evidence for antisense transcription through the *HTT* locus. In this paragraph we will review the importance of these findings.

3.1 Htt RNA gain of function in HD

Trinucleotide expansion disorders occur either in untranslated genomic regions (UTRs) resulting in a toxic RNA gain of function or loss of gene function, or in coding regions resulting in a gain of toxic protein function (Orr & Zoghbi, 2007). Until recently, it was believed that HD is solely caused by a toxic gain of function of the polyQ protein and to a lesser extent, loss of wild-type function. However, recent evidence suggests that the mutant CAG repeats of the HTT RNA transcript could also have toxic properties (Fig. 1). This RNA toxicity is caused by the long hairpin structures of the expanded RNA that result in abnormal interactions with double stranded RNA-binding proteins.

The CAG repeat hairpin in the HTT transcript was found to be stabilized by the flanking (CCG)n repeat (de Mezer et al., 2011). The resulting double stranded CAG RNA hairpin formed intranuclear foci that co-localized with the muscleblind-like 1 (MBNL1) splicing factor (Jiang et al., 2004). Altered MBNL1 function is implicated in RNA toxicity of CUG repeat expansion disorders such as myotonic dystrophy type 1 (DM1) (Kanadia et al., 2003). DM1 is caused by a CTG repeat expansion at the 3' UTR of the *DMPK* gene. The CTG repeats are known to form stable hairpin structures that are toxic by causing abnormal alternative splicing by MBNL1 binding and sequestering in nuclear foci (Fardaei et al., 2001; Kanadia et al., 2003). Similar to expanded CUG repeats in DM1, synthesized expanded CAG repeats also resulted in abnormal alternative splicing in both transiently transfected and

patient-derived cells (Mykowska et al., 2011). The RNA toxicity modifier MBNL1 was also found to be involved in another polyQ disease, namely spinocerebellar ataxia 3 (SCA3). MBNL1 was found to be up-regulated in a *D. melanogaster* model of SCA3. The neurodegenerative disorder SCA3 is caused by a CAG repeat expansion in the *ATXN3* gene, which results in the expression of a polyQ containing ataxin-3 protein. Upregulation of the *D. melanogaster* homolog of MBNL1 (*mbl*) was found to enhance pathogenic ataxin-3 protein induced toxicity, as well as pathogenic mutant htt protein induced toxicity (Li et al., 2008).

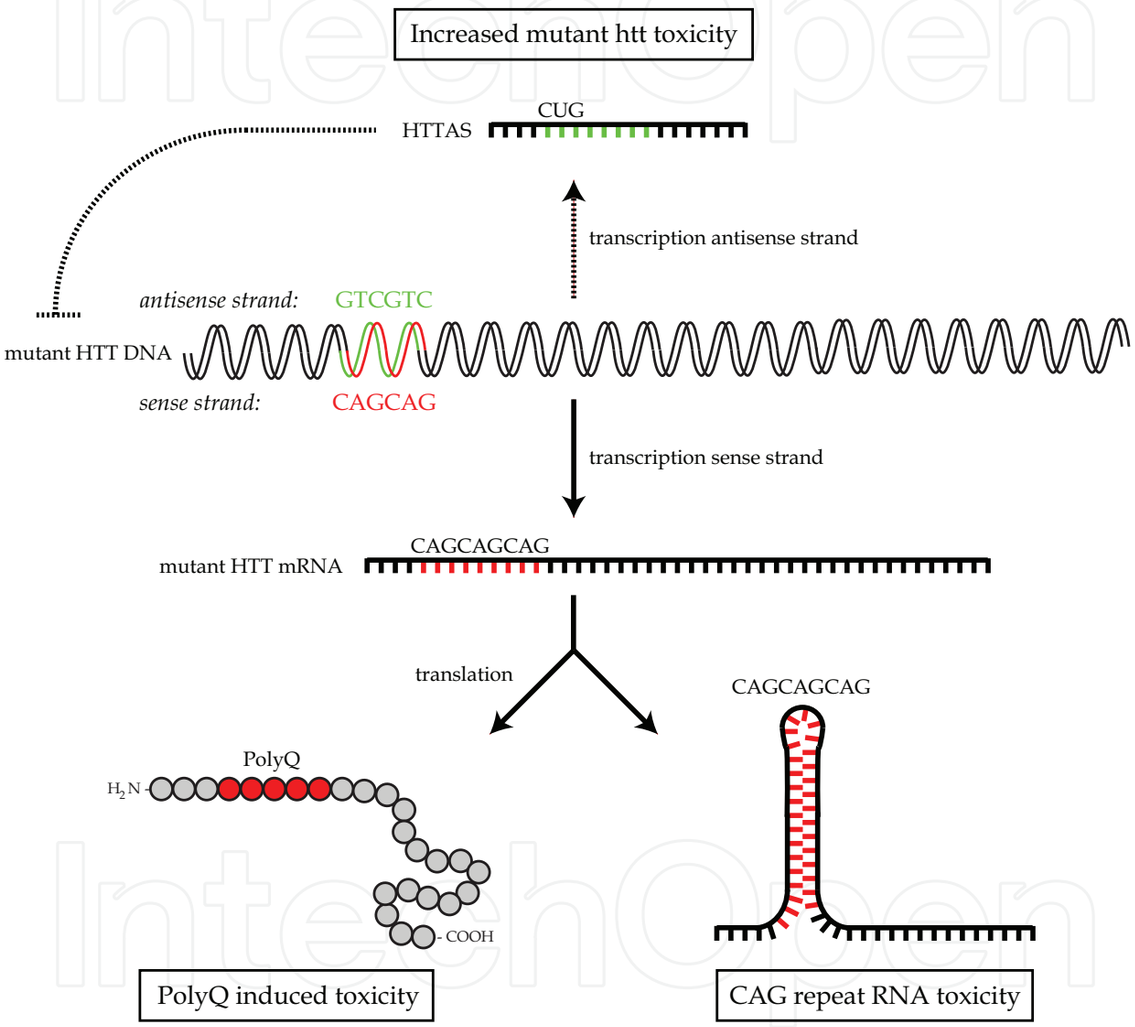

Fig. 1. Schematic representation of modes of huntingtin toxicity in HD. Mutant htt transcription results in mutant HTT mRNA which can form double stranded hairpins through the expanded CAG repeat and adjacent CCG repeat. The CAG hairpin is involved in MBNL1 induced alternative splicing and toxicity. The main pathological process in HD involves the translation of expanded CAG repeat-containing mRNA into a toxic polyQ protein. Antisense transcription through the *HTT* gene results in a HTT antisense transcript (HTTAS). This HTTAS regulates HTT sense levels. In HD there is lower expression of HTTAS, resulting in increased levels of the HTT sense transcript and increased mutant htt toxicity.

HD and SCA3 transgenes with a CAG repeat interrupted by CAA codons (expressing an identical polyQ protein as compared to a pure CAG repeat) showed only a mild phenotype, indicating the importance of the expanded pure CAG repeat for the toxic phenotype. Interestingly, both full CAG repeats and CAA interrupted CAG repeats showed similar levels of protein inclusions, indicating that the phenotype severity does not correlate with the number of inclusions (Li et al., 2008).

Recently, transgenic mice expressing a GFP construct with 200 CAGs in the 3′ UTR resulted in reduced GFP levels as compared to animals with 23 CAG repeats in their 3′ UTR of the GFP construct (Hsu et al., 2011). Furthermore, these CAG_{200} mice showed nuclear RNA foci and a reduced breeding efficiency, which supports the gain of RNA toxicity hypothesis.

Transgenic *Caenorhabditis elegans* (*C. elegans)* expressing various CAG repeat lengths in the 3′ UTR of a GFP gene showed a length-dependent toxicity. Worms with an 83 CAG repeat did not show any phenotype, whereas *C. elegans* expressing 200 CAGs died within a few days. Both 125 CUGs and 125 CAGs co-localized in nuclear foci with *C. elegans* MBNL1 homolog CeMBL and overexpression of CeMBL partly reversed the CAG 125 induced phenotype (Wang et al., 2011).

In contrast to the above studies, there is also evidence that the CAG repeat RNA is not toxic. Expression of a cDNA construct with 79 CAG repeats in the 3′ UTR did not induce cell death, whereas a construct expressing 79 CAGs in the coding region did induce cell death (Ikeda et al., 1996)**.** This was also found in two other polyQ disorders, spinocerebellar ataxia 1 (SCA1) and spinobulbar muscular atrophy (SBMA). A SCA1 mouse model with impaired nuclear localization signal in ataxin-1 did not show nuclear inclusion bodies and did not display the disease phenotype (Klement et al., 1998). Furthermore, impairing nuclear localization of the androgen receptor (AR) in SBMA by castration showed marked improvements of disease pathology, also suggesting that the pathology is mainly caused by gain of toxic protein and not RNA (Katsuno et al., 2002). A *D. melanogaster* model of CAG toxicity expressing a repeat construct with a premature termination codon before a 93 CAG repeat, did not show any phenotype (McLeod et al., 2005). Based on these results, it was suggested that the toxicity in CAG triplet repeat disorders was exclusively the result of expanded polyQ protein gain of function.

From the above we can conclude that not only gain of toxicity by expanded polyQ protein, but also RNA toxicity from the expanded CAG repeat could be involved in HD pathology. However, the size of the CAG repeat is critical for RNA pathogenicity.

3.2 HTT antisense transcription

A large proportion of the genome can produce transcripts from both strands (Katayama et al., 2005). It has become clear that antisense transcripts are involved in triplet repeat disorders and bidirectional transcription has thus far been identified in DM1, spinocerebellar ataxia 8 (SCA8), and HD like 2 (HDL2) (Moseley et al., 2006; Wilburn et al., 2011).

In SCA8, which is caused by a CTG repeat expansion in a transcribed but not translated *ATXN8OS* gene, it was thought that the expanded CTG repeat caused RNA toxicity (Koob et al., 1999). Unexpectedly, bacterial artificial chromosome (BAC) transgenic SCA8 mice showed 1C2 positive inclusion bodies. The 1C2 antibody specifically recognizes expanded

polyQ tracts, which are the hallmark of polyQ disorders. A novel transcript called ataxin-8, which encodes a polyQ protein, was expressed from the opposite strand, suggesting polyQ induced toxicity (Moseley et al., 2006). A BAC HDL2 mouse model with a pathogenic CTG repeat on the sense and expanded CAG repeat on the antisense strand at the *Junctophilin-3* locus showed both RNA toxicity caused by its expanded CUG repeat as well as protein toxicity by its polyQ translated expanded CAG repeat (Wilburn et al., 2011). These findings suggest that triplet repeat disorders can involve toxic gain of function of both protein and RNA by bidirectional transcription.

Recently, two natural HTT antisense (HTTAS) transcripts were identified at the HD locus (Chung et al., 2011). HTTAS was found to be 5′ capped, poly A-tailed and contained 3 exons. There were two different isoforms identified of which one enclosed a functional promotor and the CTG repeat. The HTTAS containing the short CTG repeat was found to be widely expressed in multiple tissues. Remarkably, expanded CTG repeat containing HTTAS was strongly reduced in HD brains. The authors state that HTTAS acts as a negative regulator for HTT transcript expression as knock-down of HTTAS resulted in higher htt levels and overexpression of HTTAS resulted in lower HTT levels (Chung et al., 2011). This negative regulating property on HTT of HTTAS could potentially have a clinical implication by overexpressing HTTAS in HD patients, thereby alleviating pathogenicity by lowering htt levels.

4. RNA modulating therapies in HD

Although the *HTT* gene was identified in 1993, there are no treatments to cure or even slow down the progression of the disease. Most therapeutic strategies under investigation are targeting one of the many altered cellular processes caused by toxic mutant htt. Targeting a single cellular process might be inadequate to be clinically beneficial.

A more effective approach would be to reduce the expression of the causative *HTT* gene and thereby inhibiting all downstream toxic effects. Recent advances to inhibit the formation of mutant polyQ proteins using RNA modulating therapies, such as RNA interference (RNAi) and antisense oligonucleotides (AONs) look promising for HD (Sah & Aronin, 2011). RNAi is an endogenous cellular process involved in transcriptional regulation and acts as cellular defense mechanism against exogenous viral components. RNAi by introducing small interfering RNA (siRNA), short hairpin RNA (shRNA), or artificial micro RNA (miRNA), is increasingly used as a potential therapeutic tool to reduce expression of target transcripts. Specific knock-down is also achieved by introducing modified single stranded AONs that can hybridize to the target RNA, which is subsequently degraded or its translation blocked.

The most frequently used htt RNA modulating strategies for HD are: Knock-down of total htt RNA levels by targeting both wild-type and mutant htt and allele-specific reduction of mutant htt RNA only (Fig. 2).

4.1 Gene therapy to lower both htt alleles in HD

Since htt has many important wild-type functions, one of the key questions that needs to be answered for htt lowering strategies to become successful is how much htt is needed for normal function, or rather, how much can htt levels be reduced before adverse effects

become apparent. Below we will first describe the studies describing lowering of both wild type and mutant htt, followed by the different approaches for allele specifically lowering mutant htt only.

Various synthetic oligonucleotides with different modifications and backbones have been used in rodents to partially lower htt expression. A partial reduction of both normal and mutant htt by 25 to 35% using shRNAs was found to be well-tolerated in wild-type rats up to 9 months without signs of toxicity or striatal degeneration (Drouet et al., 2009). Total silencing using artificial miRNAs for both wild-type and mutant htt of 75% within the striatum of a transgenic HD mouse model showed reduced toxicity, extended survival, and improved motor performance, 3 months after treatment (Boudreau et al., 2009).

Striatal injection of non allele-specific artificial miRNA in wild-type mice resulted in 70% reduction of htt levels. The high murine htt transcript reduction was sustained without adverse side effects up to the end of their study, which was set at 4 months (McBride et al., 2008). Since htt lowering strategies will be most beneficial for patients when administered over many years, the long-term safety needs to be assessed. Therefore, simultaneously lowering transcript levels from both alleles can only be applied once the role of wild-type htt in the human brain is elucidated in more detail. Moreover, to date it is not known if there is equal transcription from both the mutant and wild-type htt allele. Lowering total htt transcript levels by 70% does not necessarily mean an equal reduction of both alleles by 70%.

4.2 Allele-specific reduction of mutant htt in HD

As described in previous paragraphs, endogenous htt expression is important for normal cellular function and an ideal strategy for an autosomal dominant disorder as HD would be to specifically target the mutant allele and thereby maintaining as much wild-type htt protein as possible. Suppression of 50% to 80% using siRNA specific for human mutant htt in transgenic rodent models of HD for 4 months was found to improve motor and neuropathological abnormalities and prolonged longevity in HD mice (Harper et al., 2005; Wang et al., 2005). These studies showed that lowering mutant htt without reducing wild-type htt levels, resulted in an improved pathology. These results favored an allele-specific htt lowering approach without altering the expression of endogenous wild-type htt expression. Various studies have shown that a pronounced decrease of mutant htt levels, with only minor reductions in wild-type htt is feasible using allele-specific oligonucleotides. The different approaches, their advantages and disadvantages will be discussed in the following paragraph.

4.2.1 Targeting associated SNPs in HD

Single nucleotide polymorphisms (SNPs) are DNA sequence variations that occur when a single nucleotide is different between the two alleles of a gene. One way to distinguish between the wild-type and polyQ disease-causing allele is to target such a SNP that is unique to the mutant transcript using siRNAs (Miller et al., 2003). siRNAs are known to discriminate between transcripts that differ at a single nucleotide and various studies have shown specific reduction of mutant htt mRNA using siRNAs directed against different SNPs. The first evidence of allele-specific silencing in HD using using SNP specific RNAi was obtained in human cells overexpressing htt transgenes (Schwarz et al., 2006). The first prove of principle of

endogenous mutant htt silencing using SNPs in fibroblasts derived from HD patients was acquired in 2008 (van Bilsen et al., 2008). Extensive genotyping revealed a group of 22 SNPs 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 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 was found to be located in exon 67 of the *HTT* gene. This SNP is strongly associated with the mutant allele and 48% of the total Western HD population was heterozygous at this site (Pfister et al., 2009).

Most of the heterozygous SNPs linked to the expanded CAG repeat in exon 1, are found remote downstream from the CAG repeat in exons 25 up to 67 (Lombardi et al., 2009; Pfister et al., 2009). To determine in HD patients whether they are heterozygous and if yes, which SNP belongs to the expanded CAG repeat, a technique called SNP linkage by circularization (SLiC) was developed (Liu et al., 2008). By circulating the DNA, the CAG repeat and SNP site were brought together, making it easy to link the SNP to the expanded CAG repeat using a single PCR.

Although the selectivity 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 siRNAs. Furthermore, for HD patients that do not exhibit any of the most frequent SNPs a different treatment needs to be developed.

4.2.2 Targeting the expanded CAG repeat in mutant HTT

Another approach to achieve allele-specific silencing is based on the common denominator of all HD patients; their expanded CAG repeat. The 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. The first prove for allele discrimination by targeting the CAG repeat was achieved in HD human fibroblasts using a 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 incompatible for CAG repeat directed allele-specific silencing (Hu et al., 2009). Other chemical modifications and oligomers show much higher specificity for expanded CAG repeat transcripts. Single stranded peptide nucleic acids (PNA), locked nucleic acids (LNA), and AONs with a 2'O methyl addition and phosphorothioate backbone targeting CAG repeats have been used to specifically reduce expanded HD transcripts *in vitro* in patient derived skin and blood cells (Hu et al., 2009; Evers et al., 2011). However, 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 after transfection of patient cells with LNAs and AONs with 7-mer CUG repeats was more pronounced in the average HD CAG repeat length. Furthermore, other endogenous CAG repeat containing transcripts with important cellular functions were unaffected by the tested CUG oligonucleotides (Hu et al., 2009; Evers et al., 2011).

The main advantages of LNAs and AONs are that they are single stranded and do not show toxicity *in vivo*. Systemic delivery of modified AONs in Duchenne muscular dystrophy (DMD) boys carrying specific deletions in the DMD gene induced the synthesis of novel, internally deleted, but likely (semi-) functional, dystrophin proteins without clinically apparent adverse event (Goemans et al., 2011).

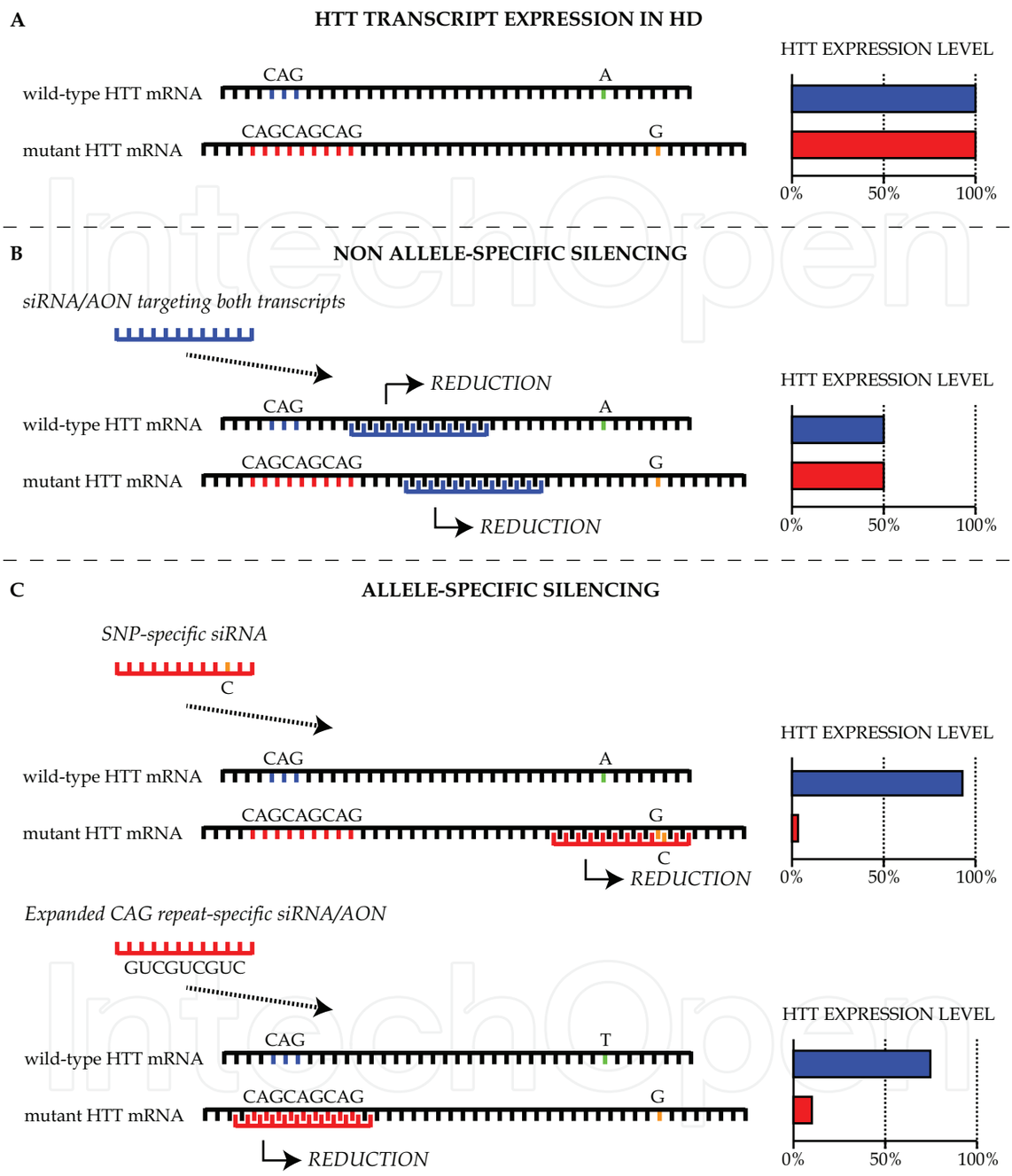

Fig. 2. RNA modulating therapeutic approaches for lowering htt. Two different HTT RNA modulating strategies used for HD are: A) Non allele-specific reduction of total HTT RNA levels by targeting a sequence that is identical in both the wild-type and mutant HTT transcript. B) Allele-specific reduction of mutant HTT RNA by targeting a unique heterozygous SNPs only present in the mutant transcript or C) Allele-specific reduction targeting the expanded CAG repeat on the mutant HTT transcripts.

Likewise, the use of only a single AON was suggested to be effective as treatment of various polyQ diseases (Hu et al., 2009; Evers et al., 2011). One expanded CAG repeat targeting AON was found to specifically reduce the expression of mutant ataxin-1 and ataxin-3 mRNA levels in SCA1 and 3, respectively, and mutant atrophin-1 in dentatorubral-pallidoluysian atrophy (DRPLA) in patient derived cells (Evers et al., 2011).

Although these results are promising, extensive research is needed to elucidate the mechanism used by those oligonucleotides to induce selective silencing and to assess specificity and safety. Likewise, the full potency of this allele-specific treatment will be revealed when the first *in vivo* results are obtained.

5. RNA modulating therapies in other neurodegenerative diseases

AONs have also been used for the treatment of neurodegenerative disorders and are found to be taken up by neurons when delivered into the cerebral lateral ventricles. Here are some examples showing therapeutic benefit in animal models and/or clinical trials.

5.1 Prevention of mutant protein translation

We will first focus on the neurodegenerative disorder amyotrophic lateral sclerosis (ALS) where RNA modulating therapeutics are used to reduce transcript levels of disease causing protein. The RNA modulating therapeutics to treat ALS are currently tested in a phase I clinical trial.

The progressive neurodegenerative muscle weakness disorder ALS is a caused by loss of motor neurons in the brain and spinal cord (Al-Chalabi & Leigh, 2000). The first mutations linked to the familial form of ALS (fALS) were found in the *superoxide dismutase 1 (SOD1)* gene. Mutated SOD1 is known to be toxic and prone to aggregation. Only approximately 1% of ALS cases is the result of mutations in the SOD1 enzyme (Bossy-Wetzel et al., 2004).

RNA modulating therapies that have been used in ALS were designed to block the translation of SOD1. In a transgenic mouse model of ALS, 2'O methoxyethyl modified AONs were used to lower mutant SOD1 levels by binding and subsequent RNase H mediated breakdown of SOD1 transcripts. Continuous ventricular infusion of the SOD1 targeting AON significantly slowed disease progression (Smith et al., 2006). The first results of a phase I study testing the safety of this SOD1 targeting AON in patients with fALS caused by mutant SOD1 are expected at the end of 2011. The outcomes of this phase I trial will be vital for future trials with RNA modulating therapies in HD.

5.2 Modulating pre-mRNA splicing

RNA modulating therapeutics are also used to modulate pre-mRNA splicing events in spinal muscular atrophy (SMA) using modified AON *in vivo*.

SMA is an autosomal recessive neuromuscular disorder caused by loss of function of the *survival motor neuron 1 (SMN1)* gene. This homozygous deletion of SMN1 results in degeneration of motor neurons in the anterior horn of the spinal cord and lower brain stem (Bowers et al., 2011). Depletion of SMN1 is not embryonic lethal because of the presence of the almost identical *SMN2* gene. However, due to a point mutation in an intron the SMN2

transcript is not correctly spliced. The majority of SMN2 transcripts are therefore lacking exon 7, which results in a truncated protein and lower expression of a functional SMN protein (Lorson et al., 2010).

Current therapeutic strategies are aimed at modulating alternative splicing of SMN2. Transfecting fibroblasts with an AON blocking intronic splicing silencers in intron 7 of SMN2 were found to result in inclusion of SMN2 exon 7 (Singh et al., 2006). Injection of differently modified AONs into the brains of SMA mouse models resulted in increased exon 7 inclusion and subsequent elevated SMN protein levels. The AON treated SMA mice displayed increased muscle size and extended survival (Williams et al., 2009; Hua et al., 2010; Passini et al., 2011).

Another modulating pre-mRNA splicing strategy involves the addition of a functional moiety to the AON to replace the missing splicing enhancer protein, thereby enhancing the inclusion of exon 7 by the splicing machinery (Cartegni & Krainer, 2003; Skordis et al., 2003). Several *in vivo* studies have shown increased SMN2 protein levels after intraventricular injection of splicing factors recruiting AONs (Dickson et al., 2008; Baughan et al., 2009).

The AONs to treat SMA show promising results *in vivo* and the progression in therapeutics will be monitored closely. Results regarding delivery of the AON to the brain in humans and how well the AON is tolerated will be very useful for the development of RNA modulating therapeutics for HD.

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

One major challenge of AON therapies for neurodegenerative disorders is delivery of the AON to the target organ. In the following paragraph we will describe in short the blood brain barrier function and how this impairs the uptake of peripherally administered drugs. We will focus in particular on the limitations and possibilities of AON delivery to the brain and will speculate on future clinical applications.

6.1 Blood brain barrier

A unique feature of the brain is that it is separated from the blood by the blood brain barrier (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 separates largely 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 were able to directly visualize the BBB.

The BBB is a major challenge in central nervous system (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 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 cerebrospinal fluid (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 at least 3 times CSF circulation, allowing continuous drainage of the brain's interstitial fluid.

6.2 Crossing the blood brain barrier

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 & 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 among 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 ATP-binding cassette transporters, drives substances away 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 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 one 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.

7. Conclusion

To date there is no treatment to prevent or even slow down the progression of HD. Considerable research has been performed to gain more insight into HD pathology. Next to the well-known toxic gain of polyQ protein function, loss of wild-type function and a toxic gain of expanded CAG repeat RNA was also suggested recently, and needs to be examined in more detail.

Recent results using SNP specific siRNAs and CAG targeting AONs look promising both *in vitro* and *in vivo*. To develop an effective HD therapy, it is likely that a combination of different RNA modifying approaches will be optimal to lower mutant htt levels. Extensive research is required to rule out toxic off-targets effects and elucidate the exact mode of action of these RNA modulating therapeutics. Ongoing clinical trials for other neurodegenerative disorders, such as in ALS, will give us more insights in the potential of RNA modulating therapeutics.

8. Acknowledgement

MME and WvRM are supported by the Center for Biomedical Genetics (the Netherlands), AtaxiaUK (United Kingdom), Dutch ataxia charity ADCA-VN (the Netherlands), and IOP Genomics (the Netherlands). The HD related research of RV and YT received financial support from the Cure Huntington's Disease Initiative (New York, USA) and Prosensa BV (Leiden, the Netherlands).

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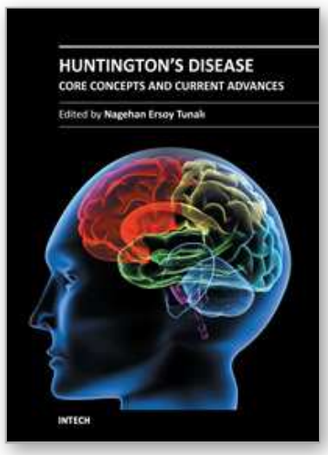

Huntington's Disease - Core Concepts and Current Advances
Edited by Dr Nagehan Ersoy Tunali

ISBN 978-953-307-953-0
Hard cover, 554 pages
Publisher InTech
Published online 15, February, 2012
Published in print edition February, 2012

Huntington's Disease is one of the well-studied neurodegenerative conditions, a quite devastating and currently incurable one. It is a brain disorder that causes certain types of neurons to become damaged, causing various parts of the brain to deteriorate and lose their function. This results in uncontrolled movements, loss of intellectual capabilities and behavioural disturbances. Since the identification of the causative mutation, there have been many significant developments in understanding the cellular and molecular perturbations. This book, "Huntington's Disease - Core Concepts and Current Advances", was prepared to serve as a source of up-to-date information on a wide range of issues involved in Huntington's Disease. It will help the clinicians, health care providers, researchers, graduate students and life science readers to increase their understanding of the clinical correlates, genetic aspects, neuropathological findings, cellular and molecular events and potential therapeutic interventions involved in HD. The book not only serves reviewed fundamental information on the disease but also presents original research in several disciplines, which collectively provide comprehensive description of the key issues in the area.

How to reference
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Melvin M. Evers, Rinkse Vlamings, Yasin Temel and Willeke M. C. van Roon-Mom (2012). Ameliorating Huntington's Disease by Targeting Huntingtin mRNA, Huntington's Disease - Core Concepts and Current Advances, Dr Nagehan Ersoy Tunali (Ed.), ISBN: 978-953-307-953-0, InTech, Available from: http://www.intechopen.com/books/huntington-s-disease-core-concepts-and-current-advances/ameliorating-huntington-s-disease-by-targeting-huntingtin-mrna-

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