STUDIES OF FACTORS AFFECTING INTRACELLULAR TOXICITY OF THE SCA7 DISEASE PROTEIN ATAXIN-7: FOCUS ON ATAXIN-7 DEGRADATION AND OXIDATIVE STRESS

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Studies of factors affecting the intracellular toxicity of the SCA7 disease protein Ataxin-7: Focus on Ataxin-7 Degradation and oxidative stress

Licentiate thesis

XIN YU
To my family
List of publications

This thesis is based on the following manuscripts referred to in the text as paper I and paper II.

I. Xin Yu, Abiodun Ajayi, Narasimha Rao Boga and Anna-Lena Ström. The SCA7 disease protein ataxin-7 is cleared by both autophagy and UPS. (Manuscript)

II. Abiodun Ajayi, Xin Yu, Lindberg S. Langel Ü, Anna-Lena Ström. Oxidative stress contributes to aggregation and toxicity in SCA7 polyglutamine disease model. (Manuscript)
Abstract

Spinocerebellar ataxia type 7 (SCA7) is one of nine neurodegenerative disorders caused by expansion of CAG/polyglutamine repeats. Proteins carrying expanded polyglutamine (polyQ) domains are suggested to be resistant to degradation and aggregate. Furthermore, a negative correlation between aggregation and toxicity has been shown. So far, little is known about the turn-over rate and degradation of the SCA7 disease protein ataxin-7 (ATXN7) and how this protein induces cellular toxicity. For the studies in this thesis work, we constructed stable inducible PC12 cell lines expressing GFP-tagged ATXN7 with 10 or 65 glutamines (Qs). Using these cell lines, we studied the turn-over of ATXN7 and the relationship between mutant ATXN7 and oxidative stress.

We showed that ATXN7 with a normal glutamine repeat (ATXN7Q10-GFP) has a short half-life and is mainly degraded by the UPS. In cells expressing expanded ATXN7 (ATXN7Q65-GFP), aggregation and reduced viability was observed. The aggregation increased the half-life of mutant ATXN7. For expanded full-length ATXN7, UPS was still the main degradation pathway; however autophagy also played a role in clearance of soluble ATXN7 fragments and possibly in aggregated ATXN7 material. Moreover, activation of autophagy reduced the level of aggregation and ameliorated the toxicity in cells expressing mutant ATXN7. From this study, we could get the conclusion that although expansion of the polyQ repeat increases the stability of expanded ATXN7, the protein can still be degraded via both UPS and autophagy. Furthermore, stimulation of autophagy could ameliorate the expanded ATXN7 toxicity and could therefore be a potential therapeutic approach for SCA7.

Regarding the role of oxidative stress we showed that expression of mutant ATXN7 leads to increased ROS levels and oxidative stress. Treatment with an antioxidant or blockage of NADPH oxidase complexes (NOX) decreased ATXN7 aggregation, the levels of ROS and ameliorated ATXN7 induced toxicity. Based on these results, we suggest that mutant ATXN7 cause increased ROS production from NOX and antioxidants treatment and or inhibition of NADPH-oxidase might potentially be used as a therapeutic strategy in SCA7.
## Contents

1. **Introduction** ................................................................. 1  
   1.1 Spinocerebellar Ataxia type 7 (SCA7) ......................... 1  
      1.1.1 Clinical manifestations and neuropathology of SCA7 ... 2  
      1.1.2 The SCA7 gene and ATXN7 protein ....................... 2  
   1.2 PolyQ diseases. ........................................................... 4  
      1.2.1 Mechanisms of polyQ toxicity ............................. 5  
      1.2.2 Pathological polyQ species ................................. 6  
      1.2.3 Protein degradation pathways ............................. 8  
         1.2.3.1 Ubiquitin-proteasome system (UPS) ................. 8  
         1.2.3.2 Autophagy .................................................. 9  
      1.2.4 Oxidative stress ................................................... 11

2. **Aims of the studies** .................................................... 14

3. **Methodological considerations** .............................. 15  
   3.1 Cell lines ................................................................. 15  
      3.1.1 HEK 293T cell .................................................... 15  
      3.1.2 Stable inducible PC12 cell line (Tet-off) ............... 15  
   3.2 Cell treatment ......................................................... 16  
   3.3 Analysis of aggregated ATXN7 ................................. 17  
   3.4 WST-1 cell viability assay ....................................... 17  
   3.5 Free radical assays .................................................. 18  
   3.6 GSH assay ............................................................... 18

4. **Results and discussion** ............................................... 19  
   4.1 Expanded ATXN7 in PC12 cells aggregates and shows toxicity. 19  
   4.2 Expanded ATXN7 is stabilized through aggregation ........ 19  
   4.3 Mutant ATXN7 could be degraded by UPS and autophagy .... 20  
   4.4 Expanded ATXN7 expression does not enhance autophagy, however activation of autophagy ameliorates expanded ATXN7 toxicity ................................................................. 21  
   4.5 Antioxidants ameliorate the toxicity of mutant ATXN7 ....... 21  
   4.6 Cellular antioxidative system is affected by mutant ATXN7 ... 22  
   4.7 Expanded ATXN7 affects ROS production from NADPH-oxidase complex .................................................. 22

5. **Conclusions** ............................................................ 23

6. **Acknowledgement** ..................................................... 24

7. **References** ............................................................... 25
Abbreviations

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akt</td>
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<td>Atg</td>
<td>Autophagy-related protein</td>
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<td>Mammalian target of rapamycin</td>
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<td>Polyglutamine</td>
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<td>peroxisome proliferator-activated receptor [PPAR]-γ coactivator 1 α</td>
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<td>Reactive oxidative species</td>
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<td>SCA</td>
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<td>STAGA</td>
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<td>Spinal and bulbar muscular atrophy</td>
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1. Introduction

1.1 Spinocerebellar Ataxia type 7 (SCA7)

Spinocerebellar ataxia type 7 (SCA7) is an autosomal dominant neurodegenerative disease characterized by progressive cerebellar ataxia and optical dysfunction due to a selective loss of neurons within the cerebellum, brainstem and retina [1, 2].

Previously, SCA7 has been referred to as autosomal dominant cerebellar ataxia type 2 (ADCA II) or olivopontocerebellar atrophy type 3 (OPCA III). SCA7 is a rare ataxia representing 2% of all Spinocerebellar Ataxias and this disease is reported to occur in less than 1/100,000 people [3]. However, SCA7 is the most diagnosed ADCAs in Sweden and Finland [4]. SCA7 is caused by the expansion of a CAG repeat in the first coding exon of the SCA7 gene, which encodes a protein named ataxin-7 (ATXN7). The expansion of the CAG repeat results in an expanded polyglutamine (polyQ) domain at the N-terminus of ATXN7, which then aggregate and form nuclear inclusion bodies [5]. The threshold length of polyQ expansion is negatively correlated to the onset age of SCA7 which ranges from a few months to more than 75 years [2, 6-8].

Except SCA7, there are eight other neurodegenerative diseases caused by polyQ domain expansions all sharing similar features [9, 10], and they are commonly defined as polyQ diseases. Besides common features, these polyQ diseases also have unique properties which are believed to be due to the context of the different mutant pathological proteins. So far we know that one of the unique characters of SCA7 compared to other ataxias is the patient blindness, which starts with the loss of blue/yellow color discrimination, for review see [11]. In order to understand more about the factors influencing the toxicity of polyQ proteins, we use SCA7 as a model. We started by addressing the question how protein degradation systems handle both wild-type and mutant ATXN7 in mammalian cells. In this work, we also focused on answering the question on how expanded ATXN7 toxicity relates to oxidative stress.
1.1.1. Clinical manifestations and neuropathology of SCA7

SCA7 is an autosomal dominant inherited disease. An earlier age of disease onset and a more severe progression of the disease between successive generations can generally be observed in families with SCA7. The severest condition is the infantile onset in which the infant can die within a few months.

Most SCA7 patients show similar symptoms, such as movement impairment, wide-based gait and dysarthria, for review see [7, 8]. Neuropathology studies have revealed that the common pathology for SCA7 patients include atrophy of the retina, cerebellum, brainstem, spinal cord and spinocerebellar tracts [2, 11, 12]. In the retina, the atrophy of choroids, degeneration of pigment epithelial cells, bipolar cells, ganglion cells and both rod and cone photoreceptor cells are generally observed [2, 12]. Under the microscope, the loss of neurons in cerebellar Purkinje and granule cell layers as well as in the dentate nucleus are commonly detectable. Both an apparent loss of neuron in the inferior olivary and spinal cord neurons in the anterior horn are also generally detectable [2, 11, 12]. In SCA7 patients, the cerebral cortex and the basal ganglia are believed not to be primary sites of pathology, and only a slight atrophy of the cerebral cortex and substantia nigra have been observed [2, 11, 12]. Other symptoms, such as increased reflexes, extensor plantar reflexes, lower limb spasticity, decreased vibration sense, dysphagia, sphincter disturbances and oculomotor symptoms have also been recorded in 25-75% patients [7, 8]. Impaired hearing, postural tremor, Parkinsonian symptoms, facial myokymia, mental deterioration nystagmus and scoliosis are also reported in a few patients. [7, 8].

A lot of evidences showed that SCA7 results in neurological symptoms, however there are also some non-neurological symptoms reported especially in infantile cases where muscle hypertonia and patent ductus arteriosus have been observed [8, 13, 14].

1.1.2. The SCA7 gene and ATXN7 protein

The SCA7 gene was first cloned in 1997 [5]. The gene encodes an 892-amino acid protein named ATXN7 with a predicted molecular weight of 95 kDa [5]. So far there are two isoforms of ATXN7 are detected, ATXN7a and b.
In healthy individuals, the CAG repeat in the SCA7 gene ranges from 7 to 17 repeats. When the CAG repeat length expands to more than 39, the individual will develop clinical symptoms. The onset age of patients are reversely correlated to the expansion length of the CAG repeat. A more severe expansion is generally accompanied with visual problems as the first symptom, whereas low repeat containing patients usually present with ataxia [5, 8, 15, 16].

ATXN7 is reported to be expressed in various tissues and not only in the nervous system. In central nervous system, ATXN7 expression has been observed both in affected regions (such as cerebellar cortex and retina) and non-affected regions (such as hippocampus) [17-19]. ATXN7 is localized both in the cytoplasm and the nucleus, and a hallmark of SCA7 disease is the formation of ATXN7 positive nuclear inclusion bodies. [5, 17-19]

The aggregation of mutant ATXN7 is believed to be due to the expanded polyQ domain and could be affected by the context sequence. A schematic structure of ATXN7 is shown in Fig. 1. Besides the polyQ domain, there are other functional domains identified including 3 potential nuclear localization signals (NLS) (aa378-393, 704-709, and 834-839) and a nuclear export sequence (NES), which means this protein can shuttle both in and out of the nucleus [20]. The cleavage of full length ATXN7 at Asp residues 266 and 344 by caspase-7 have been reported and shown to affect the localization and cellular toxicity. This effect might because of the removal of NLS and or NES of polyQ domain context [21]. There are also four proline-rich regions in ATXN7, which are predicted to be SH3-binding sites, a motif homologous to the phosphorylation recognition site of arrestins and two regions homologue to a bona fide subunit (Sgf73) of the yeast histone acetyltransferase complex SAGA. Although the exact function of ATXN7 is still not known, it was reported as a homolog of the yeast Sgf73p, a component of the SAGA (Spt/Ada/Gcn5/acetyltransferase) chromatin remodeling complex, and verified to be a subunit of human STAGA (SPT3-TAF(II)31-GCN5L acetylase) and TFTC (GCN5 and TRRAP) complexes [22, 23]. The mutation of ATXN7 induce a gain-of-function disease, however disruption of the normal function of ATXN7 have been reported as well [24, 25]. Thus the pathological mechanisms of polyQ proteins are still debated.
An alternative isoform of ATXN7 has been detected, SCA7b, which was found to be more restricted to the brain. The only difference between SCA7a and b is a small sequence close to the C terminus. In neurons, ATXN7b was only observed to localize in the cytoplasm and possibly not form inclusion bodies in SCA7 patients [26]. The identification of ATXN7b confirmed the hypothesis that the properties of polyQ domain could be modulated by the context sequence.

**Fig.1 The schematic structure of ATXN7.**

The 892-aa sequence of ATXN7 contains a nuclear export sequence (NES, black rectangle) and 3 potential nuclear localization signals (NLS, light gray rectangle). The arrows show the potential caspase-7 cleavage sites.

### 1.2 PolyQ diseases.

Besides SCA7, other polyQ diseases such as Dentatorubral-pallidoluysian atrophy (DRPLA), Spinobulbar muscular atrophy (SBMA), Huntington’s disease (HD) and SCA1-3, 6 and 17 are also caused by expanded polyQ domains. All of these polyQ diseases are believed to be gain-of-function diseases.

The hypothesis that polyQ diseases might share a common gain-of function pathological mechanism is based on the similar features they have. 1) The CAG repeat is the only similar DNA sequence observed in all these polyQ pathological protein encoding genes. 2) Expanded polyQ proteins aggregate in cytoplasm or in the nucleus of neurons. 3) In most polyQ diseases, the threshold above which the polyQ tracts cause diseases is around 35-40. 4) Eight out of nigh polyQ diseases are autosomal dominant diseases. These similar features indicate that these diseases might be gain-of-function diseases. When expressing the hypoxanthine phosphoribosyl transferase (HPRT) protein with an expanded polyQ domain in transgenic and knock-in mice, these mice developed neurological phenotype, which confirmed this hypothesis [27-29]. Additionally, not only human/mice, but also other species
such as drosophila, *C.elegans* and aplysia are affected by the toxicity of expanded polyQ domains [30-32].

Besides gain-of-function, the mutation in these polyQ proteins might also lead to loss-of-function disease contributions. This hypothesis was supported by the research of Zuccato’s group in 2001 and Gervais’s group in 2002. In Zuccato’s study, the function of normal huntingtin to up-regulate BDNF was blocked by expansion of polyQ [33], and in Gervais’s research, the binding of Huntingtin to HIP-1 was interrupted by the expansion of the polyQ domain [34]. Loss-of-function effects due to polyQ expansion was also suggested in SBMA [35]. In SCA7 the loss-of-function contribution has also been suggested especially in infantile cases with extreme repeat sizes where additional symptoms such as muscle hypertonia and heart problems were observed [14].

1.2.1 Mechanisms of polyQ toxicity.

The studies on polyQ diseases have been done for decades, however, there are still two important questions not clearly answered yet: 1) what species of these polyQ proteins are toxic; 2) how does polyQ domain expanded proteins induce cytotoxicity.

In polyQ diseases, the polyQ domain expanded proteins could misfold into soluble monomers and further dimerize, and form oligomers and fibrils, which subsequently leads to the formation of inclusion bodies (Fig.2). Among these intermediate mutant protein species, which one is the predominant pathological species is still unknown. In recent days, the post-translational modification and or proteolytic cleavage of polyQ proteins are reported to modulate the toxicity of polyQ expanded proteins, which makes this question even more complicated [36]. Inclusion bodies are believed to be toxic, however, debating reports show it might be other misfolded species for instance monomers or oligomers that generates the cellular toxicity. In regarding to the question how mutant polyQ protein induce toxicity, many mechanisms have been shown to be tightly related to the pathological process in polyQ diseases, such as gene transcription dysfunction, apoptosis induction of ER stress, energy metabolism disruption, excitotoxicity, oxidative stress, protein degradation
deficiency etc.. But the exact mechanisms leading to the toxicity is still unknown. [25, 31, 37-44]

In our group, we mainly focused on the role of protein degradation pathways in the clearance of monomeric and aggregated ATXN7 and the role of oxidative stress in SCA7 pathology.

1.2.2 Pathological polyQ species

In polyQ diseases, inclusion bodies are a hallmark and was previously believed to contribute to the cytotoxicity. The inclusion bodies are suggested to be the toxic species by physically impairing the axonal transport or nuclear functions [45]. The inclusion bodies formation in polyQ diseases is believed to be a multistep dynamic process, as figure 2 shows.

Fig.2. Schematic of possible aggregation pathways for polyQ proteins

In the aggregation process, a), mutant monomers with expanded polyQ domain are supposed to misfold into β sheet structured monomers. b) The misfolded monomers further dimerize or catalyze the transition of other random folded monomers into structured oligomers. C) These oligomers or dimers trap other free monomeric or oligomeric polyQ proteins into fibers and subsequently inclusion. “n” denotes the number of polyQ units within a disordered fibril or aggregate.

Besides polyQ protein, other proteins were also detected in these inclusion bodies for instance proteasome subunits and caspases. This suggested that the sequestration of these proteins might result in the loss of normal function by these proteins followed by dysfunction of the neurons. [46, 47]. Recently, more and more studies have shown that the inclusion bodies might not be the main toxic material formed by expanded polyQ proteins. Several reports suggest that there is a lack of significant association between inclusion body formation and
cell death or dysfunction *in vitro* and *in vivo* [48-50]. The inclusion bodies have even been suggested to be protective by sequestering reactive misfolded polyQ protein species [51]. Cells with inclusions survived much longer than cells with soluble oligomers [52]. In differentiated neuronal cell model, the oligomers but not the inclusion bodies have been shown to be cytotoxic [52, 53]. In fact, whether all the polyQ expanded pathological protein inclusions affect cells in the same way is still unknown.

Other potential toxic polyQ conformation could be the “soluble” polyQ species. During the inclusion body formation process, there are many polyQ protein species detectable, such as misfolded monomers, misfolded dimers and or oligomers, as the figure 2 shows. The polyQ protein oligomers are composed of monomers in a rapid and dynamic transitional process. The purified beta-strand polyQ monomers have been applied to cells to analyze their cytotoxicity. However, it is still difficult to say whether it is really the monomeric or the soluble oligomeric mutant polyQ proteins which are toxic since the transition between different forms happens all the time [54]. A mouse model with SBMA showed tight correlation between oligomers and symptoms [55].

Besides the expanded polyQ domain, the toxicity of causative gene products of polyQ disease could be modulated by post-translational modification or proteolytic cleavage. Phosphorylation at the Serine 421 by the Akt pathways was observed in mutant Htt to abrogate its proapoptotic activity through the neuroprotective effects of IGF-1, and the phosphorylation by Akt at serine 215/792 of androgen receptor reduced the mutant protein toxicity by altering binding of expanded androgen receptor to androgens and the translocation to the nucleus. In contrast, enhanced toxicity after the phosphorylation of the ATXN1 at serine 776 was reported due to the stabilization of mutant ATXN1 against degradation [56-59].

Ubiquitination and the SUMOylation of mutant polyQ proteins can have opposite effects. SUMOylation was suggested to compete with the ubiquitination by binding to the same modification site on polyQ proteins and this binding could interrupt the UPS degradation of modified polyQ proteins, therefore stabilize the mutant polyQ proteins from clearance resulting in elevated aggregation and impairment of UPS function. According to the previous studies, the toxicity of Htt or
ATXN1 was reported to be enhanced corresponding to the level of SUMOylation [60, 61]. ATXN7 has been shown to be a new substrate for SUMOylation both in vitro and in vivo, which modulated the aggregation properties and the cytotoxic effects of mutant ATXN7 [62].

Proteolytic cleavage of mutant polyQ protein could be considered as another type of modification. The cleavage of polyQ proteins is believed to enhance cellular toxicity by affecting cellular localization and acceleration aggregation. These hypothesis was supported by the studies on HD, SCA3, SBMA and SCA7 [21, 55, 63, 64].

1.2.3 Protein degradation pathways

Newly synthesized proteins are monitored in regards to proper folding and correct assembly [65-73]. Aberrant proteins would be recognized and guided to protein degradation pathways. These aberrant proteins could be induced under cellular stress conditions or due to mutations like in polyQ diseases. The failure of aberrant protein clearance would lead to cytotoxicity in mammalian cells. Protein degradation pathways are the basic cellular mechanism to clear away aberrant proteins [74-82]. In fact not only small proteins, but even big protein conformations like aggregates or even organelles can be degraded by intracellular protein recycling pathways. There are two main mechanisms responsible for protein degradation in mammalian cells: the ubiquitin-proteasome system (UPS) and the autophagy.

1.2.3.1 Ubiquitin-proteasome system (UPS)

The UPS system is believed to mainly degrade short-lived and small molecular sized proteins in cytoplasm and nuclei in mammalian cells. In the UPS pathway, initially, ubiquitin is activated by ubiquitin-activating enzyme (E1) and transferred onto a substrate protein by ubiquitin conjugating enzymes (E2) and ubiquitin ligases (E3) [83]. This ubiquitination result in the attachment of a single ubiquitin molecule to the single/multi lysine residues and or even synthesis of ubiquitin chains. Many substrate proteins with a polyubiquitinated chain are subsequently degraded by the 26S proteasome, which consists of the catalytic 20S complex and the regulatory 19S particles. Substrates heading for degradation either are delivered to the
proteasome by soluble ubiquitin receptors or recognized by the intrinsic ubiquitin-binding activity of the 19S particle. See review [84]. After the poly ubiquitin chain is disassembled, the substrate will be unfolded and fed into the 20S subunit. In the cavity of the 20S subunit, the substrate will be proteolytically cleaved and small peptides which can be reused finally released from the proteasomes. Ubiquitination could also directly regulate protein function in a proteolysis-independent manner, via mono-, multi- or poly-ubiquitinated proteins. The UPS system is shown in Fig.3 [85].

PolyQ proteins have been reported to be resistant to proteasomal degradation, and disrupt global UPS activity [86, 87]. In other studies, it suggested that global UPS activity in vivo was not affected in the brains of polyQ disease transgenic mice [88-90]. ATXN7 interacts with a portion of the 19S subunit [91], and the polyQ expanded form of ATXN7 was suggested to impair the proteolytic function of the ubiquitin-proteasome system [92]. However, whether pathological polyQ proteins impair UPS is still debated.

Fig.3 Ubiquitin proteasome system mediated protein degradation.

1.2.3.2 Autophagy

The other predominant protein degradation pathway is autophagy. In contrast to UPS, autophagy only occurs in the cytoplasm and can degrade organelles and cytoplasmic material as well as damaged proteins. Three types of autophagy have been identified;
macroautophagy, microautophagy and chaperone mediated autophagy (CMA) [12]. Macroautophagy (here referred as autophagy) is the major form of autophagy and degrades cytosolic material via formation of a double membrane structure called autophagosomes. A series of autophagy related proteins (Atg) involved in this process. Genetic analysis in yeast has uncovered several Atg genes, many of which have mammalian orthologs [93].

The autophagy process can be classified into four continues steps: 1) **Autophagy induction**: In this step, the mTOR pathway is a key regulator. The inhibition of mTOR initiates autophagy by activating Atg1/ULK1. ULK1 is a homologue of Atg1 in mammalian cells [94]. The inactivation of mTOR leads to downstream dephosphorylation events resulting in transcriptional activation of autophagy genes [95, 96]. Most evidence showed that rapamycin is an inhibitor for mTOR pathway. Additionally, mTOR pathway is reported to be modulated by many other pathways such as p53, PI3K/Akt and MAPK. See review [97]. There is also mTOR independent pathway could regulate autophagy, although the exact mechanism is still not clear, see review [98].

2) **Autophagosome formation**: In this step, cytosolic proteins and organelles which will be degraded are engulfed by a double membrane vesicle, which is believed to be initiated from the endoplasmic reticulum. Many Atg proteins are involved in the formation of these vesicles. During the initiation process of autophagosomes, Atg6 (Beclin 1 in mammalian cells), induces phosphatidylinositol 3-phosphate production by recruiting the class III phosphatidylinositol 3-kinase [99]. The elongation of autophagosome membranes involves two ubiquitin-like molecules, Atg12 and Atg8/LC3. This process is accompanied by two associated conjugation processes. In one conjugation process, Atg7 and Atg10 covalently link Atg12 with Atg5 and subsequently recruit Atg16 to form pre-autophagosomal structures. In the other conjugation process, after LC3 cleavage, the lipidation of LC3 with phosphatidylethanolamine to generate LC3 II is mediated by Atg7 and Atg3. After that LC3-II associates with the autophagosome membranes and will be recruited into matured autophagosomes. See review [100].
3) **Autophagosome fusion.** During this step, the autophagosome fuses with the lysosome and the contents of the autophagosome are released into the lysosome for degradation [95, 101].

4) **Autophagosome breakdown.** Following the fusion of autophagosome and the lysosome, the autophagosome membrane will be broken down by the lysosomal proteases [95, 102]. The supposed autophagy is depicted in Figure 4, edited from [103].

Degradation of some expanded polyQ proteins via macroautophagy have been observed [104, 105] and enhancing this pathway was shown to ameliorate toxicity in some, but not all polyQ disease models [106, 107]

![Fig.4. Schematic autophagy pathway.](image)

### 1.2.4 Oxidative stress

Oxidative stress is reported to occur in many neurodegenerative diseases such as Alzheimer’s disease, Parkinson’s disease and Huntington’s disease, see review [108]. However, there are few reports on oxidative stress in SCA7. Oxidative stress is believed to be due to the imbalance of reactive oxygen species production and antioxidative defense system activity. In mammalian cells, the ROS are generated by the incomplete reduction of oxygen and are produced at low levels under normal physiological conditions as a result of mitochondrial respiration and a number of other processes. Many endogenous antioxidants such as glutathione, free amino acid and antioxidative enzymes (SODs, catalase and GSTA3 for example) can be used to remove reactive oxygen species. SODs remove superoxide by catalyzing its dismutation to produce H$_2$O$_2$ [109]; Catalase reduces
H2O2 into water [110]; GSTA3 is suggested to involve in the generation of glutathione, see review [111]. When the antioxidant molecules or mechanisms are overwhelmed, the cell might accumulate ROS which leads to the oxidative stress and cell death as figure 5 shows [76,106].

![Fig.5 Schematic model of oxidative stress generation.](image)

The central nervous system is particularly vulnerable to ROS possibly due to the high oxygen utilization rate. Extracellular antioxidant drugs have been applied to ameliorate oxidative stress and supposed to be an interesting therapeutic strategy for ROS related neurodegenerative diseases [112]. Antioxidants such as coenzyme (CoQ10), creatine, N-acetyl cysteine (NAC), vitamin C/E, thiols and taurosodeoxycholic acid (TUDCA), are reported to ameliorate the toxicity from expanded Htt and ATXN1 therefore prolong the life of transgenic mice [113].

In polyQ diseases, oxidative stress has been mostly studied in HD models. There are four suggested pathways related to the ROS production in polyQ diseases: 1) Mitochondria respiration chain dysfunctions. In Huntington’s disease, the protein complexes involved in the mitochondria respiration chain was suggested to be affected directly by mutant Htt. Postmortem brain samples of HD patients showed decreased activity of mitochondrial respiratory complex II, III and IV [114]. 2) Aberrant transcriptional regulation. PGC-1α, peroxisome proliferator-activated receptor [PPAR]-α coactivator 1 α, is a key transcriptional co-regulator on mitochondrial respiration and oxidative stress defense modulation [115]. Mutant Htt was reported to
block the PGC-1α promoter by the inhibition of the CREB transcriptional activator. See review [116]. 3) Activation of membrane NOX. PolyQ expanded protein was observed to colocalize to the lipid raft with gp91, a subunit of membrane NADPH-oxidase complex and suggested stimulate the production of superoxide from NOX [117]. 4) Inclusion bodies was suggested to be centers of ROS generation [118]. The possible oxidative stress induction mechanisms in polyQ diseases are briefly described in Fig 6 [118-122].

Fig.6. Supposed ROS production in polyQ diseases

a) polyQ expanded protein could interact with membrane NADPH oxidase complexes in lipid rafts. The activation of NADPH complex leads to the elevation of superoxide and intracellular hydrogen peroxide. b) The expanded polyQ protein might interrupt the antioxidants function, and down-regulates the level of enzymes or antioxidants, such as catalase and glutathione, thus elevates the ROS level. c) PolyQ protein might disrupt the respiration chain by affecting protein complex II and or III in mitochondria membrane and release ROS. d) The polyQ protein inclusion bodies could become centers for ROS generation also.

Additionally, we should always keep one point in mind, which is oxidative stress is possibly linked to many other toxic effects in our cells. Take UPS as an example, sever or sustained oxidative stress have been shown to impairs the function of the UPS and decreases intracellular proteolysis, see review [123].
2. Aims of the study

This thesis is primarily focusing on the role of protein degradation pathways and oxidative stress in the cellular toxicity of mutant ATXN7 in a novel SCA7 cell model.

**Paper I:** This study shows how efficiently the wild-type and expanded ATXN7 are degraded via UPS and or autophagy pathways, and whether the pharmacological activators of autophagy could ameliorate the expanded ATXN7 toxicity.

**Paper II:** Here we aimed to answer the question whether the expanded ATXN7 expression causes oxidative stress and through which mechanisms.
3. Methodological considerations

The methods used in this thesis are described in each contributed paper; consequently, the theoretical and methodological aspects will not be depicted here. The selections below are valid for both papers if no special emphasis is mentioned.

3.1 Cell lines

In both papers, HEK293T cells and the stable neuronal PC12 cells expressing ATXN7 under the control of an inducible promoter were used as models.

3.1.1 HEK 293T cell

Human Embryonic Kidney 293T cells are transformed cancer cells originally derived from human embryonic kidney cells. We use HEK293T cells because it is a human cell line and it is one of the easiest cells to grow and transf ect. Additionally, ATXN7 is widely expressed and could be detected in kidney cells, which indicates that HEK293T cell might be a good model for SCA7 studies.

3.1.2 Stable inducible PC12 cell lines (Tet-off)

PC12 is a neuronal cell line derived from a pheochromocytoma of the rat adrenal medulla [124]. Commercial PC12 Tet-off cell lines (Clontech) were transfected with FLQ10-pTRE-tight or FLQ65-pTRE-tight plasmid constructs. In our PC12 cells, a pTet-off regulator plasmid coexists with our transfected constructs, which encodes the tetracycline-controlled transactivator (tTA). When doxycycline is present, the binding of the tTA protein to the Tet-Response Element (TRE) is inhibited thus the expression of our target protein, ATXN7, which under the control of the TRE element is off. However after the removal of doxycycline, the suppression of TRE is released and the ATXN7 will be expressed. See Figure 7. In contrast to the Tet-off system, the Tet-on system in other researches expresses target proteins always need the doxycycline to be present in order to express the target protein. We preferred the Tet-off system because in the Tet-off system the doxycycline is removed to express ATXN7, which will
avoid potential effects generated by doxycycline [125]. In the screening of constructed PC12 colonies, we tried to pick the cell lines which had a similar expression level of ATXN7 as endogenous ATXN7 to avoid over-expression effects.

Fig. 7. ATXN7 expression in our PC12 cell lines (Tet-off system)

3.2 Cell treatment

In paper I, pharmacological inhibitors/activators were applied to inhibit autophagy at different levels. NH₄Cl works by neutralizing lysosomes thereby interrupting the fusion of autophagosomes and lysosomes. NH₄Cl inhibits all types of lysosomal proteolysis [126-128]. 3-MA inhibits autophagy by suppression of class III PI3K activity and has been widely used as an autophagy inhibitor [129, 130]. Autophagy pathway could be enhanced by Rapamycin and Trehalose via different pathways. Rapamycin activates autophagy by binding to mTORC1 complex to interrupt the inhibition effect on autophagy initiation [131]. Although the exact mechanism of trehalose activating autophagy pathway is still unknown, the mechanism of trehalose to stimulate autophagy is mTOR independent [132]. Epoxomycin was used for UPS inhibition because of its high specificity compared to latacystin or other inhibitors [133]. Unfortunately, specific activators of UPS are rarely found except one example, IU1, which is developed recently [134].

In paper II, oxidative stress could be induced either by adding reactive oxygen species or by inhibition of antioxidative defense system. In this research, H₂O₂ treatment was used to increase cellular ROS levels
and buthionine sulphoximine (BSO) treatment was used to specifically inhibit GSH biosynthesis [135]. NAC (N-acetyl-L-cysteine) was applied as an antioxidant. In cells, glutathione is an important antioxidant, and NAC is an essential precursor for glutathione and is cell permeable [136].

To inhibit NADPH-oxidase complex, gp91ds-TAT (a peptide inhibitor conjugated to a cell penetrating peptide named TAT), apocynin and AEBSF (4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride) were used [117, 137]. Apocynin specifically inhibits the release of superoxide anion by NADPH oxidase by blocking migration of p47phox to the membrane, critically involved in initiating assembly of the functional NADPH oxidase complex [138]. AEBSF is a less specific NOX inhibitor compared to gp91-TAT and apocynin.

3.3 Analysis of aggregated ATXN7

The filter trap assay is based on antigen-antibody interactions and mainly applied to analyze aggregated ATXN7 material. In this method, cells are lysed and the pellet fraction contains aggregated material is filtered through a nitrocellulose membrane with a pore size of 0.2 µm in diameter using vacuum aspiration. Any aggregated material larger than 0.2 µm in diameter will be trapped on the membrane. The nitrocellulose membrane is then probed with primary antibody and corresponding secondary antibody to detect ATXN7. The secondary antibody is coupled to horseradish peroxidase (HRP) which can be visualized by enhanced chemiluminescence (ECL). The filter trap assay can be used to analysis relative small aggregates together with big inclusion bodies. Another advantage is this method is that it makes the quantification analysis of aggregated material easier and more objective.

Another method to analysis aggregated material is immunostainings and microscopy, which is superior to filter trap assay regarding analysis of the localization of inclusion bodies.

3.4 WST-1 cell viability assay

To detect how induction of ATXN7 expression and different treatment of our stable ATXN7 expressing PC12 cells affect cellular toxicity, WST-1 assay was applied. In viable cells, the tetrazolium salt WST-1
can be cleaved by mitochondrial succinate-tetrazolium reductase to derive the water soluble formazan, which can be quantified spectrophotometrically. The signal detected is corresponding to the viability. WST-1 assay was chosen because it is friendly to work with and the dye is easier to dissolve than many other mitochondrial succinate-tetrazolium reductase based cell viability assays.

3.5 Free radical assays

The total ROS as analyzed with a DCHF-DA assay. DCF is a dye for measuring hydroxyl, peroxyl, or other reactive oxygen species within cells. In this assay, a fluorogenic probe 2’, 7’-dichlorodihydrofluorescein diacetate (DCFH-DA) is allowed to diffuse into cells and thereafter be deacetylated by cellular esterases to non-fluorescent 2’, 7’-dichlorodihydrofluorescin (DCFH), which is rapidly oxidized to 2’, 7’-Dichlorodihydrofluorescein (DCF) by ROS. The fluorescence intensity from DCF corresponds to the ROS level within the cell. The assay is sensitive and the generated signal is stable [139].

To study the ROS produced by mitochondria, MitoSOX assay was applied. In this assay, the cationic triphenylphosphonium substituent is responsible for the rapid electrophoretically driven uptake of the probe in actively respiring mitochondria. Oxidation of MitoSOX red indicator by superoxide, but not by other ROS- or reactive nitrogen species (RNS)–generating systems, results in hydroxylation of hydroxyethidium, which binds to mitochondrial nucleic acids, resulting in specific bright red fluorescence in mitochondria [140]. We choose MitoSOX is due to the live-cell permeability and the selectively targeting to the mitochondria.

3.6 GSH assay.

To detect the glutathione level, the GSH-Glo™ Glutathione Assay was used. This assay is a luminescence-based assay in which a luciferin derivative is converted into luciferin in the presence of glutathione, and glutathione S-transferase (GST). The signal generated is corresponding to the amount of glutathione present in the cells. This assay was used because of the sensitivity and the stability of generated signals [141].
4. Results and discussion

4.1 Expanded ATXN7 in PC12 cells aggregates and shows toxicity (Paper I & II)

In order to study which factors can influence the toxicity of polyQ expanded ATXN7, we constructed stable inducible PC12 cell lines FLQ10 and FLQ65 expressing ATXN7 with 10 (ATNX7Q10-GFP) or 65 (ATNX7Q65-GFP) glutamines respectively.

In FLQ65 cells, induction (Doxycycline removal) lead to expression of mutant ATXN7 and aggregation as well as cytotoxicity is expected. Induction of ATNX7Q10-GFP did not produce any inclusion bodies or cellular toxicity. We also detected truncated N-terminal ATXN7 fragments similar to the size as previously reported by Yong et al, which are supposed to be generated after caspase-7 cleavage of ATXN7 [21]. For the expanded ATXN7, full length monomeric ATXN7 and inclusion bodies are predominately localized in the nucleus similarly as seen in human patients [24].

4.2 Expanded ATXN7 is stabilized through aggregation. (Paper I)

To investigate the stability of ATXN7, the half-lives of Q10 and Q65 mutant ATXN7 were analyzed.

We found that full-length as well as fragments from ATNX7Q10-GFP had a short half-life approximately 7.3 hours and 5.1 hours. After turn-off of further ATNX7Q10-GFP expression, almost all of the full-length and truncated ATXN7 were cleared away within 12 hours.

In mutant ATXN7, Q65-GFP expressing cells, both full-length and truncated monomeric ATXN7 showed an initial elevation after further expression had been turned off. However, after that, a rapid clearance was observed. During this clearance, an elevation of ATXN7 aggregated material was observed. This indicates that the expanded ATXN7 species is rapidly cleared not by degradation but by aggregation. The half-live for aggregated ATXN7 was estimated to 34.3 hours. Our results showed that the aggregated mutant ATXN7 species are much more stable than monomeric species (fig2.C, Paper I) and consistent with previous reports [142, 143].
4.3 Mutant ATXN7 can be degraded by UPS and autophagy (Paper I)

To investigate the degradation pathways for endogenous ATXN7 and ATXN7Q10 or Q65-GFP, we used inhibitors/activators of autophagy and UPS. Our results indicated that UPS is the main clearance pathway for endogenous ATXN7 and Q10-GFP in both PC12 cells and HEK293T cells. Autophagy is only present in the cytoplasm and since wild-type ATXN7 is mainly localized to the nucleus, this pathway is not available to degrade wild-type ATXN7.

Expanded polyQ proteins have been suggested to be resistant to UPS degradation [144, 145]. However, after UPS inhibition, increased levels of soluble full-length and truncated ATXN7Q65-GFP as well as aggregated ATXN7 material were detected in our cells (Fig. 3A, Paper I). This suggests the UPS can degrade mutant ATXN7. This finding is consistent with the study of Latouche et al, showing increased number of ATXN7 inclusions in HEK cells treated with a UPS inhibitor and supported by other studies showing that polyQ proteins can be degraded by UPS [146, 147].

The level of soluble full-length ATXN7Q65-GFP in FLQ65 cells was not affected by autophagy inhibition in any experimental setup. However, elevated levels of a 45 kDa Q65 fragment could be detected. These data suggest that autophagy does not degrade the full-length ATXN7Q65-GFP, but contributes to the degradation of truncated mutant ATXN7 as previously reported [36]. The inhibition of autophagy with NH₄Cl and or 3-MA also resulted in the accumulation of aggregated ATXN7 material. On the contrary, the activation of autophagy with rapamycin decreased the level of aggregated ATXN7 material without significantly affecting the levels of monomeric full-length or truncated ATXN7. These data suggest that autophagy could also clear away aggregated ATXN7 material as previously shown in Huntington model [148].

Additionally, similar decreased cell viability was observed after UPS and autophagy inhibition. The only form of ATXN7 corresponding to the level of cellular toxicity is the aggregated material, which indicates that the aggregates might be the main toxic contributors.
4.4 Expanded ATXN7 expression does not enhance autophagy, however activation of autophagy ameliorates expanded ATXN7 toxicity (Paper I)

Since the aggregated ATXN7 material could mainly be cleared by autophagy, activation of autophagy might be a potential therapeutic strategy. Activation of autophagy has been shown to ameliorate disease in some [106, 149, 150], but not all [107] polyQ diseases. In our PC12 cell model, no significant elevation of autophagy activity could be observed during the induction of mutant ATXN7 expression for 12 days (Fig.7, Paper I). However, pharmacological activation of autophagy with rapamycin rescued cell viability to the same levels as in non-induced PC12 cells (Fig.9, Paper I). The activation of autophagy with rapamycin did not affect monomeric full-length ATXN7 level which suggested that the effect of rapamycin was really through the activation of autophagy and not by interrupting protein synthesis of the polyQ protein as have been reported before [151]. Why the PC12 cells do not enhance the autophagic activity to counteract the ATXN7 toxicity is unclear. Failure to activate autophagy has been reported in several other neurodegenerative disorders [152] and the mechanism behind this needs to be further studied.

4.5 Antioxidants ameliorates the toxicity of mutant ATXN7. (Paper II)

Oxidative stress has been implicated in the pathology of several neurodegenerative diseases. Few studies can be found on the role of oxidative stress in SCA7. To study the role of oxidative stress in SCA7 we used the same PC12 cell model described above. In this model, induction of mutant ATXN7 expression leads to a progressive elevation of ROS level. A correlation between increased ATXN7 expression and ROS levels as well as viability could be observed. No increase in ROS levels was found in cells expressing wild-type ATXN7 (ATXN7Q10-GFP) (Fig 2, Paper II). The fact that ROS levels increase before any toxic effects can be observed in our SCA7 model suggests that oxidative stress could contribute to the cell death. This suggestion is further supported by the observation that treatment with an anti-oxidant reducing the ROS levels also rescued the cell viability. Antioxidants were previously reported to ameliorate cellular toxicity induced by expanded polyQ proteins in many diseases, see
review [153]. Additionally, according to the previous research by Underwood et al., the inhibition effect of different antioxidant on cellular autophagy is suggested through various pathways, and this should be considered before selecting antioxidants [154].

4.6 The Cellular antioxidative system is affected by mutant ATXN7. (Paper II)

Under oxidative stress conditions, cells try to keep the redox homeostasis by up-regulating anti-oxidative mechanisms. In our study we observed an increased expression of SOD1 and GSTA3. GSTA3 is an enzyme that detoxify oxidatively damaged molecules for instance lipid peroxidation products [155].

In contrast, a decrease in the expression of catalase was observed in our results (fig 6, Paper II), which could compromise the clearance of $H_2O_2$. The decreased expression of catalase was previously observed in a HD model [156]. Interestingly, in the HD model, a decrease of SOD level was observed, which indicated that mutant polyQ protein might affect oxidative defense systems differently. Taken together these data suggests that the cell is up-regulating parts of the antioxidative defense system but can’t thoroughly handle the increased ROS production induced by mutant ATXN7 expression.

4.7 Expanded ATXN7 increase ROS production from NADPH oxidase complexes. (Paper II)

In order to determine if increased ROS production play a role in the oxidative stress in our cells, we investigated ROS production from mitochondria and NOX. We could not see any increase in mitochondrial ROS, however the inhibition of NOX with gp91ds-TAT, apocynin or AEBSF prevented the ATXN7Q65-GFP induced elevation of ROS and aggregation. Furthermore, apocynin or gp91ds-TAT treatment ameliorated the ATXN7 toxicity. This suggests that ATXN7 cause oxidative stress by activating NOX and this is consistent with a recent study by Bertoni et al [117], showing that expanded polyQ stretch of Htt leads to NOX activation therefore results in oxidative stress. In conclusion, our results suggested that the NADPH-oxidase could be a potential therapeutic target for SCA7.
5 Conclusions

This thesis has provided new data regarding the half-lives and degradation of wild-type and mutant ATXN7. Furthermore we have discovered that oxidative stress plays an important role in ATXN7 toxicity.

**Paper I:** New stable inducible neuronal PC12 cell lines expressing ATXN7 were constructed as a tool to detect the clearance rate of both wild-type and mutant ATXN7. With these tools, we got the following conclusions:

- The polyQ domain expansion stabilize mutant ATXN7 from degradation through aggregation.
- The wild-type ATXN7 is mainly degraded by UPS.
- Both UPS and autophagy can degrade monomeric expanded ATXN7; however autophagy mainly contributes to the degradation of truncated and aggregated ATXN7.
- Mutant ATXN7 expression does not up-regulate autophagy in our model.
- Induction of autophagy could ameliorate cellular toxicity induced by mutant ATXN7 expression.

**Paper II:** Using the same cell model, we got the following conclusion:

- Expanded ATXN7 induce oxidative stress which contributes to the cellular aggregation and toxicity.
- Increased ROS production from NADPH-oxidase complexes contribute to the oxidative stress.
- Decreased catalase level could contribute to oxidative stress.
- Pharmacological antioxidant treatments could ameliorate the cellular toxicity induced by mutant ATXN7 expression.

Hopefully, this knowledge will help us to take further steps to understand how expanded ATXN7 toxicity is generated and which factors could influence its toxicity. This will be very useful for the development of potential therapeutic approaches for SCA7 and other polyQ diseases.
6. Acknowledgements:

It is a pleasure to thank the many people who made this thesis possible.

It is difficult to overstate my gratitude to my Ph.D. supervisor, Dr. Anna-Lena Ström. With her enthusiasm, inspiration, and great efforts to explain things clearly and simply, she helped to make this research fun for me. Throughout my thesis-writing period, she provided encouragement, sound advices, good teaching, good company, and lots of good ideas. I would have been lost without her support. The respect for you in my heart is much more than any verbal description.

I wish to thank my friends in our group. Ajayi, Narasimha and Anne, thank you for your grateful ideas and supports.

I would like to thank the many people who have given me a lot of help since I arrived in Sweden. Marie-Louise and Siv, thank you for always answering my boring questions patiently, and provided me the first sleep place in Sweden. I wish to thank all the teachers in this department for giving many good suggestions and ideas on my study and private life.

I am special grateful to Tom and Johan for your experienced help on my lab work and student life here. Linda and Kariem, thank you for your explanation on licentiate preparation, Santosh and Ellinor for the technique discussions. Special thanks to Jessica and Andres for the positive attitude on research and life. Thanks Henrik for giving the computer support. Grateful thanks to Staffan, Rania and Veronica for the happy lab assistant work together. And thanks all the people in this department.

I wish to thank my entire extended family for providing a loving environment for me, my parents, my wife and my brother. Special thanks to my son, who is the supporting angel for my whole life.

Lastly, and most importantly, I wish to thank my brother, Ming Yu. We grow up together, we fight with each other and we believe and support each other. To them I dedicate this thesis.
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