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DISSERTATION

Investigation of the Role of Collapsin Response Mediator 4 in Huntington's Disease

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Nomenclature

4C8	Anti huntingtin Antibody
AC	ADF/Cofilin
AC40	Anti Actin Antibody
AD	Alzheimer's Disease
ADF	Actin depolymerising factor
ADP	Adenosine diphosphate
AFM	Atomic Force Microscopy
ALLN	Acetyl-L-Leucyl-L-Leucyl-L-Norleucinal
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BDNF	Brain Derived Neurotrophic Factor
BSA	Bovine Serum Albumine
CAG	Tree-nucleotide codifying for Glutamine
CAG53b	Anti huntingtin Antibody
cAMP	Cyclic adenosine monophosphate
CNS	Central Nervous System
CRE	cAMP response element
CREB	cAMP response element binding protein
CRMPs	Collapsin Response Mediator Proteins
DMEM	Dulbecco's Modified Eagles medium
EGFP	Enhanced-green-fluorescent-protein
F-Actin	Filamentous Actin
FBS	Fetal Bovine Serum
G418	Geneticin

GIT1	G protein-coupled receptor kinase interacting ArfGAP 1
GST	Glutathion-S-Transferase-t
HAP-1	Huntingtin Associated Protein- 1
HD	Huntington's Disease
HD1	Anti huntingtin Antibody
HIP-1	Huntingtin Interacting Protein
His	poly-Histidin
Htt	Huntingtin
IgG	Immunoglobuline G
LTP	Long term potentiation
MAB5374 mEM48	Anti huntingtin Antibody
MAP1	Microtubule associated protein 1
MAP2	Microtubule associated protein 2
Mhtt	Mutant huntingtin
mRNA	Messenger ribonucleic acid
MSN	medium spiny neurons
NIIs	neuronal intranuclear inclusions
NP-40	Nondiet P
NRSF	neuron restrictive silencer factor
PBS	Phosphate-buffered saline
PC12	Pheochromocytoma 12 cells
pcDNA3.1-HD1Q68	Vector encoding huntingtin with 68 Poly Glutamine
PFA	Paraformaldehyde
PFN2	Profilin-2
Phalloidin	Filamentous Actin Marker
PonA	Ponasteron A
Q	Glutamine
R6/2 tg	R6/2 transgenic
REST	repressor-element-1 transcription factor
ROS	reactive oxygen species
RT	Room Temperature
S18	Ribosomal protein
SDS	Natriumdodecylsulfat
SH3GL3	SH3-domain GRB2-like 3
Tau	Tubulin associated unit
TUC-4	Anti CRMP4 Antibody

1 Summary

Huntington's disease (HD) is an inherited neurodegenerative disorder characterized by the accumulation in the affected neuronal population of N-terminal huntingtin (htt) fragments bearing elongated polyglutamine (polyQ) tracts. Several lines of evidence indicate that the process of htt misfolding and aggregation is associated with cytotoxicity in HD models and patients. Thus, the identification and characterization of proteins that can modulate this process is critical for understanding HD pathogenesis and therapy development. Here, we report a depletion of collapsin response mediator protein 4 (CRMP4) protein levels in a cell culture model of HD. CRMP4 belongs to the collapsin response mediator protein family that mediates many aspects of neuronal development and plasticity by regulating cytoskeleton dynamics.

Cell-free and cell-based assays demonstrate that CRMP4 reduces polyQ-mediated htt aggregation and toxicity. Furthermore our results show that this effect may be linked to a rearrangement of Actin cytoskeleton. Indeed both, CRMP4 and filamentous Actin (F-Actin), are recruited into the intracellular inclusions formed by mutant huntingtin (mhtt), while overexpression of CRMP4 partially detached F-Actin from the mhtt aggregates. CRMP4 is cleaved by Calpain-1 when mhtt is expressed. This result links to previous findings showing the same proteolytic cleavage of CRMP4 when cells were subject to excitotoxicity. The proteolysis of CRMP4 may therefore be a marker of neuronal toxicity. This work suggests a functional link between mhtt and CRMP4 and a role for CRMP4 as a new modulator of mhtt. Further studies are important for understanding whether CRMP4 can help deciphering the early cellular pathomechanisms in HD.

2 Introduction

Neurodegeneration is the umbrella term for the progressive loss of structure or function of neurons, including death of neurons. Many neurodegenerative diseases including Parkinson's, Alzheimer's, and Huntington's occur as a result of neurodegenerative processes. As research progresses, many similarities appear which relate these diseases to one another on a sub-cellular level. Discovering these similarities promises therapeutic advances that could lessen many diseases simultaneously. Parallels between different neurodegenerative disorders include atypical protein assemblies as well as induced cell death. [Rubinsztein and Huntington, 2006; Bredesen et al., 2006] Neurodegeneration can be found in many different scales of neuronal circuitry ranging from molecular to systemic. Many neurodegenerative diseases are caused by genetic mutations, most of which are located in completely unrelated genes. In many of the different diseases, the mutated gene has a common feature: a repeat of the nucleotide triplet encoding for the amino acid glutamine (PolyQ). Diseases showing this mutation are known as polyglutamine diseases [Thompson, 2008; Marsh et al., 2009]. I have focused my attention on one specific polyglutamine disease, called Huntington's disease.

2.1 Huntington's disease

Huntington Disease's (HD) is one of the most common inherited genetic diseases that affect 4 to 10 out of 100,000 individuals. It is an autosomal dominant neurodegenerative disorder that was named after George Huntington of Pomeroy, Ohio, who described a hereditary movement disorder in 1872 in the Medical and Surgical Reporter.

The first complete description of the main clinical features and the hereditary nature of the disease come from Charles Oscar Waters [Waters, 1842]:

> It consists essentially in a spasmodic action of all the voluntary muscles of the system, of involuntary and more or less irregular motion of the extremities, face and trunk... The disease is markedly hereditary... The first indications of its appearance are spasmodic twitching of the extremities, generally of the fingers, which gradually extend and involve all the involuntary muscles. This derangement of muscular action is by no mean uniform; in some cases it exists to a greater, in other to a lesser extent, but in all cases gradually induces a state of more or less perfect dementia. When speaking of the manifestly hereditary nature of the disease, I should perhaps have remarked that I have never known a case of it to occur in a patient, one or both of whose ancestor were not, within the third generation at farthest, the subject of this distressing malady...

(The Huntington's disease paper was given at Middleport, Ohio, and published later in the Philadelphia Journal *The medical and surgical reporter*, [Huntington, 1872]).

As described for almost all neurodegenerative diseases, also HD is generally a late onset disease, though juvenile variants can occur. HD is also called Huntington chorea, from the Greek word for *dance*, referring to the involuntary movements that develop as the disease progresses. HD causes progressive loss of cells in areas of the brain responsible for some aspects of movement control and mental abilities. A person with HD gradually develops abnormal movements and changes in cognition (thinking), behavior and personality. Although the disorder itself is not fatal, complications reduce life expectancy to around 20 years after the onset of symptoms. The symptoms of HD fall into three categories: motor or movement symptoms, personality and behavioral changes and cognitive decline. The severity and rate of progression of each type of symptom can vary from person to person. Early motor symptoms include restlessness, twitching and a desire to move about. Handwriting may become less controlled, and coordination may decline. Later symptoms include:

- Dystonia, or sustained abnormal postures, including facial grimaces, a twisted neck, or an arched back

- Chorea, which refers to brief, repetitive, jerky, or dancelike uncontrolled movements caused by muscle contractions that occur as symptoms of several different disorders. The presumed cause of choreic movements is imbalance of dopamine in a set of struc-

tures within the basal ganglia. The basal ganglia belong to a larger part of the nervous system that controls the muscles responsible for normal movement.

- Slowness of voluntary movements, inability to regulate the speed or force of movements, inability to initiate movement and slowed reactions
- Difficulty speaking and swallowing due to involvement of the throat muscles
- Localized or generalized weakness and impaired balance ability
- Rigidity, especially in late-stage disease

Personality and behavioral changes include depression, irritability, anxiety and apathy, and these symptoms often precede the onset of the motor abnormalities [Maio et al., 1993; Kirkwood et al., 2000]. The person with HD may become impulsive, aggressive or socially withdrawn.

Finally cognitive changes include loss of ability to plan and execute routine tasks, slowed thought, and impaired or inappropriate judgment. Short-term memory loss usually occurs, although long-term memory is usually not affected. The person with late-stage HD usually retains knowledge of his environment and recognizes family members or other loved ones, despite severe cognitive decline. HD is one of the several unrelated disorders that lead to imbalances of dopamine in the basal ganglia [Leavitt and Hayden, 2006; Savani and Login, 2007].

2.2 Roles of striatum and cortex impairment in Huntington's disease pathogenesis

During the 160 years since its first description, extensive effort was made by the scientific community in order to shed light on the causes and the mechanisms underlying HD. The research was performed in post mortem brain tissues of HD patients and led to the discovery of considerable shrinkage of the brain and a selective neurodegeneration in the corpus striatum. Henceforth scientists concluded that striatal neurons are the most vulnerable to the mutation causing HD, and that their loss can account for the motoric, psychiatric, and cognitive features of the disease.

Recent discoveries of a degeneration occurring in the deep layers of the cerebral cortex [Vonsattel et al., 1985] now explain cognitive and behavioral impairments better. They show that cortical-related impairments occur prior to the motor striatal ones, and thus that they could also be responsible for the striatal impairments:

1 Cortex and striatum are functionally connected by organized cortico – striatal projections, which serve their function through the excitatory neurotransmitter glutamate. In the 90s, the discovery of perturbations in the synthesis of glutamate by cortico-striatal neurons was one of the first indications of a cortical impairment beyond the striatal degeneration. Indeed, after cortico-striatal connections become dysfunctional, striatal neurons degenerate due to abnormal regulation of glutaminergic excitation [McGeer and McGeer, 1976; Spencer and Havlicek, 1974]. In cortico-striatal neurons of HD patients early changes can be observed. A hallmark of the disease is the accumulation of truncated fragments of a protein called huntingtin, henceforth called mutant huntingtin (mhtt), in the different compartments of the cells. Numerous cortico-striatal neurons projecting to the striatum present accumulation of this protein along the axons [DiFiglia et al., 1997] and abundant dendritic abnormalities [Sotrel et al., 1993].

2 Furthermore, mhtt was found to accumulate more abundantly in the cortex than in the striatum – independent of the pathological grade of the disease; and in early stages of the disease only the cortex presents inclusions while no sign of mhtt accumulation is found in the striatum [Sapp et al., 1999].

3 A further evidence of cortical involvement in the onset of the disease is the observed transcriptional down-regulation of brain derived neurotrophic factor (BDNF), leading to striatal degeneration [Zuccato et al., 2001]. In HD patients BDNF levels, as well as the transport of BDNF along the cortico-striatal connections, are strongly reduced. Indeed mice lacking BDNF mimic the expression profile of human HD [Strand et al., 2007], suggesting that striatal neurons suffer of similar insults in a BDNF-deprived environment.

4 Finally, analyses of post-mortem brains from patients at different stages of the disease showed no correlation between the extent of neuronal loss in the cortex and the degree of atrophy in the striatum, suggesting that the cortical degeneration cannot be a consequence of the

loss of trophic supports from target cells [DiFiglia et al., 1997].

These findings correlate well to the clinical manifestation of the disease, since the early display of cognitive and behavioral disturbances is probably related to impairment of cortical function, which appear before the motor deficits [Lawrence et al., 1998]. Thus, cortical neuronal dysfunction may underlie the initiation of the HD phenotype. Since the findings listed above indicate a major involvement of cortical mechanisms in the etiology of HD, I focused my investigation of the molecular basis of HD on the analysis of the cortical impairments

2.3 Genetic and molecular pathogenesis of HD

2.3.1 Huntingtin and HD

Huntingtin (htt) is a 350 kDa protein codified by the IT15 gene located on chromosome 4p16.3. The gene presents an uninterrupted CAG (**C**ytosin, **A**denin, **G**uanin) tract of trinucleotides repeats in the first Exon; this tract is located 17 codons downstream of the ATG codon.

The mutation underlying HD is an expansion within the CAG region [Mangiarini et al., 1996; Aronin et al., 1995; Trottier et al., 1995b,a]. The CAG triplet codifies for Glutamine, therefore the mutation leads to the expression of an abnormally expanded polyglutamine tract [Group, 1993]. Because of this, HD can be included in a larger group of nine neurodegenerative diseases caused by expanded CAG repeats (for instance: Dentato-rubral and Pallido Luysian atrophy (DRPLA), Spino and Bulbar muscular atrophy (SBMA), and several forms of Spino cerebellar Ataxia (SCA). All these disorders are characterized by selective neuronal death in specific and partially overlapping regions of the brain (including basal ganglia, brain stem nuclei, cerebellum, and spinal motor nuclei). The genes causing them do not share homology regions, beside the polyglutamine stretch itself. The length of the polyglutamine is different in each disease but is generally in the range of 35 to 45. All the mutant proteins, which are ubiquitously expressed in the brain, appear to undergo a conformational change and aggregate in the cells, forming characteristic inclusion bodies [Aronin et al., 1995; Landwehrmeyer et al., 1995].

Concerning htt, the number of CAG repeats present in the Exon 1 can vary between 17 and 20 in healthy humans [Myers, 2004]. A PolyQ in the range of 27 – 35 repeats normally

does not result into pathology, but the instability of the repeats can lead to 36 or more CAG, when transmitted through the paternal line. This is known as *anticipation phenomenon*: the CAG repeats have a very high instability and tend to expand further when passed to the next generation. Commonly patients with adulthood onset of the disease, meaning 30 – 45 years, show a PolyQ expansion in the range of 40 – 50 repeats. Patients with a juvenile onset show a range of 50 or more repeats. The juvenile form of HD is characterized by an early onset and a stronger anticipation phenomenon due to the growing instability of the CAG repeats.

Although a correlation between the numbers of PolyQ repeats and the age at onset of the disease can be observed, incomplete penetrance is registered in individuals with 36 – 41 repeats [McNeil et al., 1997; Quarrell et al., 2007]. In 70% of the cases a higher number of PolyQ correlates with an earlier insurgence and a more severe disease [Schilling et al., 2007; Rubinsztein et al., 1997]. Deviations from this correlation were also observed and related to other modifier genes and environmental factors [Rubinsztein et al., 1997; Wexler et al., 2004].

2.3.2 The structure of wild type and mutated Huntingtin

Both the wild type and mutant forms of the htt protein are expressed mainly in the cytoplasmic compartment of the cell, while only a small portion of the protein can be found in the intranuclear space. In the cytoplasm htt associates with the plasma membrane, the endoplasmatic reticulum (ER), the Golgi apparatus, the mitochondrion, the autophagic vesicles and the microtubules, and its location varies depending on the developmental stage and activity of the cell.

Most of the research on htt studied the PolyQ region. This region was for long considered to be the most important functional part of the gene since an elongated PolyQ region was found in all HD patients [Group, 1993]. To the contrary recent findings suggest that the PolyQ region might not be essential for understanding the roles of htt and that other regions of the gene might represent a more important challenge to identify its functions [Harjes and Wanker, 2003; Clabough and Zeitlin, 2006]. Huntingtin presents the following functional domains:

1 The first 17 amino acids of the N-terminus have been identified as a nuclear localization signal (NLS) and include a binding site for the nuclear exporter Trp (translocated promoter region) [Cornett et al., 2005]. The mutation of htt, being exactly within this sequence of

amino acids, interferes with the binding to Trp, therefore causing accumulation of htt in the nuclei [Xia et al., 2003; Cornett et al., 2005].

2 The poly-Q stretch, which in its mutated form causes HD, is located directly C-terminal of the NLS and is followed by a proline-rich domain CCG; this region is important for ubiquitination and post-translational modifications, suggesting a role in regulating the half-life and nuclear export as well as the toxicity of mhtt [Jeong et al., 2009]. Furthermore this region is important for htt and mhtt-interaction partners that bind through their SH3 domain [Zuchner and Brundin, 2008].

3 The gene has 28 – 36 HEAT repeats [Li et al., 2006; Takano and Gusella, 2002]. HEAT repeat domain is a protein domain found in a number of cytoplasmic proteins including the four that give rise to the acronym HEAT (**H**untingtin, elongation factor 3 (**E**F3), protein phosphate 2A (PP2**A**), and yeast P13 kinase (**T**OR1). HEAT repeats form rod-helical structures, which are important for protein-protein interactions. Furthermore these structures are common for proteins involved in intracellular transport and chromosome segregation [Andrade and Bork, 1995]. Independent studies showed a further role of the HEAT sites in directing htt to various intracellular membrane-bound organelles, such as plasma membrane, mitochondria, endosomal and autophagic vesicles, the Golgi apparatus and the endoplasmatic reticulum (ER) [Kegel et al., 2005; Rockabrand et al., 2007; Atwal et al., 2007].

4 The C-terminus of htt contains a NES sequence (nuclear export signal) [Xia et al., 2003], indicating that the protein is exported to the cytoplasm to exercise its functions.

The analysis of the htt protein sequence and its mutations suggest its involvement in cytoplasmatic pathways such as cytoskeleton reorganization and post-translational modifications.

2.3.3 Huntingtin inclusions and neurodegeneration

Fragments of mhtt have been found in the brains of HD patients and the fact that mhtt undergoes cleavages events was confirmed using a stable inducible cell model of HD (pheochromocytoma 12 cells) [Schilling et al., 2007]. Interestingly, the wild type protein is also cleaved

by many intracellular proteases including caspase 1, 3, 6, 7, 8 and Calpain [Suopanki et al., 2006; Kim et al., 2001; Hermel et al., 2004]. The physiological importance of these cleavages is still unknown. However, for mhtt it has been widely shown that the full-length protein is less toxic than its N-terminal fragments [Gafni et al., 2004; Graham et al., 2006]. Indeed these fragments are considered to be responsible for the formation of nuclear and perinuclear inclusions [DiFiglia et al., 1997], in humans and in mouse models for HD [Davies et al., 1997; Scherzinger et al., 1997], and for the activation of the apoptotic pathway, leading the cells to death.

A major step in the field of HD was the creation of a mouse model (R6 line), by inserting the Exon1 of the human htt gene into mouse embryonic stem cells, under the control of the human htt promoter [Mangiarini et al., 1996]. The R6 lines were created with Exon1 encoding for up to 150 repeats, and although the mice express a construct encoding only the 3% of the full-length htt protein, they display an abnormal phenotype recalling some features of the human Huntington's Disease. The R6/2 mice show an early onset of motor symptoms (5–6 weeks), severe weight loss, general brain atrophy, clasping, tremor, convulsions and a premature death at 13–15 weeks. In addition, neuropathological analysis shows the presence of inclusions/aggregates in the brain already at 4–8 weeks of age. Thus, the R6/2 mouse model is a valuable tool to study the progression of the disease [Davies et al., 1997; Lunkes et al., 1998].

While the toxicity of htt fragments was assessed, the role of inclusions in neurodegeneration remains under debate. In this regard two opposite lines of thoughts were developed:

1 Some evidence suggests that the inclusions of mhtt may prolong the survival of the cells, independent of the location where they form, by reducing the toxic intracellular levels of diffuse soluble mhtt [Arrasate et al., 2004; Ross, 2004]. For instance R6 mice show distributed and prominent intranuclear inclusions of mhtt, but just minor evidence of apoptosis. In support of this hypothesis there are experiments on striatal rat neurons transfected with mhtt. After treatment with BDNF and CNTF (ciliary neurotrophic factor) the neurons showed decreased apoptosis but increased mhtt positive aggregates [Saudou et al., 1998].

2 The opposite theory is based on a direct correlation between inclusions formation and toxicity [Davies et al., 1997]. In particular mhtt sequesters (segregates) many different proteins in

the inclusions, like chaperones, ubiquitin and proteasome-associated proteins as well as transcription factors and cytoskeletal proteins, which would induce an impairment of the degradation system of the cell, possibly leading to neurodegeneration [Ciechanover and Brundin, 2003; Muchowski and Wacker, 2005; Landles and Bates, 2004]. The formation of inclusions along the processes of neurons could furthermore explain why only some neuronal processes selectively degenerate while soma and other processes remain intact. Similarly, selective synaptic loss could depend on the formation of protein inclusions inhibiting the intracellular transport of material otherwise needed to maintain synaptic function.

2.3.4 Physiology and pathology of Huntingtin

The anti-apoptotic effect of htt An essential role of htt for embryonic development was shown. Knockout experiments (mice with an inactivating mutation of the htt gene) show in case of homozygosis embryonic lethality between day 8 and 9. The absence of htt was paralleled by an activation of the apoptotic pathway [Duyao et al., 1995; Zeitlin et al., 1995]. This supports a role of htt as an anti-apoptotic factor. Further evidences show that overexpression of htt in cell models protects against apoptotic insults like starvation, mitochondrial toxins and expanded PolyQ [Rigamonti et al., 2001]. Yet the mechanisms by which htt acts as an anti-apoptotic factor are is not fully understood. Htt may directly inhibit caspase 3 [Zhang et al., 2006] or could act indirectly through the binding to the pro-apoptotic protein Hip-1 (huntingtin-interacting protein 1), which together with HIPP-1 (Hip-1 protein interactor) activates pro-caspase-8 [Gervais et al., 2002].

Transcriptional regulation Studies on models of HD showed that mutated forms of htt lead to impairment of the transcriptional machinery [Harjes and Wanker, 2003; Li and Li, 2004; Kaltenbach et al., 2007]. More in detail, it was shown that mhtt interferes with the transcriptional machinery by inhibiting Sp1, CREB and TFII and TFIIF, thereby controlling the genes downstream of these transcription factors. Although most of the proteins with at least 20 PolyQ were found to be transcription factors, this cannot be the case of htt, because the protein is mainly expressed in the cytoplasm. Yet it could explain why mhtt impairs the transcriptional machinery of the cells. This could be due either to interference of mhtt with htt functions, or the loss of functional htt.

A relevant example of the influence of htt/mhtt on the transcriptional regulation is the control on BDNF transcription, one of the most studied aspects of HD's molecular mechanisms. Over-expression of htt was shown to increase the transcription of BDNF, while mhtt expression leads to decreased BDNF levels [Zuccato et al., 2001, 2003]. In detail the regulation is due to cytoplasmic binding of htt to the complex REST-NRSF[1], and consequent promotion of its translocation into the nucleus with activation of downstream gene transcription. These are channel proteins, receptors for neurotransmitters, BDNF, enzymes for synthesis, structural, and functional proteins involved in the production of synaptic vesicles. However, the mhtt fails to bind the REST-NRSF complex resulting in inhibition of transcription.

In agreement a function of htt in the shuttling and signaling of transcriptional regulators between the nucleus and the cytoplasm was shown and is supported by the presence of HEAT repeats, common for proteins with shuttling functions [Takano and Gusella, 2002; Xia et al., 2003; Truant et al., 2006].

Endocytosis and Vesicle trafficking Recent studies aiming at identifying httinteraction partners have shown that htt interacts with proteins involved in endocytosis and actin cytoskeleton assembly, e.g. PFN2 (Profilin 2) or MIM (HIP13), chaperons, ubiquitin and components of the proteasome [Goehler et al., 2004; Harjes and Wanker, 2003; Ross, 2004; Wanker et al., 1997; Singaraja et al., 2002]. By Western blotting htt was detected in synaptosomal membrane fractions and by immunoelectron microscopy in the membrane of vesicles [DiFiglia et al., 1995; Sharp et al., 1995]. When htt was absent, the vesicles and mitochondria trafficking along neuronal processes were inhibited [Caviston et al., 2007; Gauthier et al., 2004; McGuire et al., 2006], while expressing mhtt inhibited the axonal transport of BDNF, which controls neurotrophic support and endurance of neuronal cells [Gauthier et al., 2004]. Studies aiming at defining the function of htt in endocytosis and vesicle trafficking showed that mhtt interacts and sequesters in the inclusion proteins necessary for vesicle formation and internalization [Velier et al., 1998; Li et al., 1996]. Among the proteins sequestrated by mhtt in the inclusions (Clathrin, ï¿œ-adaptin, PACSIN1, and Dynamin), a well-studied example is HIP-1 (Huntingtin interacting protein 1) [Hussain et al., 2001; Wanker et al., 1997; Meriin et al., 2003]. Direct evidence that HIP-1 is involved in endocytosis comes from HIP-1

[1] complex in the cytoplasm repressor-element-1 transcription factor, neuron restrictive silencer factor

knock-out (HIP-1 -/-) mice, which show defects in assembly of endocytic protein complexes on liposomal membranes [Bhattacharyya et al., 2008]. Furthermore, the interaction of HIP1 with htt is stronger than that observed with mhtt [Hackam et al., 2000]. The binding of htt with HIP 1 seems to be dependent on the length of the PolyQ.

Another important protein sequestrated by mhtt is HAP1 (Htt associated protein), a cytosolic protein important for the retrograde movement of endosomes. Indeed HAP-1 mediates the binding of htt to the dynein/dynactin microtubule-based motor complex, a regulator of the retrograde cellular trafficking [Li et al., 1996; Wanker et al., 1997].

Through the binding with both, HIP1 (involved in actin assembly) and HAP1 (involved in microtubule-based transport), htt might mediate the interactions between actin and microtubule cytoskeleton. Altogether, these studies suggest that htt may have multiple roles in receptor-mediated endocytosis that include actin stabilization, clathrin assembly, and intracellular signaling. At the same time htt appears to be involved in the microtubule- dependent retrograde movement of endosomes. This suggests a crucial role for htt in the cross-talk between the actin microfilament network and the tubulin microtubule cytoskeleton, which is thought to underlie many basic cellular processes important during brain development and adult life. If components of this network become abnormally regulated, this cross talk can have dramatic consequences for neurons [Fulga et al., 2007]. The role of mhtt-mediated impairment of cytoskeleton functions for the pathogenesis of HD remains to date not fully understood.

2.4 The Collapsin Response Mediators

The need of exploiting the cytoskeleton mechanisms influenced by a disease, like HD, resulted in a better understanding of the cytoskeleton function as well. Indeed a recently discovered family of proteins seems promising in showing new insights in the relationship between cytoskeleton and disease.

The family of Collapsin Response Mediator proteins consists of five cytoplasmatic phosphoproteins (CRMPs) [Quinn et al., 1999, 2003].CRMPs are now known to be composed of five homologous cytosolic proteins (CRMP1-5); all of the family proteins are phosphorylated and have their expression peaks from the late embryonic stage to postnatal day 15 (P15), with the highest levels being observed in the forebrain and lesser levels in the cerebellum

[Wang and Strittmatter, 1996]. After this peak in developmental expression, at least CRMP-1 and CRMP4 are constitutively expressed at lower levels throughout adulthood [Wang and Strittmatter, 1996]. The persistent expression of CRMP-1 and CRMP4 in the hippocampus and the cerebellum throughout adulthood implies that they may play a role in adults.

CRMPs bind tubulin heterodimer, whereas phosphorylation of CRMPs by Rho/Rho-associated coil-forming protein kinase, Cdk5, and glycogen synthase kinase-3β(GSK-3β) lowers their binding affinity to tubulin [Fukata et al., 2002a; Uchida et al., 2005]. The CRMPs are also called: dihydropyrimidinase related proteins (DRPs), TOAD64/Ulip/CRMPs (TUC), and unc33 like phospho-proteins (Ulip).

The sequence of the CRMPs shares almost 60 % homology with the human dihydropyrimidinase and with the neuritic outgrowth and guidance related product of the unc-33 gene of C.elegans [Byk et al., 1996; Wang and Strittmatter, 1996; Quinn et al., 2003]. However, no enzymatic dihydropyrimidinase activity has been found for the CRMPs. The CRMPs are known to oligomerize in the brain, forming tetramers and interestingly hetero-oligomerization among the CRMPfamily members is preferred [Wang and Strittmatter, 1997; Leung et al., 2002].

Among the five familiy members CRMP1 – 4 share 70–80 % of amino acid identity [Wang and Strittmatter, 1997], while CRMP5 is less conserved with 50 % identity with the other members [Fukada et al., 2000].

The first member of the family to be discovered in 1995 was Collapsin Response mediated protein 2 (CRMP2), named CRMP62 / TOAD-64 [Minturn et al., 1995a]. It was identified as a mediator of the collapsin-1 / semaphorin 3A cascade [Goshima et al., 1995], a family of guidance cues regulating dendritic growth and axon guidance during neuronal development [Goshima et al., 1995; Cnops et al., 2006]. Subsequently Collapsin Response Mediator Protein 4 (CRMP4), also known as dihydropyrimidinase-like protein 3 (DPYSL3), dihydropyrimidinase related protein 3 (DRP-3) TUC-4 and Ulip-1, was identified as a protein regulated by nerve growth factor (NGF) or retinoic acid, during neuronal differentiation [Minturn et al., 1995b; Byk et al., 1996; Gaetano et al., 1997]. The other two members CRMP1 and CRMP3 were identified by homology to CRMP2 and CRMP4 [Wang and Strittmatter, 1996].

Roles of CRMP4

Neurogenesis Quinn et al. found in 1999 that CRMP4 is highly upregulated during early corticogenesis in the embryonic rat brain. In accordance, CRMP4 is strongly expressed during brain development and its expression reaches a peak during the first post-natal week, then decreasing until the adulthood [Charrier et al., 2003]. In adulthood the expression of CRMP4 is restricted to the regions where neurogenesis occurs, e.g. dentate gyrus of the hippocampus and the granular layer of the olfactory bulb. Increased levels of CRMP4 have been found in motor neurons after sciatic nerve lesions, pointing to a possible role of CRMP4 in neuronal regeneration [Minturn et al., 1995a].

Cytoskeleton dynamics The interaction of CRMP4 with cytoskeletal proteins tubulin and actin suggests an important role in cell assembly [Rosslenbroich et al., 2005; Franken et al., 2003]. Among the CRMPs, CRMP2 and CRMP4 proteins have been shown to co-localize with F-Actin in growth cones of different types of neuronal cells [Goshima et al., 1995; Minturn et al., 1995a; Yuasa-Kawada et al., 2003]. CRMP4 organizes F-Actin into tight bundles, important for cellular migration [Rosslenbroich et al., 2005]. Actin polymerization and dynamics are clearly important in any cytoskeleton rearrangement and for maintaining morphology and survival of the cell. Loss of actin bundling is a relevant event occurring in growth cone collapse, a process that correlates with a profound actin rearrangement in the peripheral domain of growth cones [Zhou and Cohan, 2001].

The expression of CRMP4 is enriched in the growth cone where it partially colocalizes with synaptic vesicle protein 2 (SV2) and Intersectin, involved in regulation of Actin dynamics [Hussain et al., 2001; Quinn et al., 2003; Fulga et al., 2007]. In addition, experiments in which the N-terminal variant of CRMP4 was over-expressed show that CRMP4 induces an increase in neurite branching [Quinn et al., 2003]. Furthermore Rosslenbroich et al. demonstrated in 2003 a localization of the protein in lipid rafts, regions known to have an important role in maintaining the growth cone and in synaptic plasticity.

CRMP4 is a target of Calpain, and experiments in primary neuronal cultures have shown that, in the presence of glutamate excitotoxicity and oxidative stress, Calpain is responsible for the truncation of CRMP4 [Kowara et al., 2005]. The proteolysis mediated by Calpain under normal conditions has a role in synaptic plasticity, long-term potentiation, migration,

proliferation, differentiation and apoptosis [Chan and Mattson, 1999; Vanderklish and Bahr, 2000], all of which are impaired in the context of degeneration. Calpain is also involved in the inhibition of neuritic outgrowth, by proteolytic activation of different kinases and destruction of lipid raft scaffolds resulting in growth cone collapse [Shea, 1994; Rosslenbroich et al., 2003]. The degradation of CRMP4 mediated by Calpain could, therefore, be associated with the disruption of the lipid rafts scaffold in the induction of growth cone collapse [Kowara et al., 2006].

These results show an important link between CRMP4 and cytoskeleton. In particular, CRMP4 is known to control Actin dynamics and to participate to exo-/endocytosis, neurite outgrowth, branching and synaptic plasticity.

Since cytoskeleton dynamics could be important in the pathogenesis of HD (See subsection 2.3.4 on page 17), and given the connections of CRMP4 with cytoskeleton dynamics (listed above), I have focused my attention on the role of CRMP4 in HD molecular mechanisms.

2.5 Cytoskeleton alteration and neurodegeneration

The cytoskeleton defines the highly asymmetrical shape and structural polarity of neurons, essential for neuritogenesis, cell signaling, and physiology. A rearrangement in the Actin cytoskeleton identified as Actin dynamics, allows neurite outgrowth in unpolarized neurons. The way Actin dynamics is induced or inhibited by regulating factors is to date not known. Several proteins have been identified and show the ability of inducing changes in the structure of Actin

The proteins of the ADF (adenosine diphosphate)/Cofilin (AC) family bind Actin after the release of inorganic phosphate (Pi). Pi is released when monomers of globular Actin (G-Actin) assemble in filamentous structures (F-Actin). The AC complex binds cooperatively F-Actin, inducing a twist in the filament conformation that enhances the subunit turnover from the minus end and promotes filament disassembly. The structure modification occurring when the AC complex binds F-Actin removes the phallodin-binding site and results in the loss of dye staining [Minamide et al., 2000]. Furthermore profilin (PFN), a G-Actin binding protein, catalyzes the exchange of Actin-bound ADP to ATP, priming the monomer of G-Actin for polymerization. Indeed the inhibition of PFNIIa during neuronal polymerization

promotes destabilization of the Actin cytoskeleton and thereby neurite formation [Yarmola and Bubb, 2009]. Interfering with Actin dynamics and stability has been shown to have dramatic consequences in migration, axon formation, failure in building neurites and loss of axonal tracts [Witte and Bradke, 2008; Benitez-King et al., 2004].

In neurodegenerative diseases, the cytoskeleton is often abnormally assembled leading to impairment of neurotransmission, e.g. in Alzheimer's two major lesions, namely amyloid plaques and neurofibrillary tangles, contain several cytoskeletal components, composed of paired helical filaments, with abnormally phosphorylated tau protein, a component of microtubules [Small and Duff, 2008; Jellinger, 2008; Iqbal and Grundke-Iqbal, 2008]. Furthermore Lewy bodies, markers of Parkinson's disease and Lewy Body Dementia (LBD) are primarily built of cytoskeletal proteins: synuclein, tubulin, Microtubule associated protein (MAP) 1, and MAP2 [Jellinger, 2008]. Inclusions containing the Actin-depolymerization factor (ADF), cofilin, and Actin were found in postmortem brains and in cultured hippocampal neurons of Alzheimer's Disease (AD) [Minamide et al., 2000]. Tau-induced neurodegeneration is associated with F-Actin and the formation of Actin -rich rods in models of tauopathy [Fulga et al., 2007]. In addition F-Actin and Actin-associated proteins are major constituents of Hirano's bodies in human brains. Hirano's bodies are paracrystalline rod-shaped intraneuronal inclusions observed more frequently in Alzheimer's disease and in a number of other neurodegenerative diseases, including Pick's disease [Galloway et al., 1987; Goldman, 1983]. This suggests that alteration of Actin cytoskeleton might be a critical step in tau-mediated neurotoxicity in Alzheimer's disease and related disorders [DiProspero et al., 2004] and suggests that Hirano bodies are derived from an abnormal organization of the neuronal cytoskeleton [Maciver and Harrington, 1995].

HD and Neurofilaments Concerning the implication of cytoskeleton dysfunction in HD, recent studies on postmortem brain have shown a progressive loss of neurofilaments staining in the cortex already of early stage HD patients [DiProspero et al., 2004]. The importance of neurofilaments in neuronal cells is mostly on the mechanical support and velocity conductance [Elder et al., 1998]; the loss of neurofilaments induces neuronal atrophy and reduction in axonal caliber, features that have been observed in HD tissue [Dom et al., 1976]. Even more the accumulation of neurofilamentous material was observed in dystrophic HD neurites in the

cortex of HD patients; such as the neurofilament aggregates found in other neurodegenerative diseases [Jackson et al., 1995a,b]. More specifically Nagai et al. found in 1999 that PolyQ domain proteins bind neurofilaments and disrupt their assembly.

HD, Microtubules, and Microfilaments Since the shape of dendritic branches in pyramidal cells of the cortex and medium spiny neurons of the striatum are altered in HD, the studies on alteration of the cytoskeleton machinery have focused on the Microtubules and Microfilaments. The Microtubules are polymers of α and βtubulin that, with the support of MAPs, undergo rapid changes in length and stability in an energy dependent manner. A loss of α-tubulin was found by analyzing the postmortem brain of HD patients; this loss could be explained by the sequestration of α-tubulin in the mhtt inclusions or by an alteration of the regulatory protein MAP2 [DiProspero et al., 2004; Tukamoto et al., 1997]. The expression of MAP2 is crucial for the growth direction of dendrites, and an alteration of its expression or function due to mhtt expression can impair the growth of dendritic branches, leading to the alterations observed in cortical neurons of HD's patients [Graveland and DiFiglia, 1985].

HD and endocytosis Actin is one of the major proteins of the cell and plays an important role in maintaining morphology, motility, exo- and endocytosis. Recently researchers have shown that Actin dynamics plays an important role in triggering mhtt aggregation and toxicity. 2003 Meriin et al. studied a yeast model expressing a polypeptide derived from htt with 103 repeats. They found that inclusion formation leads to a rapid cessation of endocytosis and that specific mutations associated with endocytosis can delay the polyglutamine aggregation process, suggesting that this pathway is involved in the formation of the inclusions. Endocytosis is an active cellular process responsible for the control of plasma composition, cellular signaling and nutrient uptake. In the yeast circa 50 proteins are known to cooperate for the formation of an endocytic vesicle and its scission. The process can be divided in two main steps, of which the first is driven by proteins that trigger the formation of the endocytic complex (EC) at the cytoplasmic side of the plasma membrane, proteins like Lis17/WASP. The second phase of endocytosis starts upon activation of the Arp family and leads to Actin polymerization and internalization of the vesicle. Here we find proteins like Sla1, Sla2/HIP-1, Arp2/3 and F-Actin. Meriin et al. show that when the factors involved in the first step of endocytosis become down-regulated, this leads to a reduction of htt inclusions, suggesting that

these factors are necessary for the formation of PolyQ aggregation. This result was also confirmed in HEK293T cells expressing polyglutamine with application of siRNA against WASP Meriin et al.; DiProspero et al.; Shao et al.. Furthermore loss of Profilin, an Actin regulating protein, in a pre-symptomatic stage of HD mouse models, induces changes in Actin dynamics and correlates with altered neurotransmitter disease and loss of dendritic spines [Guidetti et al., 2001; Burnett et al., 2008].

CRMP4 represents another important modulator of Actin dynamics, the protein binds to F-Actin promoting Actin bundling and maintaining. Dramatic Actin depolymerization has been observed in the absence of CRMP4 in the B53 Neuroblastoma cell line[2]. Therefore, I have concentrated my analysis on the role of CRMP4 protein in HD.

2.6 Aim of the thesis

The study of cytoskeleton alterations in the field of neurodegenerative diseases has been gaining importance by the discoveries of the last decade. Neurodegenerative diseases are highly complicated to decode mostly for their late onset: Although the disease is possibly there from birth, symptoms become visible typically after 30 years or later. Concerning Huntington's disease the genetic mutation has been discovered long ago, yet a major research break trough has not been achieved. To date we are far from being able to prevent the insurgence of the symptoms and from curing the disease. It seems that the body does not perceive large parts of damage progression until the degeneration has reached a severe stage.

Therefore it is important to learn as much as possible about the progression and the art of these alterations and its 'invisible' drivers and consequences, expressed in cellular modifications. We have several reasons to believe that the cytoskeleton's function is impaired, for the cells undergo morphological changes, observed in the curved processes of medium spiny neurons (MSN). We see inclusion formation, morphological impairments, isolated processes, synaptical dysfunctions. The brain presumably compensates these alterations until a certain point – the severe symptoms appear.

CRMPs revealed more of the cytoskeletal mechanisms. I focused my attention on a member of the CRMP family, the CRMP4. It is important for the stability of the Actin cytoskeleton

[2]Cell line originated from neuroblastoma Group B, File 53, [Rosslenbroich et al., 2005]

and crucial for the development of neuronal processes in the brain. The expression of CRMP4 in adulthood is restricted to brain regions that are important for neurogenesis and neuronal plasticity and CRMP4 is involved in the response of cells to excitotoxic stimuli. These interactions motivated me to investigate CRMP4 as possible modulator of mhtt aggregation and toxicity. In order to have a better overview on the project I would like to give a short outline on the main questions we asked: Firstly we asked whether CRMP4 expression levels are altered in the presence of mhtt in a cell and mouse model for HD. Since it is widely known that mhtt recruits specific cytoskeletal proteins in the inclusions, we analyzed whether this was the case also for our candidate. CRMP4 is indeed an important actin-binding protein like Profilin2, studies on Profilin2 show that it can modulate the formation of the mhtt-inclusion [Burnett et al., 2008], therefore we investigated whether CRMP4 also behaves in our cell model for HD as an inclusions-modulator. Finally a work by Kowara et al. in (2005) show that CRMP4 is cleaved by Calpain-1 under glutamate toxicity and oxidative stress, considering this important finding we asked whether this cleavage is also important for the interaction between CRMP4 and mhtt in our cell model for HD.

3 Materials and Methods

3.1 Materials

3.1.1 Reagents and Chemicals

Product Name	Supplier
Ponasteron A	Invitrogen
Phosphate buffered saline	Dulbecco
Bovine serum albumin	Sigma Aldrich
Lipofectamine 2000	Promega
PreScission protease, 2 mM)	GE Healthcare
Normal Mouse Serum	Dako Cytomation
RPMI	Biochrom
Fetal Calves Serum	Biochrom
Percoll	Amersham Biosciences
Sodium azide	Merck
Glutathione-agarose beads	Sigma Aldrich
Dithiothreitol (DTT) 100 mM	Roche
ALLN (LLnL, Calpain Inhibitor I)	Sigma

3.1.2 Kits

Product Name	Supplier
Caspase 3/7 assay Apo-ONE	Promega
RNeasy mini kit	QIAGEN
AMBION kit	Applied Biosystems
Cell Fractionation kit	Invitrogen
Bradford Protain Assay	Bio-Rad Laboratories GmbH

3.1.3 Antibodies

The following antibodies were used for immunofluorescence and Western blotting analyses. Rabbit polyclonal anti Htt antibodies: CAG53b and HD1 [Scherzinger et al., 1997; Davies et al., 1997], mouse anti-Htt MAB5374 clone mEM48 (Chemicon), mouse monoclonal anti-HA 12CA5 (Roche Diagnostics), rabbit anti-CRMP4 (TUC-4, Chemicon), anti-GAPDH (Sigma, St. Louis, MO), anti-CRMP2 (Abcam), anti-CRMP1, mouse monoclonal anti-Actin AC40 (Sigma), mouse monoclonal anti- b-tubulin (Sigma), and mouse monoclonal anti- Flag (Sigma). Alexa 549 and Alexa488 conjugated secondary antibodies were from Invitrogen. The antibodies CRMP1,2, HD1, CAG53b (Rabbit) were gently provided by the research group of Prof.E.E. Wanker.

Antibody	Source	Supplier	Working Dilution
mEM48	Mouse	Chemicon	1:400
HA 12CA5	Mouse	Roche Diagnosis	1:400
CRMP3	Rabbit	Abcam	1:400
CRMP4	Rabbit	Chemicon	1:400 – 1:2000
CRMP5	Mouse	Abcam	1:400
Actin	Mouse	Sigma	1:400 – 1:10000
Tubulin	Mouse	Sigma	1:400 – 1:1000
Flag	Mouse	Sigma	1:400
TexasRed Phalloidin		Molecular Probes	1:100
Ubiquitin	Rabbit	Dako Cytomation	1:500
Map2	Mouse	Millipore	1:500
TuJ1	Mouse	Covance	1:500

Antibody	Source	Supplier	Working Dilution
GFAP	Rabbit	DAKO	1:1000
α-Olig	Mouse	Mitte	1:300
CD11b	Mouse	Serotec	1:50
Alexa 488 phalloidin		Invitrogen	1:100
Alexa 549	Rabbit	Invitrogen	1:500
Alexa 488	Mouse	Invitrogen	1:500
Alexa 488	Rabbit	Invitrogen	1:500
Alexa 405		Invitrogen	1:500
Alkaline Phosphatase-conjugated secondary Abs		Invitrogen	1:10000
Vectashield DAPI mounting medium		Vector	1:1

3.1.4 Bacteria and Vectors

Plasmids were generated using commercially available Gatewayï¿œ technology (Invitrogen). The entry clones from RZPD[1]; CRMP4; IOH54070, and GAPDH; RZPDo839G08126D were shuttled into the destination vectors pTLHA1-D-48 and pFLAG-CMV2. His7-tagged and GST-tagged proteins were constructed by shuttling the entry clones into the destination vectors pDESTco and pGEX-6p-D21, respectively. The GST-Htt1Q51 plasmid was previously described [Busch et al., 2003; Horn et al., 2006].

Product Name	Supplier
E.coli BL21(DE3)pLysS strain (Stratagene)	Promega
Vetors pcDNA3.1-HD1Q68	QIAGEN
Vector pTLHA1-D-48 - PA, HA encoding htt CRMPs	Invitrogen Gateway®
Vector pDESTco	Invitrogen Gateway®
pGEX-6p-D21	Invitrogen Gateway®
pFLAG-CMV2	Invitrogen Gateway®

[1] `http://www.imagenes-bio.de/search`

Product Name	Gently provided by
CRMP4 clone IOH54070	Research group Prof. E.E Wanker (MDC)
GAPDH clone RZPDo839G08126D	MDC
GST-Htt1Q51	MDC
TAPHD510Q17	MDC
pCAG-Mcs-N1-hCRMP1-Ires-EGFP	Department of Experimental Neurology (EN)
pCAG-Mcs-N1-httexon1Q83-Ires-DsRed	EN
pCAG-Mcs-N1- httexon1Q17-Ires-DsRed	EN

3.1.5 Tools and Equipment

Product Name	Supplier
ABI-Prism7100 Sequence-detection system	Applied Biosystems
Centrifuge	Eppendorf,Germany
	Heraeus/Instruments,Germany
Incubator	Binder GmbH
Heat Plate	Heidolph, Germany
Heat Bath	GFL Germany
Light Microscope	Carl Zeiss MicroImaging GmbH, Germany
Confocal Microscope Leica DM 2500	Leica Microsystems
pH meter	WTW, Germany
Sterile Bench	Heraeus Instruments
Vortex	Jahnke and Kunkel, Germany
Image Reader	Fuji
Blotting chamber	Trans-Blot Semi-Dry Transfer Cell, Bio-Rad Laboratories GmbH
Cell culture incubator	Nuaire, COTECH
Criterion cassettes	Bio-Rad Laboratories GmbH
Criterion precast gel, 4–20 %	Bio-Rad Laboratories GmbH

Product Name	Supplier
Multi-well cell culture plates	Falcon
Nitrocellulose membrane, 0.2 mm	Bio-Rad Laboratories GmbH
Blotting chamber	Trans-Blot Semi-Dry Transfer Cell, Bio-Rad Laboratories GmbH
Cell culture incubator	Nuaire, COTECH

3.1.6 Buffers

Ammonium chloride lysis buffer Ammonium chloride 82.9 g, potassium bicarbonate 10.0 g, EDTA disodium salt 0.37 g, distilled water ad 1.0 liter, pH adjusted to 7.2 to 7.4 using 1 M NaOH or 1 M HCl, and stored at 4 °C.

Stock isotonic percoll solution Prepared by adding 9 parts of Percoll to 1 part of 10 × concentrated PBS solution. Further, 30 % and 70 % of Percoll solutions were prepared by necessary amount of adding 1 × PBS solution.

Lysis buffer mice PBS containing 1mM EDTA, 0.1 mM PMSF and 0.05 % Tween 20, lysozyme 1 mg/ml /Roche/ and DNA-se I at (10 U/ml), final concentrations.

Lysis buffer cells 50 mM Hepes at pH 7.5, 150 mM NaCl, 2 mM $MgCl_2$, 25 U/ml Benzonase, 1 % Tween20 and Protease inhibitors (Roche)

PLL-solution 5ml Poly-L-Lysin (Biochrom) 0.1 mg/ml, with 100 ml PBS (1×) without Ca^{2+} and Mg^{2+} and incubation for 1 hour at room temperature (Kappa).

$\frac{1}{2}$ **PLL-solution** 10 ml Poly-L-Lysin (Biochrom) 0.1 mg/ml with 390 ml PBS (1×) without Ca^{2+} and Mg^{2+} and incubation over night in the fridge.

Collagen-Medium A new bottle of DMEM 25 ml FKS Gold (from PAA) 5 ml Pen/Strep 5 ml Hepes 1M (Biochrom) 5 ml CollagenG (Biochrom)

NBM (Neurobasal Medium) + B27 Neurobasal medium, add 10 ml B27 (supplement from Biochrom), 5 ml Pen/Strep (Biochrom), 1.25 ml L-Glutamin (Biochrom)

Starter Medium NBM-B27 but with 500 µl of Glutamate concentrated 25 mM

N-Med 4 g of glucose, DMEM 50 ml FKS 5 ml Pen/Strep 5 ml L-Glutamine 1.25 ml Insulin 5 ml Hepes 1M (Biochrom)

Trypsin Trypsin-EDTA (10×) from Biochrom. Diluted 1:10 with PBS without Ca^{2+}

TEA buffer 1× 40 ml TEA 50×, 2 l of destilled water.

LB Medium ca.500 ml of destilled water, 10 g NaCl, 10 g Trypton (Pepton), 5 g yeast extract

Laemmli sample buffer 1 M Tris/HCl, pH 6,8 10 % (v/v) Glycerol, 2 % (w/v) SDS, 5 % (v/v) beta-mercapthoethanol, 30 µM Bromophenol blue.

Coomassie blue 0.2 % Coomassie blue, 7.5 % Acetic acid and 50 % Ethanol

Destaining solution 10 % ethanol and 0.75 % Acetic acid

AttoPhos buffer (Europa Bioproducts).

Digestion buffer 50 mM KCl, 10 mM Tris HCl, pH 8.3, 2.5 mM $MgCl_2$, 0.1 mg/ml BSA, 0.45 % v/v NP40, 0.45 % Tween 20

Permeabilization solution 0.3 % Triton X-100

Blocking solution PBS containing 2 % bovine serum albumin (BSA) and 10 % normal donkey serum (NDS).

Fixative solution 4 % PFA in PBS, pH 7.4 or 100 % methanol

Growth Medium Dulbecco's modified Eagle's (DMEM) supplemented with 5 % FCS, 100 u/ml Penicillin/Streptomycin.

Maintenance Medium DMEM with 10 % Horse Serum, 5 % fetal bovine serum, 1 % penicillin/streptomycin, 200 µg/ml G418, 2 mM glutamine and zeocin

RIPA-buffer 50 mM Tris/HCl pH 7.4; 50 mM NaCl; 10 mM EDTA;1 % IGEPAL; 0.1 % SDS; 0.25 % sodium deoxycholate

Artificial cerebrospinal fluid buffer aCSF; 134 mM NaCl, 2.5 mM KCl, 1.3 mM $MgCl_2$, 2 mM $CaCl_2$, 1.2 mM K_2HPO_4 10 mM Glucose and 26 mM $NaHCO_3$

NP40 buffer 50 mM Tris, pH 7.5, 150 mM NaCl, 1 % Nonidet P-40, 0.25 % sodium deoxycholate, 0.2 % sodium dodecyl sulfate, 1 mM EDTA, 2.5 × Benzonase (Merck) and Complete Protease-Inhibitor (Roche)

PBS 4.8 g of it 500 ml destilled water

TRITON 10X 4.5 ml of destilled water. 500 µl of triton stock. For staining: 0.3 %.

DEPC water 1ml DEPC. 1 L destilled water.

3.1.7 Software

Product Name	Supplier
ImageJ	National Institutes of Health, public domain
AIDA Image Analyzer	Raytest
SPSS version 14	SPSS inc

3.1.8 Mouse Model: R6/2

The R6/2 transgenic mice were purchased from the Jackson Laboratory and bred locally. Mice were fed food and water ad libitum and subject to a 12 hrs light/dark cycle.

Brain lysates from R6/2 tg mice aged 2, 4, and 6 weeks were generously provided by Claus Zabel (Charite Institute for Human Genetics, Charité – Campus Virchow)

3.2 Methods

3.2.1 DNA Extraction and purification

E.coli cells containing plasmid were cultured in sterilized LB-medium with an appropriate antibiotic, ampicillin (100 µg/ml) or kanamycin (30 µg/ml), ON at 37 °C. Small-scale preparations (3 ml culture) of plasmid were performed by the alkaline lysis method [Birnboim and Doly, 1979]. Large-scale (200 ml culture) preparations of plasmid DNA were performed using the Plasmid Maxi-Kit from Qiagen. All procedures for this were carried out according to the manufacturer's protocol. The concentration and purity of the DNA were determined by UV-spectrophotometry. The isolation of DNA fragments from agarose gel was performed with Quiaquick Gel Extraction Kit (Quiagen) or with NucleoSpin-Extract II-Kit (Machery-Nagel). The DNA was eluted with 10-25 µl TE and stored at -20 °C.

3.2.2 Cell Culture: PC12 cells

An inducible mutant Htt-expressing PC12 cell line was used as a model for Huntington's Disease for the *in vitro* experiments. PC12 are a stably-transfected cell line with a construct encoding truncated exon 1 (first 17 aa plus polyQ repeat) of htt, containing a range of polyQ repeats encoded by alternating CAG_CAA repeats (25-103 Qs), and fused in frame to the coding sequence for the enhanced GFP (EGFP) tag at the carboxyl terminus [Apostol et al., 2003]. The cells were maintained in high glucose Dulbecco's Modified Eagle's medium (DMEM) containing 10 % Horse serum, 5 % fetal bovine serum, 1 % penicillin/streptomycin and 200 mg/ml G418, 2 mM glutamine and zeocin at 37 °C and 10 % CO_2. Htt expression was induced with 3 µM Ponasterone A (PonA, Invitrogen) for 48 hrs. Plasmid transfection was performed using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions.

3.2.3 Immunocytochemistry

For immunocytochemistry, the PC12 cells were washed with phosphate-buffered saline (PBS) and fixed in 4 % paraformaldehyde for 15 minutes at room temperature. Unspecific binding was blocked and cells were incubated with primary antibodies against CRMP4 (rabbit anti-TUC-4 1:400, Chemicon) and Htt (mEM48 1:400, Chemicon) overnight at 4 °C. After washing with PBS, the secondary antibodies were applied for 1 hr at room temperature (donkey anti-rabbit IgG Alexa594 conjugated antibody 1:500, Invitrogen). Both induced and not-induced PC12 cells were stained for filamentous Actin, visualized with Texas red-phalloidin (Molecular Probes 1:100). Phalloidin was added after the secondary antibodies to the cells and incubated for 30 minutes. PC12 cells were transfected with pGEx-CRMP4 construct using Lipofectamine protocol (Promega). Two days after the transfection, the cells were fixed and stained with specific antibodies against CRMP4 and Htt, followed by specific secondary antibodies. The R6/2 mice 12 weeks of age were deeply anesthetized and perfused with with an ice-cold fixative solution of 4 % PFA in PBS, pH 7.44. The brains were removed, post-fixed in 4 % PFA for 4 hours and subsequently cryoprotected in 30 % sucrose. Serial sections of 30 µm were cut using a vibratome (Leica) and free-floating sections were collected in PBS for staining. The slices were permealized with 0.3 % Triton X-100 and blocked in PBS containing 2 % bovine serum albumin (BSA) and 10 % normal donkey serum (NDS). After blocking, the following primary antibodies were applied overnight at 4 °C: EM48 (1:200, Chemicon), anti-CRMP4 (1:400, Chemicon), and anti-Ubiquitin (1:500, Dako Cytomation). The secondary antibodies were applied for 1 hr at RT (donkey anti mouse IgG Alexa 549 conjugated antibody or donkey anti-rabbit IgG Alexa488 conjugated antibody, 1:500, Invitrogen). All the images were taken with the confocal microscope Leica DM 2500, and pictures were merged using the ImageJ software.

3.2.4 Quantification of htt aggregates

For the quantification of Htt aggregates in PC12 cells, the samples were lysed in lysis buffer containing: 50 mM Hepes at pH 7.5, 150 mM NaCl, 2 mM $MgCl_2$, 25 U/ml Benzonase, 1 % Tween20 and Protease inhibitors (Roche). Htt aggregates were analyzed by the filter-trap assay. The signal was visualized by fluorescence under blue light in an Image reader (Fuji) and

quantified using the AIDA program. The cells were also analyzed performing visual counts of EGFP-positive aggregates using fluorescent microscopy. At least 600 cells were counted from 3 independent experiments. Aggregation values are presented as the percentage of the cells with aggregates versus total number of EGFP-positive cells. All the images were taken with the confocal microscope Leica DM 2500, and pictures were merged using the ImageJ software.

3.2.5 Dissection and freezing of tissue

For cortex dissection in brains of 12 weeks old mice, both wild-type and transgenic mice were killed by cervical dislocation, and their brains were quickly removed and placed in artificial cerebrospinal fluid buffer (aCSF; 134 mM NaCl, 2.5 mM KCl, 1.3 mM $MgCl_2$, 2 mM $CaCl_2$, 1.2 mM K_2HPO_4 10 mM Glucose and 26 mM $NaHCO_3$), bubbled with 95 % O_2/5 % CO_2. Subsequently, the brains were sliced using a vibratome and 300 µm-thick pieces were collected in aCSF buffer. The cortex was dissected under light microscopy and rapidly frozen in liquid nitrogen.

3.2.6 SDS-PAGE and Western blotting

Frozen cortical tissues from R6/2 transgenic mice were homogenized in modified RIPA-buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.4, 1 % NP-40, 0.25 % sodium deoxycholate, 0.2 % sodium dodecyl sulfate, 1 mM EDTA, 2.5 x Benzonase /Merck/ and Complete Protease-Inhibitor /Roche/) at 1:10 w/v. The brain lysates was incubated on ice for 30 minutes and centrifuged at 6000 x *g* for 5 minutes at 4 °C. PC12 cells expressing httexon1Q25 and httexon1Q103 cells were induced with 3 mM Ponasterone A for 48 hrs. The cells were washed with cold PBS and lysed in ice-cold NP40 buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 % Nonidet P-40, containing protease inhibitors and benzonase) for 30 minutes. Total protein concentrations were determined using Bio-Rad protein assay and the lysates were adjusted to 12 µg/µl of total protein. Equal amounts of extract were analyzed by SDS-PAGE and Western blotting. Samples were diluted in an equal volume of 2x Laemmli sample buffer (1 M Tris/HCl, pH 6.8 10 % (v/v) Glycerol, 2 % (w/v) SDS, 5 % (v/v) s-mercapthoethanol, 30 µM Bromophenol blue, followed by 5 minutes at 100 °C. The proteins were separated by

SDS-PAGE and for protein staining after electrophoresis separation, the gel was incubated in Coomassie blue (0.2 % Coomassie blue, 7.5 % Acetic acid and 50 % Ethanol) for 1 hr. Afterward the gel was destained in the destaining solution consisting of 10 % ethanol and 0.75 % Acetic acid for 30 minutes. For performing western blotting analysis, the proteins separated in the gel were transferred by electrophoresis onto Protran BA 83 nitrocellulose membrane (Schleicher and Schuell). The membranes were then incubated in blocking solution for 1 hr at room temperature. Subsequently, the immunoblots were incubated with primary antibodies overnight and followed by a secondary antibody conjugated to alkaline phosphatase.

Primary antibodies were diluted in PBS, 0.05 % Triton-X-100. The following antibodies were used: anti- CRMP4 (1:2000), anti-Htt (CAG5b, HD1; 1:2000), anti-Tubulin b (1:5000) and anti-Actin (1:5000). The blots were incubated overnight at 4 °C, followed by alkaline phosphatase-conjugated secondary antibodies (1:10000).

For detection of the staining results, the membranes were incubated for 1–3 minutes in an AttoPhos buffer (Europa Bioproducts). The signal was visualized by its fluorescence under blue light in an Image reader (Fuji) and quantified using the AIDA software.

3.2.7 Protein expression and purification

All proteins were expressed in the *E.coli* BL21(DE3)pLysS strain (Promega). Each recombinant protein was tested before producing it at large scale. Bacteria from glycerol stocks containing the expression vector were plated on LB-agar plates, with the appropriate antibiotics (ampicillin, Amp at 100 μg/ml concentration), and allow to grow overnight at 37 °C. One colony was picked up and resuspended in 100 ml LB-medium containing Amp at 100 mg/ml concentration and grown at 37 °C overnight. The next day, this culture was used to inoculate 1.5 L of LB-Amp medium, containing 20 mM MOPS/KOH pH 7.9, 0.2 % glucose and 20 μg/ml thiamine. The culture was grown until optical density (OD) measured at 600 nm reached a 0.4–0.6 value. Then, the expression of recombinant proteins was induced by adding 1 mM IPTG for 4 hrs at 37 °C. The cells were then centrifuged at 4000 × *g* at 4 °C for 15 min. The bacterial cell pellet was resuspended in lysis buffer (PBS containing 1 mM EDTA, 0.1 mM PMSF and 0.05 % Tween 20, lysozyme 1 mg/ml /Roche/ and DNA-se I at (10 U/ml), final concentrations). The suspension was lysed twice using French Pressure and the lysates were centrifuged at 50 000 × *g*, 30 min, 4 °C. The tagged-proteins were purified under native con-

ditions by affinity (using Gluthatione-Sepharose for GST-tagged proteins or Ni-NTA standard protocols for His7-tagged proteins) and size exclusion chromatography. Western blotting and Coomassie Blue staining monitored the status of purified proteins.

GST-HttexQ51 with PolyQ of different lengths were expressed in *E.coli-BL21* (DE3)-pLysS (Promega) and purified to ca. 95 % homogeneity on glutathioneagarose beads (Sigma). Full-length CRMP4[2] was shuttled into the destination vector pDESTco according to Invitrogen Gateway® technology protocols. The His7-CRMP4 and His7-CRMP2 proteins were expressed in *E.coli-BL21* (DE3)pLysS (Promega), and affinity purified using affinity and size exclusion chromatography.

E.coli-BL21 cells containing plasmid pDESco-CRMP4, His7 Tag and pGex-6p-D21-CRMP4, GST Tag, with ampicillin resistance, were cultured in sterilized LB-medium with an appropriate antibiotic, ampicillin (100 µg/ml) or kanamycin (30 µg/ml), ON at 37 °C. Small-scale preparations (3 ml culture) and large-scale (100 ml culture) preparations were performed. The protein expression was induced with 1 mM IPTG (o.n 22 °C). Incubate overnight. The expression and purification of the protein was controlled by Coomassi and SDS gel analysis

[2]clone IOH54070, `http://www.imagenes-bio.de`

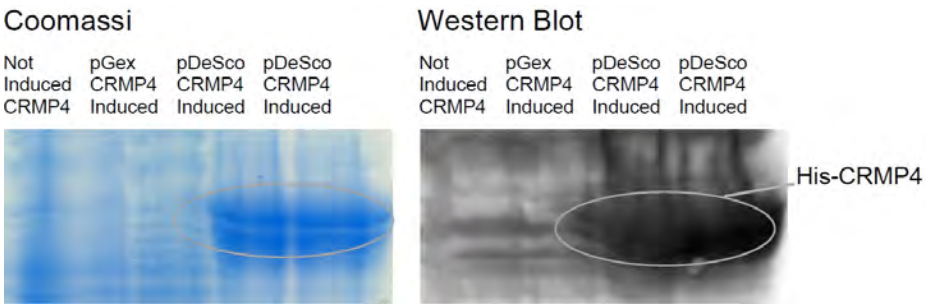


Figure 3.1: Control of CRMP4 Small Scale protein purification, by Coomassi and Western Blot Analysis

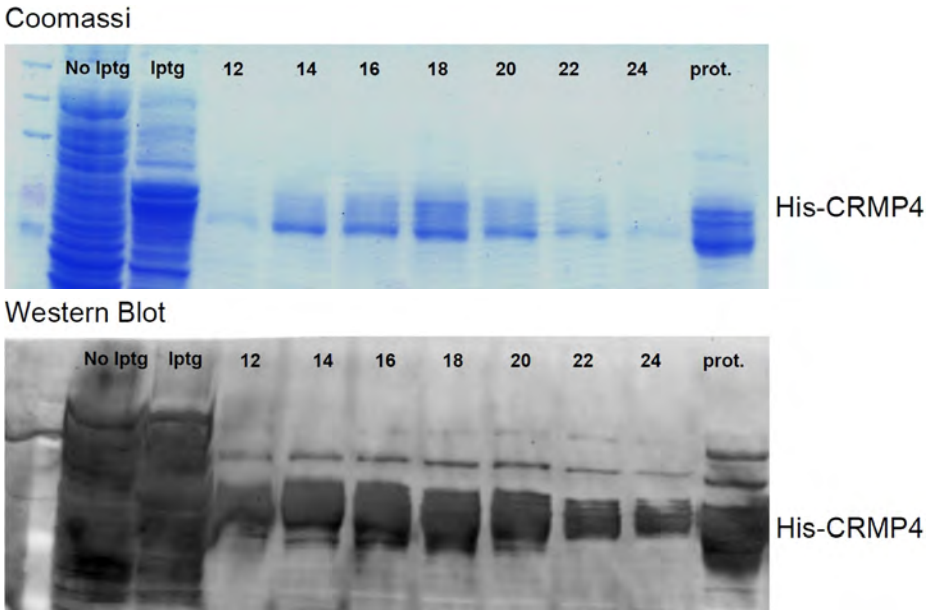


Figure 3.2: Control of CRMP4 Big Scale protein purification, by Coomassi and Western Blot Analysis

3.2.8 Filter retardation assay

To detect the formation of insoluble aggregates with high molecular weight, Scherzinger and Wanker developed 1997 a simple filter assay. The idea of developing such assay comes from the finding that the proteolytic cleavage of GST-httexon1Q51 leads to the formation of SDS-insoluble aggregates retained on cellulose acetate filter, while no insoluble aggregates are observed upon proteolytic cleavage of GST-httexon1httQ20.

GST-httexon1Q51 fusion protein [Scherzinger et al., 1997] was incubated with PreScission protease (2 mM) for 3 hrs at 8 °C to remove the GST-Tag. After cleavage the samples were incubated with or without His7-CRMP4 at 30 °C for indicated time points.

The aggregation reactions were boiled in 2 % SDS, 0.5 mM DTT for 5 min at 100 °C. Aliquots were diluted in 200 µl of 0.2 % SDS and filtered through cellulose acetate membranes of 0.2 µM pore size using a BRL dot blot filtration unit. Afterwards, two washes with 200 µl of 0.2 % SDS were performed in each well. The SDS-resistant aggregates retained on the filter were detected using anti-CAG53b or anti-HD1 huntingtin antibodies, followed by an anti-rabbit secondary antibody conjugated to alkaline phosphatase.

3.2.9 Atomic Force Microscopy (AFM)

GST-httexon1 fusion proteins were incubated with Prescission protease to induce polyQ-meditated protein aggregation [Busch et al., 2003]. Aliquots were spotted on freshly cleaved mica (Nanoworld, Germany), glued to a microscope slide and htt aggregation samples of 20 µl were adsorbed to a surface of freshly cleaved mica ($5 \times 5\,mm^2$) for 2 minutes, washed with freshly filtered deionized water ($4 \times 30\,\mu l$) and dried over night. Salts and unbound materials were removed with three washes by adding 30 µl of water and immediately absorbing excess liquid into filter paper. AFM images were recorded in intermittent contact mode using a Nanowizard II / Zeiss Axiovert setup (JPK, Berlin, Germany) II scanning probe microscope (JPK, Berlin, Germany). Overviews ($30 \times 30\,\mu m^2$) were recorded before detailed pictures of representative areas were recorded for each sample. AFM images were recorded on a using intermittent contact mode and FEBS cantilevers (Veeco, Santa Barbara, CA).

3.2.10 Cell toxicity assay

For the detection of apoptotic cells, the Apo-ONE Homogenous caspase 3/7 Assay (Promega) was used according to manufacturer's procedures. PC12 were seeded in 96 well plates after 24 hrs incubation. The cells were transfected with empty vector or with CRMP4 cDNA using Lipofectamine. The following day the cells were induced with 3 mM ponasterone. Afterwards, the cells were incubated with a bifunctional cell lysis/activity buffer containing the pro-fluorescent substrate Z-DEVE-R110, which can be cleaved by active caspase 3/7, revealing a fluorescent group. The caspase 3/7 activities were quantified in a fluorometer with excitation set at 499 nm and emission at 521 nm. The amount of fluorescent product generated was assumed to be proportional to the amount of active caspase 3/7 present in the assay.

3.2.11 Real Time Quantitative PCR

Total RNA was extracted from PC12 cells and purified using Trizol® reagent RNeasy mini kit (QIAGEN) following the manufacturer's protocol. Cell pellets were dissolved in Trizol® reagent and digested for 20 minutes at room temperature. Chloroform was added to the samples, which were vigorously mixed, and incubated for 15 minutes at room temperature. After centrifugation, the aqueous phase was transferred into new tubes, and isopropanol was added to precipitated RNA. RNA was washed with 75 % ethanol, and finally resuspended in ultrapure water. 1 µg total RNA was purified from DNA contamination by performing digestion with DNase enzyme for 30 minutes at 37 °C, and then stopped for 10 minutes at 65 °C. After the digestion step, a total of 1 µg purified RNA was transcribed to cDNA using a Reverse Transcription kit AMBION for one hour at 37 °Cand then immediately transferred to 4 °C.

Quantitative Real-Time PCR was carried out for 40 cycles (15 sec at 95 °C, 1 min at 60 °C). Expression levels of each target gene were normalized to the expression of the 18S housekeeping gene. Q-PCR reactions were performed in triplicate on an ABI Prism 7100 Sequence Detection System using Taqman Universal PCR Master Mixer (Applied Biosystems) and specific Taqman probes. Specific primers to rat CRMP4 were used (Applied Biosystems). CRMP4 transcript levels were calculated using the AACT method.

3.2.12 Co-Immunoprecipitation

For the verification of endogenous Htt-CRMP4 interactions, PC12 cell pellets from the Q103 strain and the control Q25 strain (induced and not-induced) were resuspended in the Hepes lysis buffer containing 1 % NP-40 (50 mM Hepes at pH 7.5, 150 mM NaCl, 2 mM MgCl2, 25 U/ml Benzonase and Protease inhibitors, Roche). The tubes were places on ice for 30 minutes and centrifuged for 5–10 min, at 20 000 g and 4 °C to remove insoluble cell debris. The supernatant was transferred to precooled tubes and diluted with 4× SDS-sample buffer, referred to as "initial". Before co-immunoprecipitation, GFP-Trap®M magnetic beads (Chromotek) were washed three times in lysis buffer. Afterwards, cell lysate were incubated with anti-HD1 antibody for 1 hr at 4 °C. As controls, non-immune serum, polyclonal anti- synaptobrevin or no primary antibody were used. The supernatant was diluted with 4× SDS buffer and referred to as "unbound". The beads were washed twice with ice-cold dilution buffer and resuspended in 2× SDS-sample buffer. The protein complexes were heat-denatured with 6× Laemmli sample buffer at 90 °C for 10 min and collected by magnetic separation for 2 minutes at 4 °C, referred to as "bound". The supernatant was analyzed by SDS-PAGE and Western blotting was performed with a monoclonal anti-Htt antibody (rabbit CAG53b; 1:1000, rabbit HD1) or a rabbit anti-CRMP4 antibody.

3.2.13 Transgenic mice and Genotyping

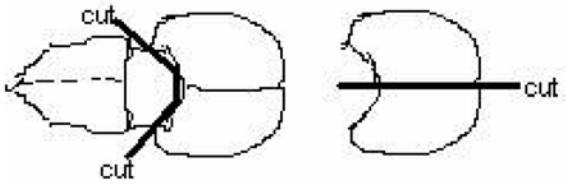
All animal experiments were performed following animal protection regulations. The R6/2 transgenic mice were purchased from the Jackson Laboratory and bred at the MDC. Mice were fed food and water ad libitum and subject to a 12 hrs light/dark cycle. The genotyping and phenotype analyses were performed as described previously [Suopanki et al., 2006]. The Biopsies of the mouse tails were digested with proteinase K (0.6 µg/ml) in 200 µl DNA digestion buffer (50 mM KCl, 10 mM Tris HCl, pH 8.3, 2.5 mM $MgCl_2$, 0.1 mg/ml BSA, 0.45 % v/v NP40, 0.45 % Tween 20) at 55 °C overnight. The genotyping of successive offspring was assessed on tail DNA using the commercial primers J1 and J2 and Expand high fidelity polymerase from Roche[3]. The amplification reaction conditions were as follows:

[3]The Jackson laboratory, `http://www.jax.org`

Step	temperature	duration
1	94 °C	3 min
2	94 °C	35 sec
3	61 °C	45 sec
4	72 °C	45 sec
5	Go to 2	15 times
6	94 °C	35 sec
7	55 °C	30 sec
8	72 °C	45 sec
9	Go to 6	35 times
10	72 °C	4 min

3.2.14 Primary Cortical Neuron Preparations

Before the culture, plates were coated with Poly-L-Lysin and Collagene. Embryos at Embryonic day 16 (E16) were dissected, and the cortex was carefully isolated after removal of the meningis. Cortex was collected and washed with PBS. Moreover the cortex was mechanically dissociated by re-suspension with a glass pipette in N-Med (see section 3.1.6 on page 31), after 15 minutes incubation at 37 °C with diluted Trypsin-EDTA.



The dissection was followed by centrifugation and re-suspension of the cells in starter medium (see section 3.1.6 on page 31). The suspension was filtered through 0.2 µm filters. A cell counting chamber was used to count cell density of cultured cells. The cells in a single-cell suspension were mixed with Trypan Blue to mark dead cells. Viable cells exclude Trypan Blue, while dead cells stain blue due to Trypan Blue uptake. Cells are loaded onto a counting chamber covered with a cover slide and then counted under a microscope. Concentration was around 3×106 cells/ml. After rinsing of plates twice with PBS, cortical cells were plated onto

24-well plates (for immunochemistry) or onto 6-well plates (for Western Immunoblotting experiments) in wells in starter medium Cultures were kept at 36.5 °C and 5 % CO_2, and were fed every four days, from the fourth day *in vitro* (DIV 4) on with cultivating medium (starter medium without glutamate) by replacing half of the medium. The condition of the cultures was assessed by light microscopy, prior to experiments. In my primary cortical cell culture system, as demonstrated by immunocytochemistry (using antibodies against glial fibrillary acidic protein (GFAP) for astrocytes, CD11b for microglia, A-Olig for oligodendrocytes and Map2/TuJ1 for neurons) neuronal purity was always higher than 70 % until DIV 12; Less than 10 % astrocytes until DIV 12, less than 2 % oligodendrocytes until DIV 12 and no microglia were present.

3.2.15 Nucleofection of neurons

Gene transfection per Nucleofection was done on the Day 0 after preparation of the primary cortical neurons. Cells were transfected following the AMAXA protocol for primary cells isolated from mouse embryonic brain. After transfection, cells were plated at high density.

4 Results

4.1 PC12 cell model of Huntington's Disease

Cell lines, which express neuronal properties, are useful model systems for studying the nervous system at the single cell and at the molecular levels. Such lines have been established from human and murine neuroblastomas and from rat central nervous system tumors. The Pheochromocytoma cell line (PC12), a well known cell model, widely used for studying polyglutamine diseases *in vitro*, was established by [Greene and Tischler, 1976] from a transplantable rat adrenal pheochomocytoma. The cell line we used is stably transfected and expresses an inducible httexon1Q103 or httexon1Q25 coupled with the enhanced-green-fluorescent-protein (EGFP-carboxyl-terminal, [Apostol et al., 2003]). The expression of httexon1-PolyQn-EGFP is induced upon addition of Ponasteron A (PonA) to the culture medium.

In order to confirm the validity of the cell model, I induced the expression of mutant (httexon1Q103) and native (httexon1Q25) forms of htt exon1, and 48 hrs later I investigated the presence of mhtt inclusions. I observed that the cells expressing httexon1Q103, but not httexon1Q25, showed some pathogenic features of Huntington's disease like the formation of mhtt inclusions in the perinuclear space, the cytoplasm and the cell processes (Figure 4.1A,B). Indeed 48 hrs post-induction, 55 % of the cells showed redistribution of mhtt into the inclusions. No EGFP expression was detected when PonA was not added to the medium of PC12 cells expressing httexon1Q103 or httexon1Q25 (Figure 4.1C).

The inclusions shared two major features with those observed in patients: they contained short fragments of the mutant protein, and they were ubiquitinated (data not shown). This implies that a processing mechanism operates in these cells to generate a fragment with high aggregation potential.

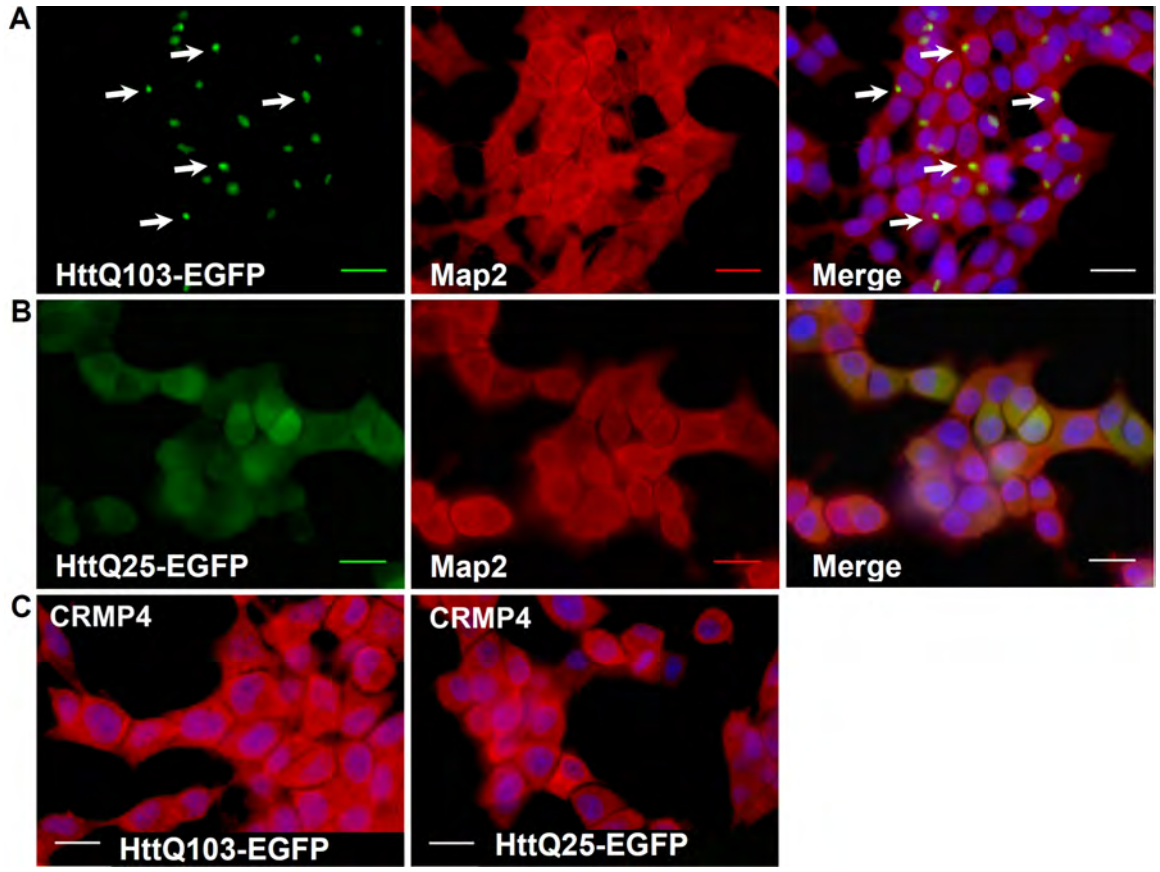


Figure 4.1: httexon1Qn expression in PC12 cells. **A** and **B** PC12 cells expressing httexon1Q103 and httexon1Q25 were analyzed after induction with PonA. EGFP expression was confirmed by fluorescence microscopy, and PC12 cells were stained with specific antibodies for Map2. Nuclei were counterstained with DAPI. **A** EGFP-positive aggregates were detected in the cytoplasm and the perinuclear space when the expression of httexon1Q103-EGFP was induced. **B** No EGFP-positive aggregates were observed with httexon1Q25-EGFP. The expression of EGFP was diffuse in the cell cytoplasm. **C** Non induced PC12 cells expressing httexon1Q103 and httexon1Q25 were analyzed. CRMP4 immunoreactivity expression was detected mainly in the cell cytoplasm and no expression of EGFP was present. Scale bar 10 µm

The formation of the aggregates can be observed also by analyzing the cells using Western blotting and filter- trap assay of total cell lysates incubated with CAG53 antibody (which binds mutant and native htt). After 48 hrs of induction with 3 μM of PonA, the maximal effect of polyglutamine expression can be observed. The cell lysates of PC12 cells encoding httexon1Q103 or httexon1Q25 were tested for the formation of SDS-insoluble aggregates. Figure 4.2A shows that the mutant htt ran at higher molecular weight compared to httexon1Q25 and formed aggregates that remained trapped in the filter.

The formation of htt-induced aggregates shown by immunocytochemistry, Western blot and cell-free assay coincides with the activation of the apoptotic pathway. Figure 4.2B shows that PC12 cells expressing httexon1Q103 have a dramatic activation of Caspase 3/7 when compared to non-induced cells expressing httexon1Q103 and cells expressing httexon1Q25 (data not shown).

All these evidences support the notion that our cell model can be used for studying Huntington's disease pathogenesis.

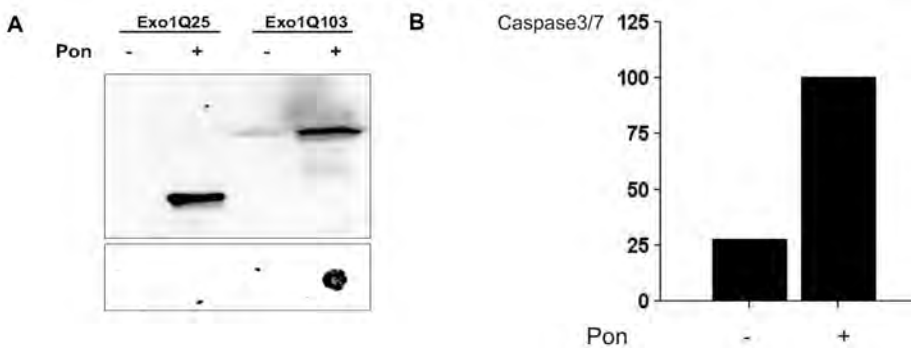


Figure 4.2: httexon1Qn expression in PC12 cells and activation of the apoptotic pathway **A** In the presence of PonA the expression of httexon1Q25 and httexon1Q103 is detactable in PC12 cells. **B** The expression of httexon1Q103 induces caspase 3/7 activation as shown in the graph.

4.2 CRMP4 is specifically down-regulated in PC12 cells

Several studies show that htt aggregation is linked to disease progression in HD and to the development of cognitive and motor symptoms [Davies et al., 1997; Sánchez et al., 2003]. Therefore, cellular target proteins of mutant htt might represent potential modulators of disease pathogenesis and serve to explain the cellular events triggering neurodegeneration.

Alterations of cytoskeleton organization have been shown to contribute to early changes in HD pathology [Zabel et al., 2008; Burnett et al., 2008]. Indeed studies on post-mortem HD brains revealed that the levels of neurofilaments and tubulin are dramatically decreased. The detrimental effect of mutant htt on cytoskeleton stability could be either triggered by a direct interaction of htt with cytoskeletal proteins or due to the loss of cytoskeletal regulator proteins, which may be sequestered in the htt-induced aggregates. Therefore, studying the interaction of htt with proteins involved in cytoskeleton organization might help to understand the cellular dysfunction in HD. The CRMP family has been connected to the regulation of cytoskeleton dynamics; more precisely, CRMP2 and CRMP4 bind to Actin and Tubulin and modulate the dynamics of microtubules and microfilaments [Rosslenbroich et al., 2005; Fukata et al., 2002a]. Therefore I decided to investigate whether the induction of mhtt, in cells stably expressing Ponasterone A (PonA)-inducible httexon1Q103-EGFP/Q25-EGFP [Apostol et al., 2006], influences the protein levels of CRMP2 and CRMP4. The expression of httexon1Q103 (or httexon1Q25) was induced for 48 hrs and the total lysates of induced and non-induced cells were analyzed by SDS-PAGE and immunobloting using antibodies specifically recognizing CRMP2 and CRMP4. When PC12 cells encoding for httexon1Q103 were used, the expression levels of CRMP4 were diminished by 70 %±10 % 48 hrs after induction with PonA, while CRMP2 expression was not influenced. A semiquantitative analysis of these results is shown in Figure 4.3. As a negative control, I analyzed PC12 cells encoding for httexon1Q25, and I confirmed that the expression levels of CRMP4 and CRMP2 remained unchanged. (Figure 4.4)

In conclusion, the reduction of CRMP4 is specific to mhtt expression, suggesting that the interaction between CRMP4 and mhtt might be involved in the cellular events related to HD pathogenesis.

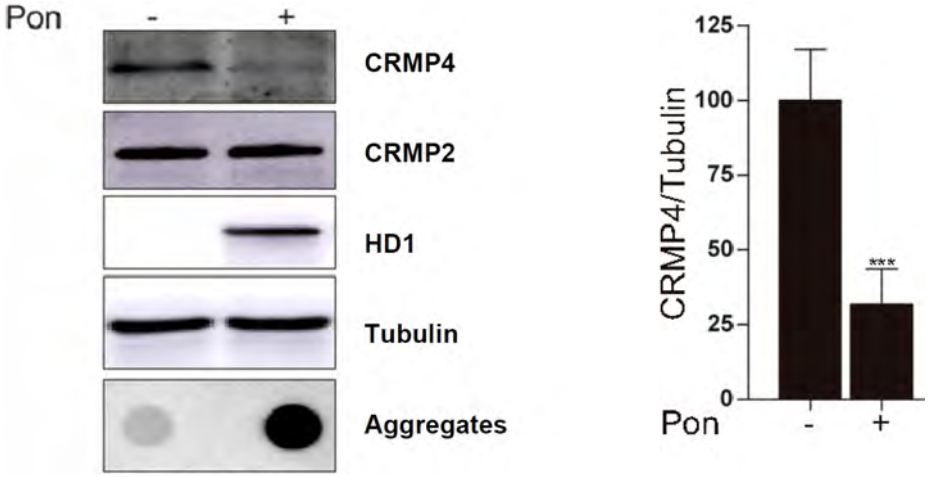


Figure 4.3: CRMP4 is down regulated in the PC12 cells expressing httexon1Q103. PC12 cells expressing httexon1Q103 were induced with PonA (+). Non-induced PC12 cells expressing httexo1Q103 were used as control (-). Cell lysates were analyzed by Western blotting using CRMP4- or CRMP2-specific antibodies. A strong down-regulation of CRMP4 expression was observed in induced PC12 cells, while no changes in CRMP2 levels or tubulin levels were detected. PonA induced PC12 cells expressing httexon1Q103 expressed mhtt as revealed by HD1 staining and formed insoluble aggregates in the filter assay. The expression of CRMP4 was reduced by 70 %±10 %, when normalized to tubulin expression. $P < 0.001$

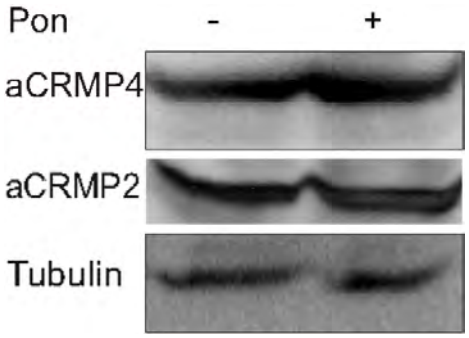


Figure 4.4: CRMP4 and CRMP2 expression is not influenced by httexon1Q25. PC12 cells expressing httexon1Q25 were induced with PonA (+). Non-induced PC12 cells expressing httexon1Q25 were used as control (-). Cell lysates were analyzed by Western blotting using CRMP4- or CRMP2-specific antibodies. No difference in the expression levels of CRMP4 and CRMP2 were observed between induced and non-induced PC12 cells expressing httexon1Q25. Tubulin expression served as control

4.3 Down-regulation of CRMP4 expression occurs at the protein level

Mutant htt has been shown to abnormally interact with several nuclear proteins and transcription factors, sequestering them into the aggregates and thereby inhibiting their transcriptional activity [Glass et al., 2000; Luthi-Carter et al., 2000]. Indeed transcriptional dysregulation is a key feature of HD [Cha, 2007], described both for HD patients and for R6/2 transgenic mice [Glass et al., 2000; Luthi-Carter et al., 2000].

In light of the importance of transcriptional dysregulation in HD, I decided to analyze whether the loss of CRMP4 protein, observed in induced PC12 cells expressing httexon1Q103, was a result of reduced CRMP4 transcription. Therefore, I isolated total RNA from PC12 cells expressing httexon1Q103, PonA-induced and non-induced. I prepared cDNA and quantified the CRMP4 transcript by Real-Time Quantitative PCR (RT-Q-PCR). I did not find any significant differences in the mRNA levels of CRMP4 in induced and non-induced httexon1Q103 PC12 cells, suggesting that down-regulation of CRMP4 occurs at the protein level (Figure 4.5).

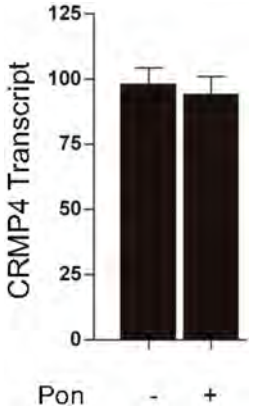


Figure 4.5: Down-regulation of CRMP4 is not detectable at the mRNA level after mhtt expression. The mRNA expression levels of CRMP4 were analyzed in PC12 cells expressing httexon1Q103, induced and non-induced. Cells were cultured for 48 hrs and the mRNA levels of CRMP4 were measured by RT-Q-PCR. PonA induced expression of httexon1Q103 (+) did not change CRMP4 mRNA levels compared with non-induced controls (-).

4.4 Co-localization of CRMP4 with mHtt aggregates in HD cell models

The expanded polyglutamine is believed to confer a new function to htt that is toxic to the cell. This hypothesis finds confirmation in the fact that the mutant protein, either in its soluble or in its insoluble form, has been shown to disrupt several intracellular pathways by abnormally interacting and sequestering their key components into the aggregates.

Since the expression of CRMP4 mRNA was unchanged by mhtt, I analyzed whether the reduction in CRMP4-protein expression could depend on a redistribution of the protein into mhtt-induced inclusions. As I previously described the induction with PonA for 48 hrs led to the sequestration of mhtt into perinuclear aggregates forming in 55 % of the cells (see section 4.1 on page 45). The aggregates were visible already 24 hrs after induction and a strong activation of the apoptotic pathway was observed at 48 hrs in these cells. By immunocytochemistry

of PC12 cells expressing httexon1Q103-EGFP, 48 hrs after induction, I observed that many htt-induced inclusions were immunoreactive for CRMP4 (Figure 4.6). Counting the number of CRMP4 +/ httexon1Q103+ inclusions, among all inclusions, I observed that CRMP4 is present in almost 40 % of the mhtt-induced inclusions.

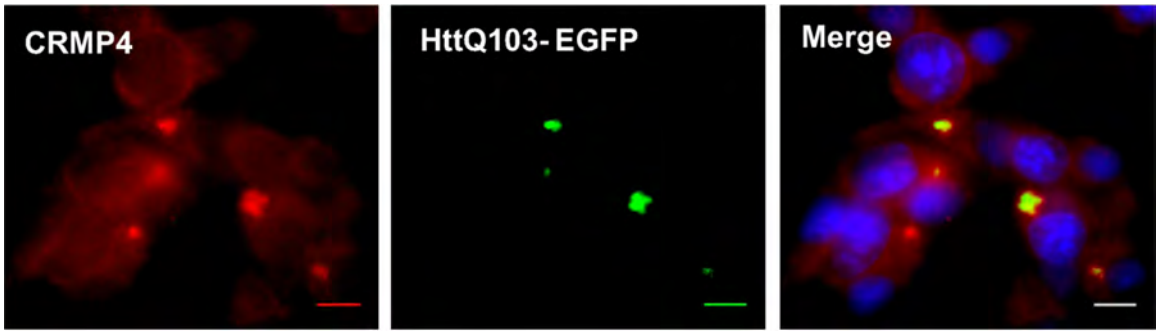


Figure 4.6: CRMP4 colocalizes with mhtt in the aggregates formed in PC12 cells. PC12 cells expressing httexon1Q103-EGFP were induced with PonA and cultured for 48 hrs. The formation of GFP+ aggregates was assessed by fluorescence microscopy, and colocalization with CRMP4 immunoreactivity was shown by overlay (CRMP4: red, EGFP: green, DAPI: blue). Scale bar 10 µm

This suggests that endogenous CRMP4 protein can partially re-locate from the cytoplasm into mhtt inclusions. Mhtt might interfere with the normal distribution and function of CRMP4 protein in the cells and thereby with the function of CRMP4-interacting partners.

4.5 CRMP4 down-regulates the formation of SDS-stable mutant htt aggregates *in vitro*

The relationship between inclusion formation and neurodegeneration remains a big debate in the field of HD research; this is due to the conflicting evidence that a direct correlation between inclusion formation and induction of toxicity can be observed [Davies et al., 1997], whereas that at the same time formation of inclusions can actually prolong the survival of the cells [Arrasate et al., 2004; Ross and Poirier, 2004].

Since CRMP4 can be incorporated into mhtt-induced inclusions, I decided to test whether CRMP4 directly influences the formation of SDS-insoluble aggregates.

To test this, a well-established cell-free aggregation assay using purified proteins was performed [Scherzinger et al., 1997; Wanker et al., 1997]. Htt protein with normal (Q20) or

pathogenic (Q53) polyQ length was produced as soluble GST-FLAG- protein and purified under native conditions by affinity chromatography [Busch et al., 2003].

The purity of the protein was assessed using SDS-Page and Coomassi blue staining (Figure 4.7).

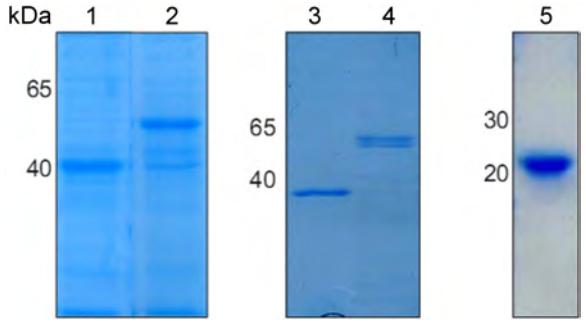


Figure 4.7: Quality assessment of proteins utilized for *in vitro* aggregation assays GSTFLAG-httexon1Q20 (lane 1), GST-FLAG-httexon1Q53 (lane 2), His7-GAPDH (lane 3), His7-CRMP4 (lane 4) and GST (lane 5).

The recombinant proteins contain a unique cleavage site for PreScission Protease® (PP) situated between the GST and the FLAG-htt-exon1 protein (Figure 4.8 A).

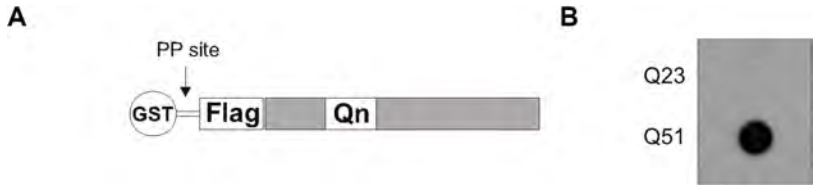


Figure 4.8: Purified GST-FLAG-httQ(n) protein: **A**. Purified GST-FLAG-httQ(n) protein possesses a PreScission protease site (PP, cartoon), **B**. aggregation of httexon1Q53, but not of httexon1Q20 can be monitored by filter-trap assay.

The absence of additional internal cleavage sites for PP was confirmed, allowing the use of these GST- and His7-proteins in the filter-trap assay. The GST increases the solubility of htt, therefore loss of GST stimulates the formation of SDS-insoluble aggregates in the case of pathological polyQ lengths. Httexon1Q53 and 20 were cleaved with PP for 3 hrs at 8 °C, and after cleavage the samples were incubated for 16 hrs at 30 °C to allow the formation of SDS-insoluble aggregates. As expected, PP-cleaved-httexon1Q53 at the concentration of 2 µM and after the loss of GST, resulted in the formation of SDS-resistant aggregates, whereas no aggregates were observed with PP-cleaved httexon1Q20 [Busch et al., 2003], (Figure 4.8B).

In order to analyze the effects of CRMP4 on mhtt aggregation, I incubated GST-httexon1Q53 with equimolar concentration of His7-CRMP4 or BSA (negative control) and PP at 8 °C for 3 hrs. Afterwards, the samples were transferred to 30 °C to allow the formation of SDS-

insoluble aggregates. The reaction was monitored after 6 and 12 hrs of incubation. CRMP4 was able to reduce the formation of SDS-insoluble aggregates by 30±5 % after 6 hrs and by 50±5 % after 12 hrs. Addition of an equimolar concentration of BSA had no effect on the formation of SDS-insoluble aggregats (Figure 4.9A).

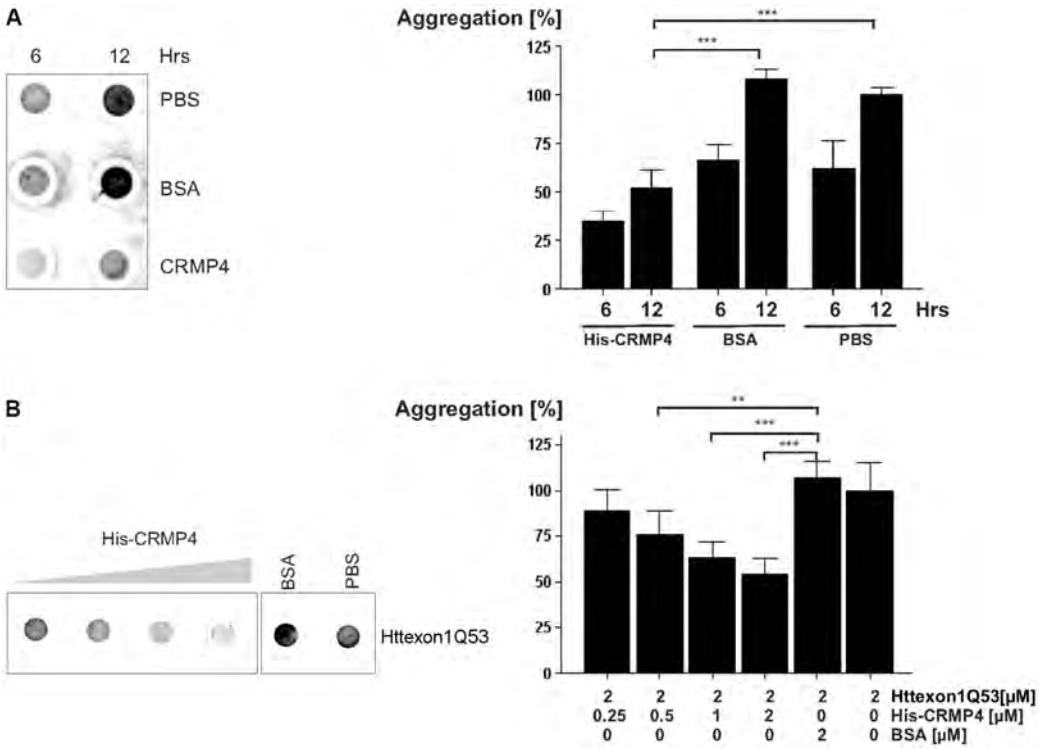


Figure 4.9: CRMP4 down-regulates the formation of polyQ-mediated htt aggregation *in vitro*. **A**. GSThttexonQ53 was incubated with either His7-CRMP4 or BSA as control, and after addition of PP to the reaction mixture, the formation of mhtt aggregates was monitored. CRMP4 reduced the amount of SDS-insoluble aggregates formed by mhtt in a time dependent manner. Addition of BSA had no effect on the aggregation process. A reduction in the amount of aggregates by 50±5 % was observed for a 1:1 molar ratio of httexon1Q53:His7-CRMP4 *** ($P < 0.001$). **B**. The effect of CRMP4 on the formation of httexon1Q53-induced SDS-insoluble aggregates was also concentration-dependent ($P < 0.001$).

In another experiment, I observed that the effect of CRMP4 on the formation of SDS-insoluble aggregates by httexon1Q53 was highly concentration-dependent (Figure 4.9B). In contrast, CRMP2 did not interfere with the formation of SDS-insoluble aggregates by httexon1Q53(Figure 4.10).

Next, I checked the effects of CRMP4 on the morphology of the mhtt-induced aggregates by using Atomic Force Microscopy (AFM). Less fibrils were formed when His7-CRMP4

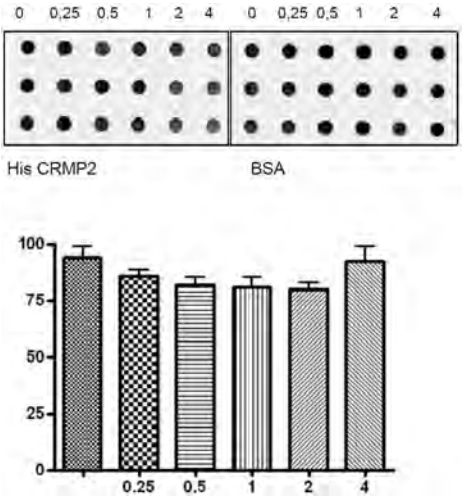


Figure 4.10: CRMP2 has no effect on the formation of polyQ-mediated htt aggregation *in vitro*. GST-httexon1Q53 was incubated with either His7-CRMP2 or BSA as control, and after addition of the PP to the reaction mixture, the formation of mhtt aggregates was monitored. CRMP2 had no effect on the formation of SDS-insoluble aggregates. The same was true for BSA. Data from 3 independent experiments were quantified and are shown in the graph.

was incubated together with httexon1Q53 protein, whereas addition of BSA to the incubation mixture did not have any effects on the morphology of the aggregates (Figure 4.11).

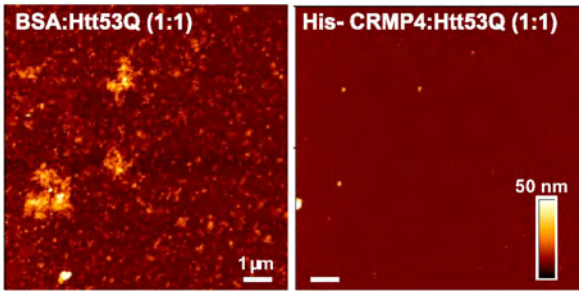


Figure 4.11: AFM analysis: CRMP4 diminished the amount of httexon1Q53-induced aggregates when used at equimolar concentrations (1:1) whereas BSA had no effect on the process of aggregation.

As it is well known that members of the CRMP family can homo- and hetero-oligomerize [Wang and Strittmatter, 1997; Leung et al., 2002], I also tested the His7-CRMP4 protein alone under the same conditions to exclude that the results were due to self-aggregation of CRMP4. No fibrillar or amorphous aggregate structures were observed under these conditions (data not shown). Together, these results demostrate that CRMP4 can suppress the aggregation of mhtt *in vitro* .

4.6 CRMP4 reduces aggregation and toxicity induced by mutant htt in PC12 cells

I observed that CRMP4 expression was strongly reduced by mhtt and that CRMP4 was sequestered in the htt-mediated inclusions formed in PC12 cells; furthermore, I found that CRMP4, but not CRMP2, directly influences the formation of SDS-insoluble mhtt aggregates in a cell-free *in vitro* system. Therefore, I decided to investigate whether over-expression of CRMP4 could modulate the formation of mhtt inclusions and the mhtt-induced toxicity in PC12 cells expressing httexon1Q103-EGFP. I transfected PC12 cells with a construct encoding for FLAG-CRMP4 and as controls I used cells transfected with empty vector encoding only FLAG and untransfected cells. 12 hrs after transfection, I induced the cells with PonA for 48 hrs. The efficiency of the transfection was assessed by Western blotting. Immunodetection with htt-specific antibodies revealed that over-expression of the target protein CRMP4 did not influence the expression of mhtt protein (Figure 4.12A).

Counting 2.000 cells in independent visual fields, there was no significant difference in the number of EGFP-positive cells with aggregates between untransfected httexon1Q103-EGFP cells and transfected cells with empty control vector. In CRMP4-transfected cells expressing httexon1Q103, the number of EGFP-positive aggregates was reduced by 40±10 % with respect to the controls (Figure 4.12B).

These results suggest that CRMP4 also reduces the aggregation of mhtt in a cell culture model of HD. Therefore, the down-regulation of endogenous CRMP4 levels, by mhtt (see chapter 4.2) might strongly increase the formation of mhtt aggregates.

Since mhtt aggregates have been shown to be toxic for the cell [Sánchez et al., 2003], I decided to test whether the decrease of mhtt aggregates induced by CRMP4 expression is paralleled by a reduction of mhtt-mediated toxicity in PC12 cells expressing httexon1Q103. The expression of mhtt in PC12 cells induces the activation of the apoptotic pathway. It is well known that different upstream pathways leading to apoptosis and cell death depend on the activation of Caspases 3/7 for final execution. In the PC12 cell model of HD, activation of Caspases 3/7 is observed upon induction of mhtt expression with PonA and this activation leads to apoptotic cell death Apostol et al., 2006. In our experiments, transfection with FLAG-

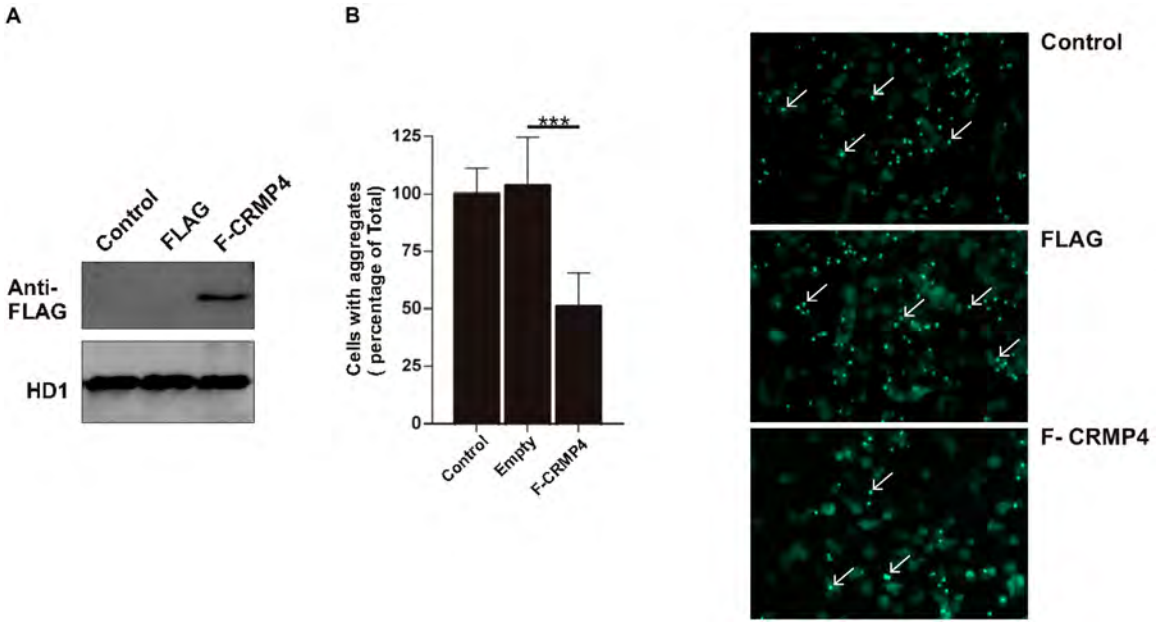


Figure 4.12: CRMP4 inhibits the aggregation of mhtt in cultured PC12 cells. **A**. PC12 cells expressing httexon1Q103-EGFP were transfected with a plasmid encoding FLAG-CRMP4 and induced with PonA. Western blot analysis with anti- FLAG and anti-htt (HD1) antibodies was performed to demonstrate the successful transfections and the unchanged levels of mhtt. **B**. PC12 cells expressing httexon1Q103 were cultured for 48 hrs, fixed, and analyzed using fluorescence microscopy. Aggregates (arrows) were counted in cells transfected with FLAG-CRMP4 and FLAG alone, non-transfected controls. CRMP4 reduced the amount of GFP positive aggregates by 40±10 %, P<0.001 (u= 3). (Representative images of the cultures are shown on the right).

CRMP4 reduced the mhtt-induced activation of Caspases 3/7 by 60±10 % compared with empty vector (Figure 4.13).

These results suggest that CRMP4 can partially rescue PC12 cells from apoptosis induced by mhtt. Consistent with our observations, CRMP4 is a potent inhibitor of mhtt-mediated aggregation and cytotoxicity in a cell model of HD.

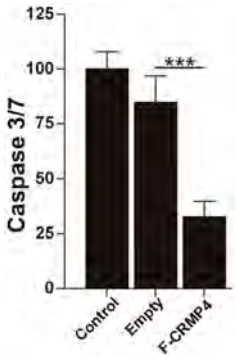


Figure 4.13: CRMP4 inhibits mhtt induced toxicity in cultured PC12 cells. Cellular toxicity was assessed by Caspases 3/7 measurements in PC12 cells expressing httexon1Q103-EGFP. Transfection with FLAG-CRMP4 reduced Caspases 3/7 activaty by 60±10 % compared with PC12 cells transfected with empty vector *** P<0.003

4.7 CRMP4 removes F-Actin from the inclusions formed by mutant htt

Reorganization of the actin cytoskeleton is important for survival and function of the cell machinery. Huntingtin binding partners are proteins directly or indirectly involved in actin remodeling. Based on the finding that CRMP4 co-localizes with mhtt and, upon over-expression, specifically mitigates mhtt-induced aggregation and toxicity, I analyzed whether CRMP4 is involved in the cytoskeleton response to mhtt. First I analyzed whether the actin dynamics was induced in PC12 cells by expression of mhtt. PC12 cells expressing httexon1Q103-EGFP contained GFP+ aggregates after 48 hrs, which stained positive with phalloidin antibody (Figure 4.14). The phallodin antibody detects F-Actin, and I found that the majority of the mhtt inclusions were positive for F-Actin (80±10 %, Figure 4.14).

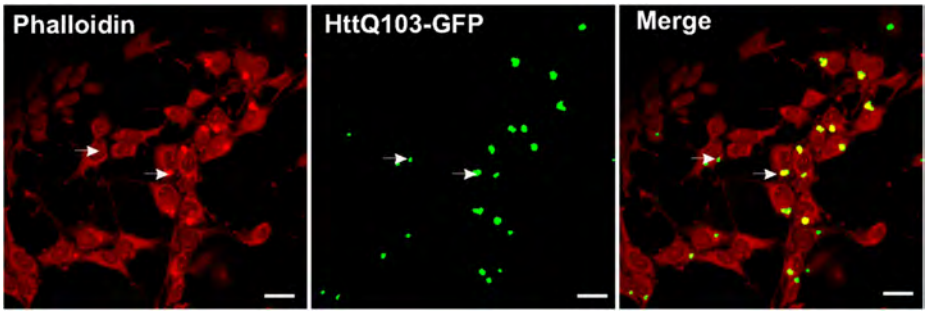


Figure 4.14: F-Actin is present in mhtt induced aggregates. PC12 cells expressing httexon1Q103-EGFP were induced with PonA and cultured for 48 hrs. Phalloidin staining was used to detect the F-Actin. 80±10 % of the GFP+ aggregates formed by mhtt were positive for F-Actin.

Next, I transfected PC12 cells expressing httexon1Q103-EGFP with a plasmid encoding FLAG-CRMP4 or with empty vector encoding only FLAG as control. 12 hrs after transfection,

I induced the PC12 cells with PonA for 48 hrs. I counted approximately 1.000 cells from independent visual fields. When I analyzed the cells transfected with CRMP4, I found a significant 90±10 % decrease in the percentage of F-Actin-EGFP positive aggregates among all EGFP-positive aggregates compared with empty vector (Figure 4.15). The results suggest a functional link between CRMP4 and the actin cytoskeleton alterations induced by mhtt.

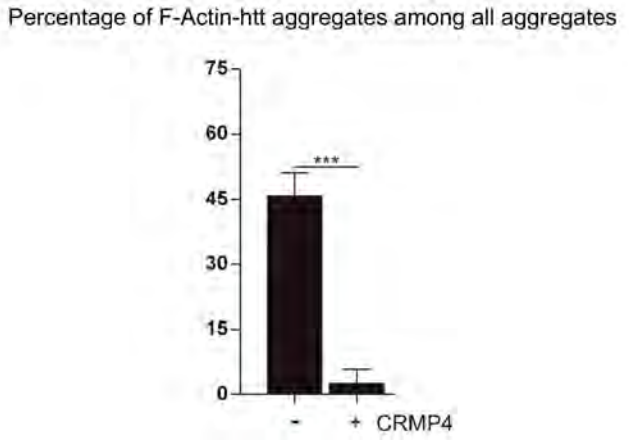


Figure 4.15: CRMP4 detaches F-Actin from the mhtt-induced aggregate. PC12 cells expressing httexon1Q103-EGFP were transfected with FLAG-CRMP4 (+) or FLAG (-) vector. After 48 hrs, GFP+ htt aggregates were assessed for staining with phalloidin. The percentage of F-Actin-htt aggregates was reduced by 90±10% in the presence of CRMP4 overexpression *** (P<0.0001) (u = 2).

4.8 Co-Immunoprecipitation shows an interaction between CRMP4 and htt in PC12 cells

I found that CRMP4 modulates the toxicity induced by mhtt and may be modulated by htt *in vivo* . Therefore, I investigated whether full length CRMP4 forms a complex with htt. In order to do that, I induced PC12 cells to express httexon1Q103-EGFP and httexon1Q25-EGFP. 48 hrs post-induction with 3 μM of PonA, total protein extract was immunoprecipitated with antisera raised against the N-terminus of huntingtin. The immunocomplexes were analyzed by SDS-PAGE and Western blotting. Next, the blots were probed with either antibodies against htt (CAG53b and HD1) or monoclonal anti-CRMP4 antibody. I found that CRMP4 co-immunoprecipitate with httexon1Q103, suggesting that full length CRMP4 protein can interact with mhtt in a cell-based assay (Figure 4.16).

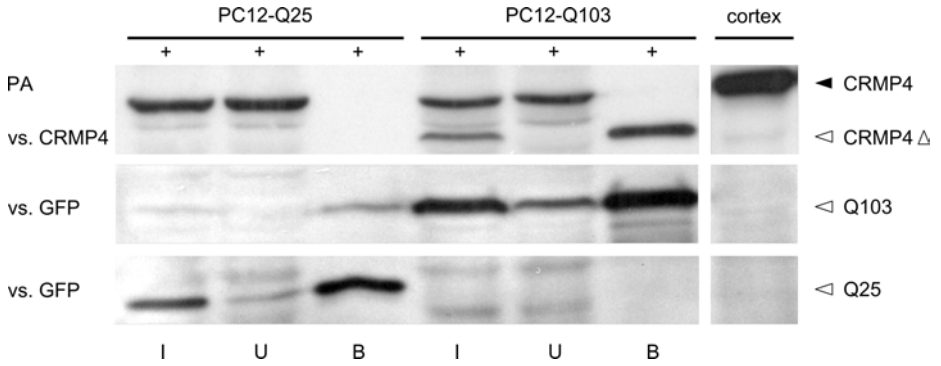


Figure 4.16: Co-immunoprecipitation of CRMP4 with httexon1Q25-EGFP and httexon1Q103-EGFP in PC12 cells. The gel shows the band for CRMP4 (62 kDa) in the initial (I) and unbound (U) fraction and no band in the bound (B) fraction for httexon1Q25. In PC12 cells with httexon1Q103 the gel shows the band for CRMP4 (62 kDa) in the initial (I) and unbound (U) fraction. The initial fraction shows a second band for CRMP4 of around 55kDa and the bound (B) fraction shows that only the shorter CRMP4 fragment directly interacts with httexon1Q103. GFP staining: the gel shows the presence of a band for httexon1Q103-EGFP (55 kDa) in the initial and bound fraction. Similarly, the gel shows the presence of a band for httexon1Q25-GFP (25 kDa) in the initial, unbound and bound fraction. Cortical lysate was used as control for the expression of CRMP4.

When analyzing PC12 cells expressing httexon1Q103 I found that the CRMP4 antibody recognized the specific CRMP4 band of 62kDa and a smaller band appearing only under these conditions. This band was not present in non-induced PC12 cells or in PC12 cells expressing httexon1Q25. When I analyzed the bound lysate, I found that only the smaller subtype of CRMP4 was present in the protein complex with mutant htt. This result led me to hypothesize that CRMP4 might undergo a proteolytic cleavage under toxic mhtt conditions.

Kowara et al. 2005 showed that CRMP4 is a target of Calpain. Glutamate excitotoxicity and oxidative stress led to cleavage of CRMP4 by Calpain in primary neuronal cultures. They used N-methyl-D-aspartic acid (NMDA) and hydrogen peroxide (H_20_2) to demonstrate that under toxic conditions, CRMP4 is present as a doublet of 62 and 60 kDa. This was not due to changes in the phosphorylation state of CRMP4, since the second band vanish after addition of adding a Calpain inhibitor. After Calpain cleavage, the full length CRMP4 expression was reduced.

We decided to investigate whether the additional band identified with CRMP4 antibody by Western blot in immunoprecipitates of PC12 cells expressing httexon1Q103 after immunoprecipitation was a cleaved form of CRMP4. In order to do that, we tested whether Calpain Inhibitors abolished the formation of the second lower band of CRMP4 (Figure 4.17), sug-

gesting that Calpain was responsible for this proteolytic cut under mhtt condition *in vitro* .

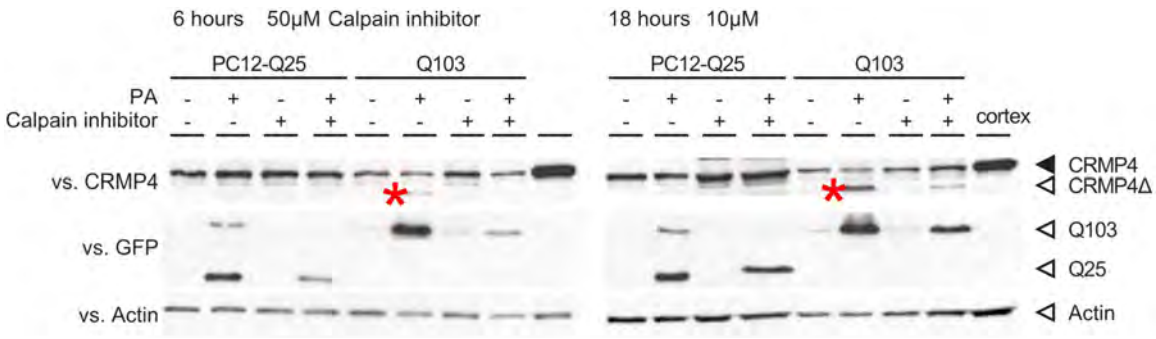


Figure 4.17: CRMP4 is cleaved by Calpain-1 in the presence of mhtt: PC12 cells were induced with 3 µM PA to express httexon1Q25-EGFP or httexon1Q103-EGFP fusion proteins. The cleaved CRMP4 fragment (CRMP4 Δ) is only present in PC12 cells expresing mutant httexon1Q103, indicated by asterisks (*). When cells were treated with LLnL Calpain inhibitor (at a concentration of 50 µM for 6 hrs or 10 µM for 18 hrs, right pannel), formation of CRMP4-Δ was inhibited.

This experiment confirmed the hypothesis that Calpain-1 is responsible for the proteolitic cleavage of CRMP4 under mhtt conditions.

4.9 Analysis of CRMP4 expression levels in R6/2 mice

A major step in the field of HD research was the creation of an HD mouse model by Mangiarini et al. [Mangiarini et al., 1996]. This mouse model, named R6 line, was created by inserting the exon1 of the human htt gene with 150 CAG repeats into mouse embryonic stem cells under the control of the human htt promoter. The mice appear normal at birth, but develop progressive motor dysfunctions, as well learning and memory deficits at around 8-10 weeks of age. In addition, brain and body weight loss have been reported. The mice die between 12 to 14 weeks of age with significant atrophy. In contrast to cell culture models like PC12 cells, which develop acute toxicity already 24 hrs after induction of httexon1Q103 expression, mouse models of HD like the R6/2, show chronic toxicity and may develop strategies to overcome the loss of proteins recruited into the inclusions. Therefore, I decided to investigate whether the modulation of CRMP4 protein levels observed in httexon1Q103 PC12 cells could be recapitulated in the HD mouse model. I performed Western blot analysis of cortical lysates in pre-symptomatic (2 weeks of age), early stage (4 and 6 weeks of age with

early signs of HD and occurrence of inclusions; [Carter et al., 1999; Lione et al., 1999] and late stage (12 weeks of age) R6/2 tg mice and the corresponding wild type littermates. The expression of CRMP4 was normalized against tubulin expression. Semi-quantitative analyses of cortical lysates from 2 weeks old R6/2 and wt mice did not show any differences in the protein expression levels of CRMP4 (Figure 4.18A).

CRMPs are known to reach a peak of expression during brain development and to decrease gradually thereafter. At 4, 6, and 12 weeks of age, the levels of CRMP4 gradually decreased in both WT and R6/2 mice with no differences between the genotypes (Figure 4.18).

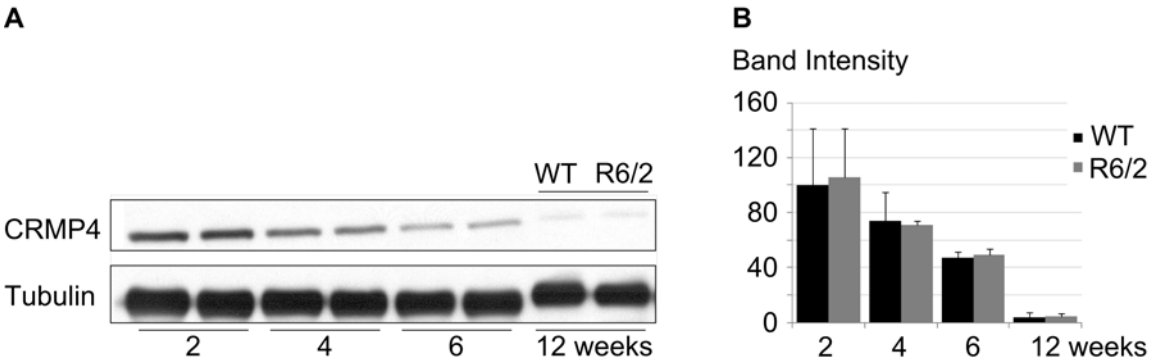


Figure 4.18: CRMP4 expression in R6/2 and wild type mice. **A**. Cortical lysates from 2, 4, 6, and 12 week-old R6/2 transgenic mice (R6/2) were analyzed. Aged-matched wild-type (WT) mice were used as controls. Western blotting with CRMP4- and tubulin-specific antibodies was performed. An age-dependent decrease of CRMP4 expression levels was observed in both WT and R6/2 mice. No differences were ound between the genotypes. **B**. Quantification of 3 independent experiments performed in triplicates.

Furthermore, no changes were observed between 12 week-old R6/2 mice and WT controls when the cortical lysates were analyzed for CRMP2 expression (Figure 4.19). As expected, CRMP2 levels were very low at 12 weeks of age in both genotypes.

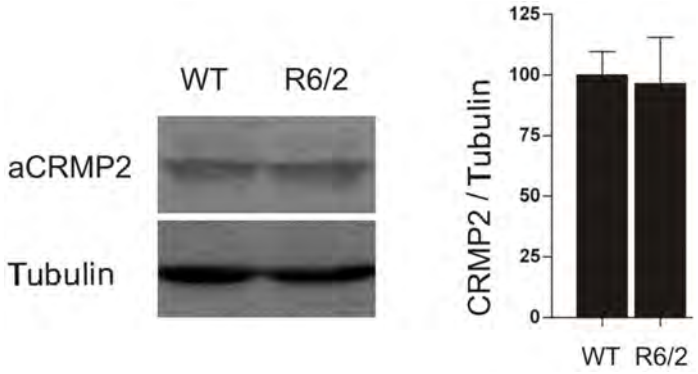


Figure 4.19: CRMP2 expression is unchanged in late stage R6/2 mice. The expression levels of CRMP2 were analyzed by Western blotting in the cortex of R6/2 mice, 12 weeks old months old and age-matched wt mice as control. No changes in the expression levels of CRMP4 were observed.

4.10 Co-localization of CRMP4 with polyQ aggregates in a mouse model for HD

The formation of intracellular htt inclusions is a hallmark of Huntington's Disease and has been reported in the brains of HD patients, in animal models and in cell-based and cell-free models of HD. Detailed analysis of htt-induced inclusions and identification of the proteins that are recruited into those aggregates revealed many interaction partners of mhtt. Htt interacts with proteins, which are involved in cytoskeleton reorganization, endocytotic pathways and the transcription- translation machinery [Wanker et al., 1997; Singaraja et al., 2002; Goehler et al., 2004; Cattaneo et al., 2005]. Changes in cytoskeleton structure have been associated with HD progression, suggesting that mhtt could vary the normal pattern of interactions, compromising important cellular processes like exo-endocytosis, trafficking of neurotransmitter vesicles and retrograde transport of proteins along the microtubules. Given the importance of CRMP4 for the cytoskeleton, I decided to investigate whether it colocalizes with mhtt inclusion bodies in R6/2 HD transgenic mice. I performed immunofluorescence microscopy analysis of parasagittal brain sections from 12 weeks old R6/2 tg mice (Figure 4.20) and from aged-matched wt controls (data not shown). CRMP4 expression in the wt mouse brain was diffusely expressed in the cytoplasm and the dendrites of neuronal cells. In the R6/2 mouse brain several prominent ubiquitinated neuronal intranuclear inclusions (NIIs) were found throughout the cortex. Furthermore, by using double labelling conventional im-

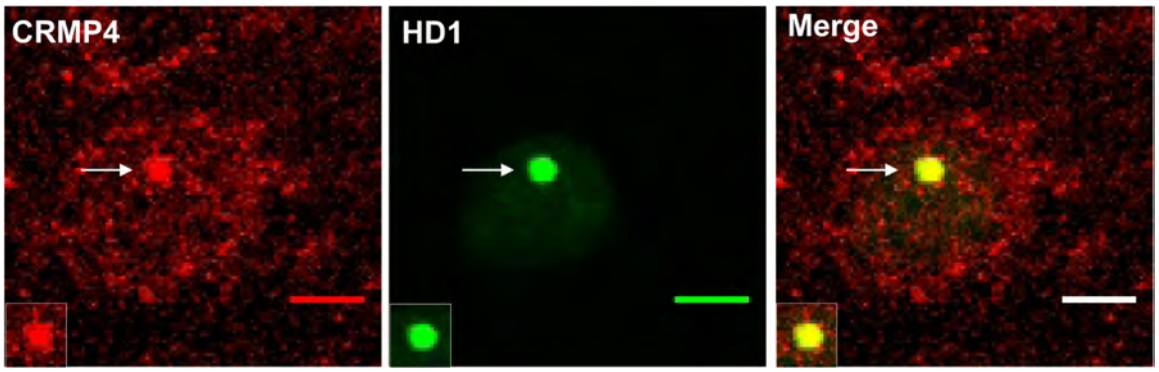


Figure 4.20: CRMP4 is present in mhtt aggregates in the R6/2 tg mouse brain. Double immunostaining for CRMP4 (red) and huntingtin (green) in the cortex of R6/2 brains at 12 weeks. The degree of colocalization is illustrated by merging htt and CRMP4 images. Scale bar: 10 µm

munofluorescence methods with specific antibodies against CRMP4 and htt, we confirmed that CRMP4 is indeed localized in NIIs.

4.11 Primary cortical neurons

I have isolated primary neurons from the cortex of 16 days old mouse embryos following the protocol described in the Methods session (see section 3.2.14 on page 43). After isolation of the cells, I kept them in culture for 12 days and semi-quantitatively characterized the populations present in my cultures. In order to count the different cells populations, I stained Neurons using Map2 and TuJ1, Astrocytes using GFAP, Oligodendrocytes using A-Olig and Microglia using CD11b. I counted the cells at 4, 6, 8, and 12 days after isolation and for each time point I counted 10 random fields. The results are:

Cell Type	Day 4	Day 6	Day 8	Day 12
Neurons (Map-2/TuJ1)	98 %	95 %	82 %	77 %
Astrocytes (GFAP)	2 %	5 %	18 %	22 %
Oligodendrocytes (A-Olig)	0 %	0 %	0 %	1 %
Microglia (CD11b)	0 %	0 %	0 %	0 %

Figure 4.21 show representative images of the different cell populations present in the cultures.

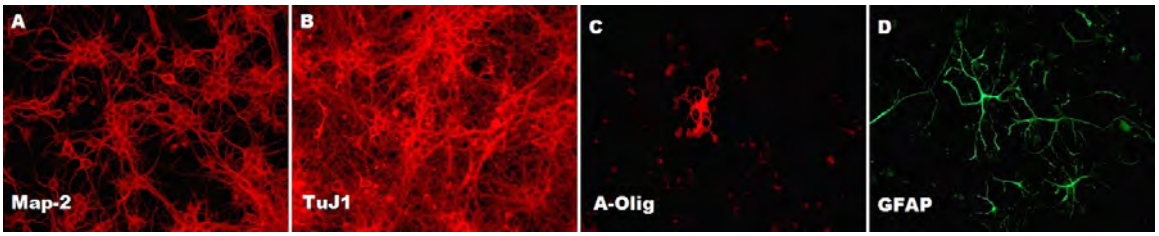


Figure 4.21: Characterization of primary neurons isolated from embryionic mouse cortex: Primary neurons are stained with Map2 and TuJ1 (**A, B**). **C** shows the presence of Oligodendrocytes stained with A-Olig antibody, these cells appear first after around 12 days of cuture. **D** Atsrocytes are stained with the GFAP antibody

4.12 Primary cortical neurons transfected with mutant htt show morphological changes of the dendrites and axons

To test whether the features of HD can be reproduced in a neuronal cell model, I have isolated primary cortical neurons from mouse embryos at embryonic day 16. After isolation the cells were transfected with pCAG-Mcs-N1-httexon1Q83-GFP and with pCAG-Mcs-N1httexon1Q20-GFP using Nucleofectoion (Figure 4.22). Five days after transfection, the cells developed long processes and networks. Interestingly, in the presence of mhtt but not wthtt, the neurons showed curved dendritic and axons processes reminescent of those described by Graveland and DiFiglia (1985) in postmortem HD brain tissue.

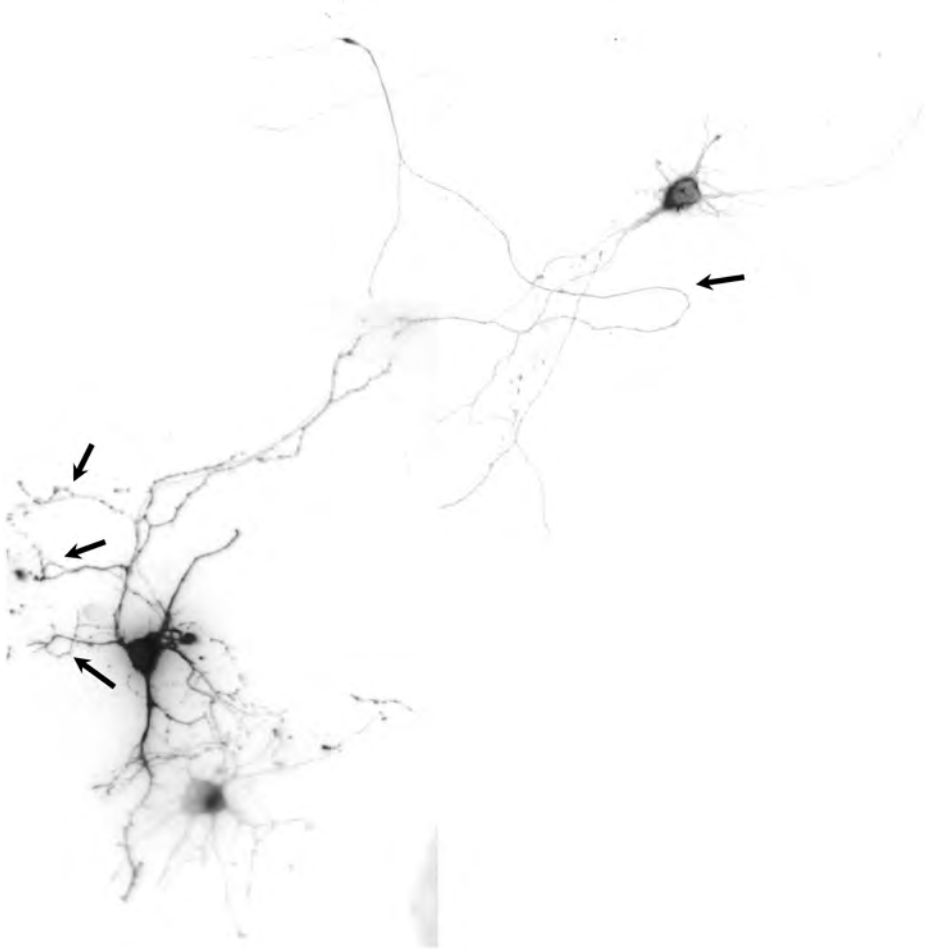
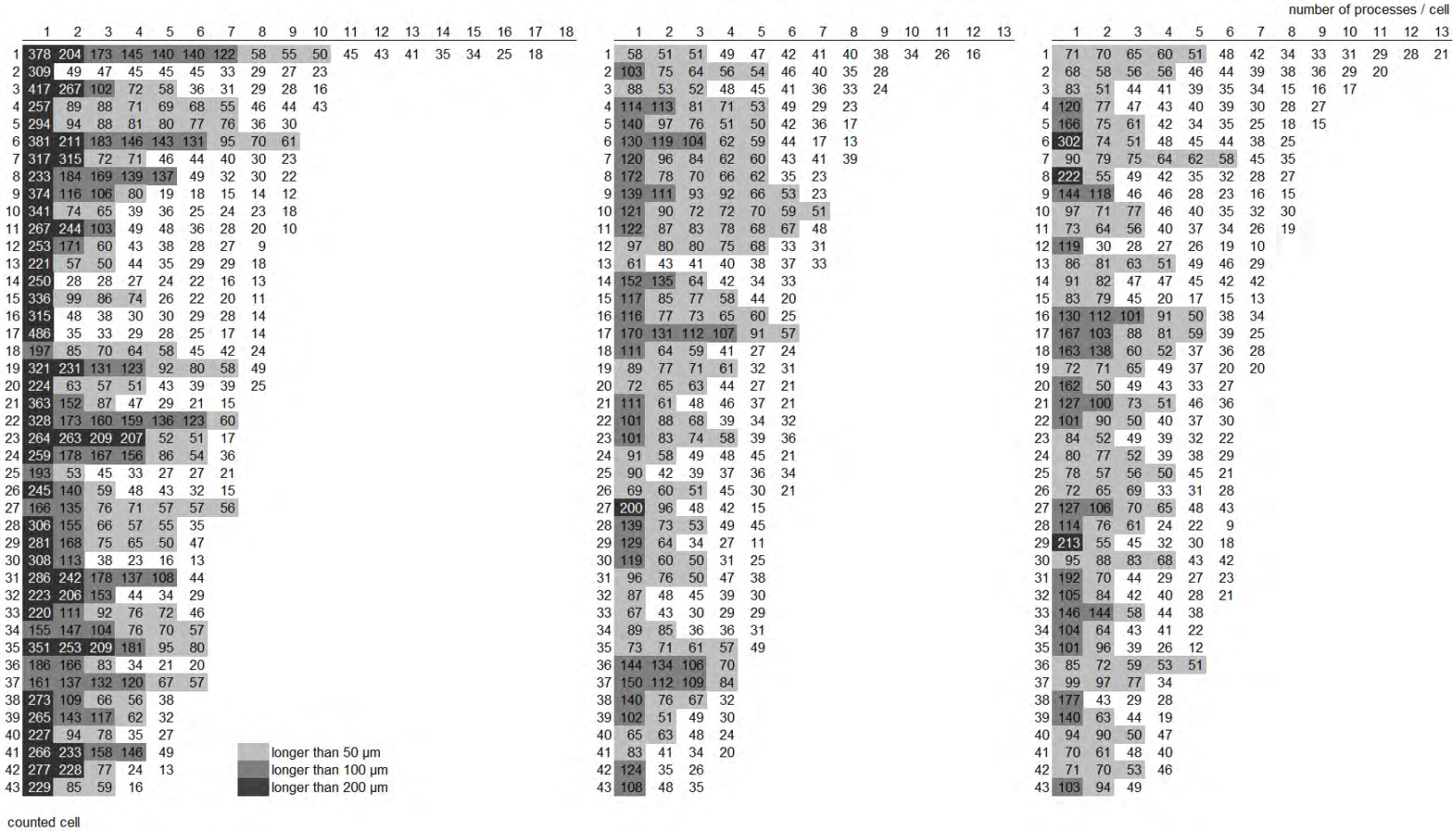


Figure 4.22: Primary Cortical Neurons 10 days after transfection with httexon1Q83-EGFP. EGFP expression (fluorescent inverted for clarity) was detected in the cell body and in the processes. The cells show morphological alterations in the growth direction of axons and dendrites (indicated by arrows) similar to the changes observed in postmortem brain from HD patients.

4.13 CRMP1 is involved in the development of neuronal processes

CRMPs are important in brain development [Byk et al., 1996] and their dysfunction is described in many diseases of the central nervous system like Alzheimer's Disease, Parkinson's Disease, Huntington's Disease, Down Syndrome and paraneoplastic neurological syndromes [Honnorat et al., 1999; Lubec et al., 1999; Gu et al., 2005; Weitzdoerfer et al., 2001; Yu et al., 2001; Castegna et al., 2002; Guidetti et al., 2001]. Much knowledge on the role of CRMPs in the development of neuronal cells comes from the study of CRMP1. CRMP1 was identified

as a signaling molecule of Sema3A [Goshima et al., 1995; Schmidt and Strittmatter, 2007; Deo et al., 2004] and the Rho kinase pathway [Fukata et al., 2002a,b]. Both CRMP1 and CRMP2 are involved in the Sema3A-induced growth cone collapse response in dorsal root ganglia (DRG) neurons [Yuasa-Kawada et al., 2003; Bretin et al., 2005; Leung et al., 2002]; in addition, the phosphorylation of CRMP1 and CRMP2 by Cdk5 and the sequential phosphorylation of CRMP2 by GSK-3 are crucial for Sema3A signaling [Uchida et al., 2005]. CRMP1 has been described as an important protein for the development of dendritic spines in the brain [Yamashita et al., 2007], and aberrations in dendritic spines represent an early neuropathological change in HD brains [Guidetti et al., 2001]. Therefore, I transfected primary neurons with or with the empty vector pCAG-Mcs-N1-hCRMP1-IRES-EGFPpCAG-Mcs-N1-IRES-EGFP using nucleofection. This resulted in an increase of CRMP1 expression not only in the axons of the cells, but also in the dendritic compartment (Figure 4.23). Immunostaining for CRMP1 also revealed a completely different intracellular distribution of the protein compared to control (Figure 4.23). Punctate immunoreactivity was observed in dendrites and axons, particularly at branching points (Figure 4.23). I also measured the lengths of the dendritic processes per cell and the number of processes per cell in three different groups: 1) Neurons transfected with CRMP1, 2) Neurons transfected with empty vector containing only the fluorescent protein (EGFP, DsRed) and 3) non-transfected neurons. CRMP1 overexpression had a striking effect on axons length, which increased by twofold compared to non-transfected neurons (Table 4.1).



counted cell	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	378	204	173	145	140	140	122	58	55	50	45	43	41	35	34	25	18	
2	309	49	47	45	45	45	33	29	27	23								
3	417	267	102	72	58	36	31	29	28	16								
4	257	89	88	71	69	68	55	46	44	43								
5	294	94	88	81	80	77	76	36	30									
6	381	211	183	146	143	131	95	70	61									
7	317	315	72	71	46	44	40	30	23									
8	233	184	169	139	137	49	32	30	22									
9	374	116	106	80	19	18	15	14	12									
10	341	74	65	39	36	25	24	23	18									
11	267	244	103	49	48	36	28	20	10									
12	253	171	60	43	38	28	27	9										
13	221	57	50	44	35	29	29	18										
14	250	28	28	27	24	22	16	13										
15	336	99	86	74	26	22	20	11										
16	315	48	38	30	30	29	28	14										
17	486	35	33	29	28	25	17	14										
18	197	85	70	64	58	45	42	24										
19	321	231	131	123	92	80	58	49										
20	224	63	57	51	43	39	39	25										
21	363	152	87	47	29	21	15											
22	328	173	160	159	136	123	60											
23	264	263	209	207	52	51	17											
24	259	178	167	156	86	54	36											
25	193	53	45	33	27	27	21											
26	245	140	59	48	43	32	15											
27	166	135	76	71	57	57	56											
28	306	155	66	57	55	35												
29	281	168	75	65	50	47												
30	308	113	38	23	16	13												
31	286	242	178	137	108	44												
32	223	206	153	44	34	29												
33	220	111	92	76	72	46												
34	155	147	104	76	70	57												
35	351	253	209	181	95	80												
36	186	166	83	34	21	20												
37	161	137	132	120	67	57												
38	273	109	66	56	38													
39	265	143	117	62	32													
40	227	94	78	35	27													
41	266	233	158	146	49													
42	277	228	77	24	13													
43	229	85	59	16														

counted cell	1	2	3	4	5	6	7	8	9	10	11	12	13
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3	88	53	52	48	45	41	36	33	24				
4	114	113	81	71	53	49	29	23					
5	140	97	76	51	50	42	36	17					
6	130	119	104	62	59	44	17	13					
7	120	96	84	62	60	43	41	39					
8	172	78	70	66	62	35	23						
9	139	111	93	92	66	53	23						
10	121	90	72	72	70	59	51						
11	122	87	83	78	68	67	48						
12	97	80	80	75	68	33	31						
13	61	43	41	40	38	37	33						
14	152	135	64	42	34	33							
15	117	85	77	58	44	20							
16	116	77	73	65	60	25							
17	170	131	112	107	91	57							
18	111	64	59	41	27	24							
19	89	77	71	61	32	31							
20	72	65	63	44	27	21							
21	111	61	48	46	37	21							
22	101	88	68	39	34	32							
23	101	83	74	58	39	36							
24	91	58	49	48	45	21							
25	90	42	39	37	36	34							
26	69	60	51	45	30	21							
27	200	96	48	42	15								
28	139	73	53	49	45								
29	129	64	34	27	11								
30	119	60	50	31	25								
31	96	76	50	47	38								
32	87	48	45	39	30								
33	67	43	30	29	29								
34	89	85	36	36	31								
35	73	71	61	57	49								
36	144	134	106	70									
37	150	112	109	84									
38	140	76	67	32									
39	102	51	49	30									
40	65	63	48	24									
41	83	41	34	20									
42	124	35	26										
43	108	48	35										

counted cell	1	2	3	4	5	6	7	8	9	10	11	12	13
1	71	70	65	60	51	48	42	34	33	31	29	28	21
2	68	58	56	56	46	44	39	38	36	29	20		
3	83	51	44	41	39	35	34	15	16	17			
4	120	77	47	43	40	39	30	28	27				
5	166	75	61	42	34	35	25	18	15				
6	302	74	51	48	45	44	38	25					
7	90	79	75	64	62	58	45	35					
8	222	55	49	42	35	32	28	27					
9	144	118	46	46	28	23	16	15					
10	97	71	77	46	40	35	32	30					
11	73	64	56	40	37	34	26	19					
12	119	30	28	27	26	19	10						
13	86	81	63	51	49	46	29						
14	91	82	47	47	45	42	42						
15	83	79	45	20	17	15	13						
16	130	112	101	91	50	38	34						
17	167	103	88	81	59	39	25						
18	163	138	60	52	37	36	28						
19	72	71	65	49	37	20	20						
20	162	50	49	43	33	27							
21	127	100	73	51	46	36							
22	101	90	50	40	37	30							
23	84	52	49	39	32	22							
24	80	77	52	39	38	29							
25	78	57	56	50	45	21							
26	72	65	69	33	31	28							
27	127	106	70	65	48	43							
28	114	76	61	24	22	9							
29	213	55	45	32	30	18							
30	95	88	83	68	43	42							
31	192	70	44	29	27	23							
32	105	84	42	40	28	21							
33	146	144	58	44	38								
34	104	64	43	41	22								
35	101	96	39	26	12								
36	85	72	59	53	51								
37	99	97	77	34									
38	177	43	29	28									
39	140	63	44	19									
40	94	90	50	47									
41	70	61	48	40									
42	71	70	53	46									
43	103	94	49										

Table 4.1: Effects of CRMP1 overexpression on the length and the number of dendritic processes in primary neurons. Cells were transfected with hCRMP1 (left column) or with empty vector (middle column). Non transfected cells (right column) served as negative control. After immunostaining with anti-CRMP1 the lenght of 43 randomly selected neurons/condition was analyzed. Error is about 0.5 µm/measurement. Overexpression of CRMP1 increased the length and number of dendritic processes compared with emty vector-transfected and non-transfected cells.

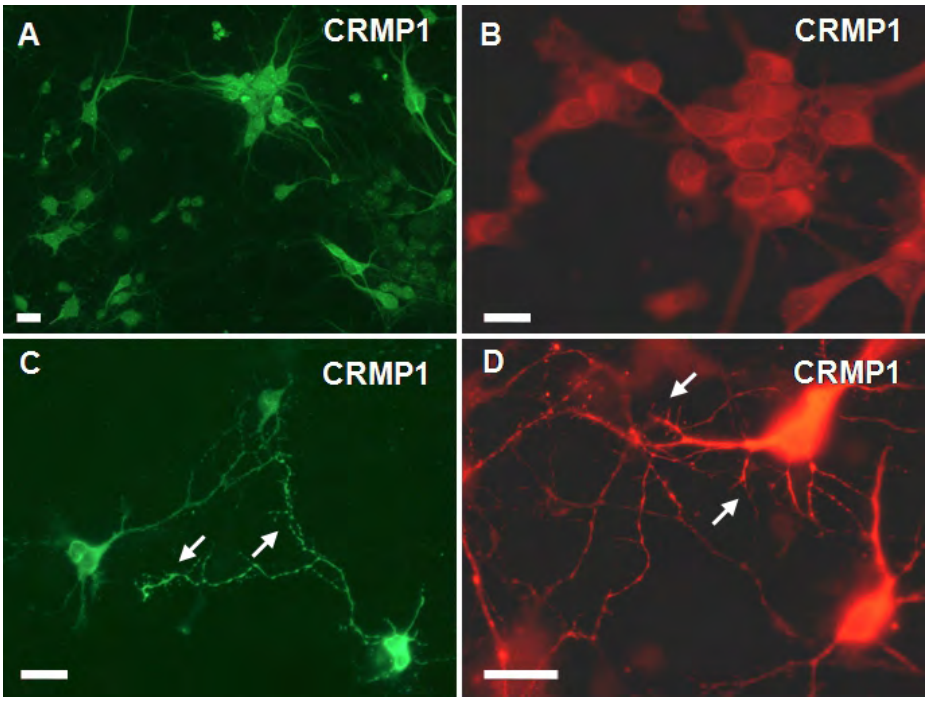

Figure 4.23: Overexpression of CRMP1 in primary cortical neurons. Primary cortical neurons isolated from mouse embryos were transfected with empty vector (**A, B**) and with hCRMP1(**C, D**). Cells were stained with a specific antibody against CRMP1 (**B, D**, red), EGFP fluorescence is shown in green (**A, C**). Overexpression of CRMP1 increased the lengths and the number of branches of axons and dendrites (indicated by arrows). Scale bar: 10 µm

4.14 Co-localization of CRMP1 with mutant huntingtin aggregates in cell and mouse models of HD

CRMP1 is constitutively expressed in primary cortical neurons as I demonstrate using immunocytochemistry. In order to test whether human CRMP1 interacts with htt *in vitro* , I generated the following expression vectors encoding htt: pCAG-httexon1Q20 and pCAG-httexon1Q83.Transfection of primary cortical neurons with pCAG-httexon1Q83 revealed the presence of mainly perinuclear huntingtin inclusions, whivh colocalized with CRMP1 immunoreactivity (Figure 4.24 **A, C**). I found inclusions along the processes, in the perinuclear compartment and in the nuclear compartment. When I stained the cells for CRMP2, I could not find any colocalization of htt and CRMP2 immunoreactivity (data not shown). Analysis of R6/2 mouse brain also revealed colocalization of CRMP1 and mhtt immunoreactivity in cortical neurons (Figure 4.24 **D, F**).

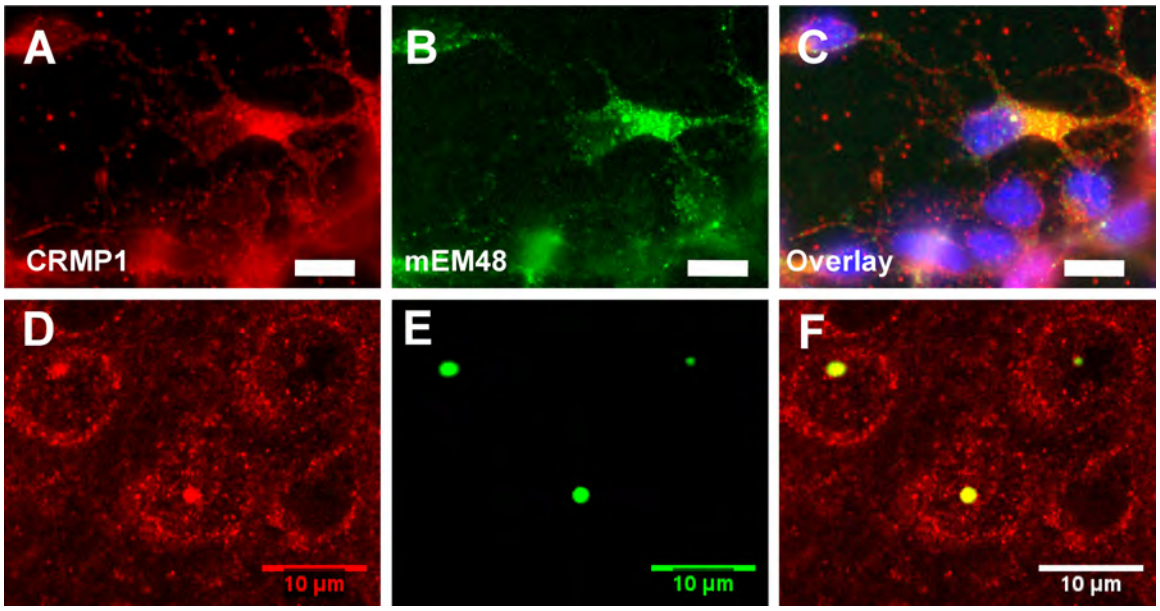

Figure 4.24: CRMP1 is present in mhtt inclusion bodies. (**A, C**) Primary cortical neurons from E16 embryos were transfected with pCAG-httexon1Q83. The formation of mhtt inclusions was monitored 96 hrs post-transfection by immunohistochemistry with mEM48 antibody (**B, E**, green) and confocal microscopy. CRMP1 immunoreactivity (**A, C**, red) colocalized with nuclear, perinuclear or intracytoplasmic mhtt inclusions as indicated by the overlay in (**C**). (**D, F**) Staining of R6/2 transgenic mouse cortex (11 weeks old) using polyclonal antibodies against CRMP1 (**D**) and htt (**E**). CRMP1 immunoreactivity is seen in mhtt-induced nuclear inclusions as shown by the overlay of confocal images in (**F**)

No co-localization of CRMP2 immunoreactivity with htt inclusions was detected in this model. I also did not find any htt, CRMP1 or CRMP2 aggregates in cortical neurons of age-matched WT mice (data not shown).

The data suggest that CRMP1 and CRMP4 might interact with mhtt and share common functional pathways in neuronal cells.

5 Discussion

5.1 HD Network

There are several ways to approach the study of genetically inherited disorders. Concerning HD, an important step forward was the development of disease networks. These networks shed light on the complexity of huntingtin, by digging out the great number of its interaction partners [Goehler et al., 2004; Kaltenbach et al., 2007]. CRMPs, and more precisely CRMP1, were identified as htt-interaction partners using the HD network [Goehler et al., 2004].Concerning our data, we found that both, CRMP1 and CRMP4, co-localize with mhtt inclusions in cell culture and mouse models of HD. The specificity of our findings is underscored by the fact that another CRMP familiy member, namely CRMP2, is not redistributed into the inclusions. The CRMP family includes five members, which share 50-70 % [Charrier et al., 2003; Deo et al., 2004]. Their functions comprise the regulation of axonal guidance [Uchida et al., 2005], neuronal cell migration [Yamashita et al., 2006], and spine morphogenesis during cortical development. Despite the sequence homology, a unique C-terminal region characterizes the CRMPs, which is a serine-rich region probably involved in the conformational changes leading to the secondary structure. Since CRMP1 and 4 interact with mhtt, while CRMP2 does not, we speculate that the binding site for mhtt is located in the unique C-terminal region of the CRMPs. CRMPs are known to form oligomers, preferentially hetero-oligomers [Wang and Strittmatter, 1997] and an avid interaction between the CRMPs requires one region in the amino half and a second region in the carboxyl half of the molecule. None of these regions alone is sufficient for ensuring a strong oligomerization among CRMPs. The importance of oligomerization is still to be elucidated, but it probably plays a regulatory role in CRMPs functions. CRMPs also undergo post-transcriptional modification, and their unique C-terminal region contains phosphorylation sites for Cdk5, Gsk3 and ROCK [Schmidt and

Strittmatter, 2007] and the specific binding sites to actin and tubulin [Wang and Strittmatter, 1997; Leung et al., 2002; Goshima et al., 1995; Cnops et al., 2006; Rosslenbroich et al., 2005; Franken et al., 2003]. The phosphorylation of CRMPs follows extracellular signals that lead to cytoskeleton modifications, which are important for neuronal development and survival. Since CRMPs are phosphorylated and bind to actin/tubulin, they transduce extracellular cues into cytoskeleton rearrangements. Besides, it is important to note that there are two pools of CRMPs: cytosolic and membrane-associated. Oligomerization, post-transcriptional modification and membrane association-dissociation are the processes responsible for the regulation of CRMP functions and they act via the unique C-terminal region [Wang and Strittmatter, 1997; Quinn et al., 1999, 2003; Yuasa-Kawada et al., 2003; Uchida et al., 2005; Yamashita et al., 2007]. An abnormal interaction between CRMP1 and CRMP4 with mhtt may therefore impair oligomerization and post-transcriptional regulation, thereby hindering cytoskeleton rearrangements and neuronal survival.

5.2 HD and the Cytoskeleton

During the last years, the study of cytoskeleton impairment in neurodegeneration has gained increased attention [DiProspero et al., 2004; Zabel et al., 2008]. With regard to Huntington's disease, many independent works show that actin regulating proteins are sequestered into mhtt-inclusions. CRMP4 is important for actin-polymerization; indeed, Rosslenbroich et al. showed in 2005 that the knock-down of CRMP4 in Neuroblastoma B53 cells induced F-actin depolymerization. They also found that CRMP4 co-localized with F-actin bundles in lamellopodia. This interaction was eliminated by addition of drugs, which destroy actin bundles, suggesting that CRMP4 specifically binds the filaments [Rosslenbroich et al., 2005]. In contrast, over-expression of CRMP4 induced a small, but reproducible decrease in cell migration, which implies that CRMP4 is a negative regulator of cell migration through its binding with F-actin[Rosslenbroich et al., 2005]. In our cell model, the PC12 cells expressing httexon1Q103 showed a dramatic down-regulation of CRMP4 protein levels, while CRMP4 mRNA levels remained unchanged. Our collaboration partners found a similar result for CRMP1 [Stroedicke et al., 2010] in preparation, while the expression of the homologous protein, CRMP2, did not show any changes. In parallel to the reduction of CRMP4, we observed that F-actin is recruited

into mhtt-aggregates, displaying a strong overlay with mhtt. We found that the equilibrium of these aggregates is broken by CRMP4. Over-expression of CRMP4 in these cells resulted in a dramatic drop in the percentage of F-actin-immunoreactive mhtt aggregates. The binding site of CRMP4 with F-actin is located in the C-terminal unique region of CRMP4, since the smaller N-terminal variants of 55kDa do not bind F-actin [Rosslenbroich et al., 2005]. Actin is important for the maintenance of cell motility, stability, vesicle trafficking and the morphology of dendritic spines. Our results suggest an important role of cytoskeleton dysfunction in HD, which is in line with previous studies showing that mhtt compromises the normal function of cytoskeleton proteins. Recently, the role of actin cytoskeleton rearrangements in HD pathogenesis was supported by the observation that drugs or mutations, which inhibit actin polymerization, strongly enhance the formation of intracellular mhtt aggregates [Meriin et al., 2007]. Already in 2003, Meriin et al. found that mhtt aggregation leads to a rapid cessation of endocytosis. Specific mutations associated with endocytosis can delay the polyglutamine aggregation process, suggesting that this pathway is involved in the formation of the inclusions [Meriin et al., 2003]. A role for htt in endocytosis has been proposed after identifying that wthtt and mhtt interact with HIP-1 [Wanker et al., 1997], a protein important for the invagination of vesicles at the plasma membrane. Moreover htt binds AP-2 [Harjes and Wanker, 2003], an adaptor protein important for the formation of the clathrin cap. Finally htt binds PACSIN-1[Harjes and Wanker, 2003], which is important for actin polymerization and leads to the detachment of the vesicle from the membrane. Several other direct interaction partners of mhtt, which are involved in the regulation of actin assembly and vesicular transport, were found to enhance mhtt aggregation, including SH3GL3 and GIT1 [Goehler et al., 2004; Sittler et al., 1998]. SH3GL3 (SH3-contaning Grb2-like protein) has a C-terminal domain (Src-homology3-domain), which is important for protein-protein interactions and binds proline-rich ligands. This domain mediates the interaction of SH3GL3 with the prolin-rich region of htt [Sittler et al., 1998]. SH3GL3 interacts more strongly with mhtt than with wthtt, perhaps because the elongated polyQ causes a conformational change with the establishment of hydrogen bounds and the formation of beta-sheets, which make the proline site more accessible. SH3GL3 is recruited into the nuclear inclusions formed by mhtt and enhances their formation in the filter trap assay [Sittler et al., 1998]. Another actin-binding protein, which also interacts with htt, is Profilin2 (PFN2). PFN2 regulates filamentous actin (F-actin) poly-

merization [Burnett et al., 2008]. PFN2 acts as a disease-modifier by ameliorating polyQ toxicity in cell culture and Drosophila models of HD [Burnett et al., 2008]. Profilin binds to the polyproline region of htt, and this binding is necessary for reducing mhtt-induced inclusions. Indeed, a Profilin mutant, which lacks this region, was not able to bind to htt and did not have the inhibitory effect on mhtt-inclusions [Shao et al., 2008]. Furthermore, Profilin binds G-actin-ADP monomers and induces the shift to G-actin-ATP and the polymerization of F-actin. Like CRMP1 and CRMP4, Profilin protein levels were also down-regulated by mhtt expression, which was observed in cell culture models and post-mortem brain samples of HD patients [Burnett et al., 2008].

5.3 CRMP4 reduces mhtt aggregation and toxicity

In my work, I found that over-expression of CRMP4 in httexon1Q103-expressing PC12 cells reduced the number of inclusions formed by mhtt and the mhtt-induced toxicity. Using a cell-free assay for detecting the formation of mhtt SDS-insoluble aggregates, we observed that CRMP4 strongly inhibited the formation of SDS-insoluble aggregates in a time- and concentration-dependent manner. In line with our results, Shao et al. (2008) demonstrated that HEK cells co-transfected with Profilin1 and mhtt exon 1 formed less mhtt aggregates. Therefore, Profilin1, like CRMP4, reduces mhtt aggregation [Shao et al., 2008]. Concerning mhtt-induced toxicity, we observed that CRMP4 has a protective effect when over-expressed in PC12 cells. As an indicator of the activation of the apoptotic pathway in these cells, we measured the induction of caspase 3/7. Over-expression of CRMP4 resulted in a significant reduction in caspase 3/7 activation when compared to control mhtt-expressing PC12 cells. This suggests an anti-apoptotic role for CRMP4 and, since CRMP4 also reduced mhtt-inclusions, a potential relationship between mhtt inclusion formation and toxicity. Interestingly, wthtt is considered to be an anti-apoptotic protein. Previous studies showed that wthtt inhibits the intrinsic apoptotic pathways of cytochrom c and pro-caspase 9. It interacts with HIP-1 and prevents it form binding to Hippi, which would activate pro-caspase 8/ caspase 3 and the apoptotic pathway [Harjes and Wanker, 2003]. The interaction of htt with HIP-1 is compromised by the polyQ mutation, inducing an increase of free HIP-1 available for Hippi. This may be one of the mechanisms for apoptotic death in HD. In addition to its direct role in apop-

tosis, htt is a substrate of the Akt kinase pathway, which induces the transcription of survival genes like BDNF. Indeed, over-expression of wthtt resulted in increased BDNF levels, while BDNF transcription and transport is reduced in HD [Harjes and Wanker, 2003]. Considering our results and the published literature, we can conclude that even if mhtt inclusions are not directly responsible for toxicity, the aggregation process appears to be linked to HD pathogenesis. CRMP4 shows a direct interaction with mhtt, and over-expressed CRMP4 might interfere with the tendency of the elongated polyQ stretch to self-assemble, thereby reducing the number of aggregates formed. The ability of CRMP4 to inhibit mhtt aggregation and toxicity is reminiscent of the role of chaperones in preventing protein misfolding. In fact, chaperone molecules like Hsp40 and 70 co-localize with the mhtt inclusions in HD. Moreover, over-expression of Hsp40 and 70 consistently suppressed the formation of polyQ inclusion bodies and their toxicity [Cummings et al., 1998; Muchowski et al., 2002; Muchowski and Wacker, 2005].

5.4 Morphological changes in HD

My experiments suggest that the over-expression of CRMP1 in primary neurons induces a re-distribution of the protein in new branches and dendrites, and an increase in the length of the dendritic processes. These findings are in line with the observation that CRMP1 -/- mice have poorly formed dendrites and dendritic spines, while the expression of axonal markers like Tau-1 and synapsin remains unchanged, suggesting that the absence of CRMP1 impairs dendritic formation [Yamashita et al., 2006, 2007; Su et al., 2007]. CRMP1 -/- mice are completely normal during their embryonic development; the only region of the brain showing alterations is the CA1 region of the hippocampus. Here, MAP2 dendritic staining was localized disproportionally in the proximal part of the dendrites, suggesting that they were not properly formed [Su et al., 2007]. Interestingly, the role of MAP2 is connected to LTP-induced cytoskeleton reorganization in the hippocampus. Furthermore, reduction of staining and protein levels of PSD95 and GAP43 was observed CRMP1 -/- mice. These findings are indicative of fewer dendritic spines and fewer growth cones, leading to impaired neuronal plasticity and cognitive dysfunctions. Indeed, CRMP1 -/- mice exhibit deficient spatial memory, as well as impaired long-term potentiation [Su et al., 2007]. Actin is also important for maintaining cel-

lular morphology. Indeed, the shape of dendrites is directly regulated by the actin cytoskeleton [Uchida et al., 2005; Fukazawa et al., 2003]. Studies on post-mortem brain revealed that the dendrites of medium spiny neurons in the striatum exhibit a variety of changes in HD with regenerative features and degenerative ones, the latter being characterized by the occurrence of terminal dendritic segments with abnormal curved morphology [Graveland and DiFiglia, 1985]. The impairment of F-actin assembly is expected to cause aberrant morphology [Fischer et al., 2000; Star et al., 2002; Burnett et al., 2008]. CRMP4 is important for F-actin stability. F-actin and CRMP4 are recruited into the inclusions formed by mhtt. My own findings suggest that over-expression of CRMP4 modulates the formation of inclusions, the toxicity of mhtt as well as the distribution of actin in the inclusions. Dendritic spines are responsible for the formation of synaptic contacts among neurons, which create the network necessary for their survival. The dynamics of this process are insured by the constant reorganization of actin in dendritic spines [Star et al., 2002]. GAP43 was recently discovered to be an interaction partner of CRMP4 [Kowara et al., 2007]. Both proteins accumulate in the lipid rafts at the inner surface of the cellular membrane and are highly expressed in neurite outgrowth. CRMP4 binds actin and tubulin, while GAP43 binds actin, alpha-actinin, talin and spectrin, all of which are involved in the maintenance of shape, motility and guidance pathways. The affinity of GAP43 for actin is increased by its phosphorylation, whereas the opposite is true for CRMP4. Both proteins may therefore act in concert, inducing opposite reorganization stimuli on the actin cytoskeleton. These findings suggest a role for cytoskeletal abnormalities in HD pathogenesis. Moreover, we propose CRMP4 as a new modulator of mhtt, important for both cytoskeleton dynamics and neurodegeneration. Future investigations could focus on elucidating the interaction of CRMP4 and CRMP1 with htt and actin. For instance, FRET analysis could be performed to study the specific protein-protein interactions by identifying the region responsible for this binding.

5.5 CRMP4 is cleaved by Calpain-1 in the presence of mhtt

To further investigate the interaction between CRMP4 and mhtt, we analyzed protein lysates of PC12 cells expressing httexon1Q103 by co-immunoprecipitation assay. We confirmed the

decrease in full-length CRMP4 protein levels. Moreover, we identified a cleavage product of CRMP4 running at approximately 60KDa on a Western blot. Interestingly, only this truncated CRMP4 variant co-immunoprecipitated with mhtt, suggesting that it mediated the interaction. Based on pharmacological inhibitor experiments, we found that this new CRMP4 variant resulted from calpain-1 cleavage. However, CRMP4 only underwent calpain-mediated proteolytic cleavage in the presence of mhtt. Although the physiological and pathological significance of this cleavage in the context of HD remains to be evaluated, this is a very interesting finding to follow up. It could well be that CRMP4, like other htt-interacting partners, is degraded under toxic mhtt conditions. A similar result was found for Profilin, whose downregulation in the context of mhtt was prevented by the addition of a proteasome inhibitor (specific for the inhibition of the proteasome and not the autophagy pathway). Kowara et al. (2005) observed the same breakdown product of CRMP4 that we found in our experiments. They studied primary neurons under NMDA and H_2O_2 toxicity, and found that the smaller CRMP4 variant, running at 60kDa, appeared 2 and 4 hours after treatment, respectively. Calpain inhibitors, but not caspase inhibitors, were successful in blocking the cleavage [Kowara et al., 2005]. Furthermore, the band was not abolished by inhibition of phosphatase activity, confirming that it was due to calpain activation. Calpains are Ca^{2+}-dependent proteases, activated by the increase of intracellular Ca^{2+} levels [Gafni et al., 2004; Cowan et al., 2008]. Ca^{2+} is a ubiquitous intracellular messenger that regulates growth, plasticity, membrane excitability and cell death; all these pathways are regulated by changes in the calcium concentration that activate channels, proteins and enzymes. Therefore, abnormal Ca^{2+} concentrations can lead to cellular dysfunction and degeneration. Indeed, Ca^{2+} and calpains (mRNA and protein levels) are increased in the cortex and striatum of HD knock-in mice and R6/2 mice [Gafni et al., 2004]. The inactive forms of calpain 5-7 and 10 are found in the cytoplasm and nucleus, while the active forms (N-terminally cleaved) are found exclusively in the nuclei. Concerning mhtt, cytotoxicty has been connected with its presence in the nucleus. Htt has an NES domain, important for keeping 95 % of the protein in the cytoplasm. The protein only translocates to the nucleus after cleavage with calpain/caspase. The accumulation of mhtt in the nuclei as observed in HD, may be a consequence of the polyQ mutation impairing the removal of the cleaved fragments from the nuclei [Gafni et al., 2004]. Wthtt and mhtt have two sites of calpain-cleavage aa468-470 and aa535-537 and one caspase site aa513-552. Cells transfected

with mhtt fragments, where the cleavage sites for calpain were removed, not only show lower amounts of proteolytic fragments of mhtt, but also a strong decrease in the mhtt aggregation and cytotoxicity [Gafni et al., 2004]. CRMPs were found to undergo calpain and caspase cleavages that generate truncated forms of the proteins under different acute conditions, e.g. brain ischemia or trauma, excitotoxic damage (e.g. NMDA) and oxidative stress [Kowara et al., 2005; Hou et al., 2006; Kowara et al., 2006; Touma et al., 2007; Zhang et al., 2007; Jiang et al., 2007]. Here, we show that CRMP4 is is cleaved by calpain under toxic mhtt conditions. Future experiments should be designed to examine the functional consequences of CRMP4 cleavage in HD pathogenesis.

5.6 HD and excitotoxic

Under normal conditions, NMDA glutamate receptors, when stimulated by the increase of intracellular Ca^{2+} levels, are responsible for changes in actin-dynamics, which promote the formation of new dendrites and spines. Indeed, newly formed spines mainly express NMDA receptors [Fischer et al., 2000]. On the other hand, AMPA glutamate receptors are present after the spine morphology has been established at synapses and has to be maintained. Therefore, glutamate has opposite effects via AMPA receptors, namely stabilization of actin [Fischer et al., 2000]. Under mhtt conditions, NMDA receptors are hyperactivated and dysfunctional. Indeed, there is evidence for impairment in the transport and uptake of glutamate (decreased mRNA levels of GLT-1 transporter) in cell culture and mouse models of HD, as well as in HD patients [Sánchez et al., 2003]. Furthermore, it is known that wthtt interacts indirectly with NMDA receptors and this interaction is mediated by PSD-95. The data suggest that wthtt participates in the signal transduction, which is important for spine morphology and synaptic plasticity. When htt is mutated, this results in a stronger binding of PSD-95 to NMDA receptors, causing their sensitization and promoting glutamate-induced neuronal apoptosis (excitotoxicity). It has been shown that the over-expression of wthtt significantly attenuates excitotoxicity; consequently, the binding of htt to PSD-95 might be important for understanding mhtt-induced toxicity [Harjes and Wanker, 2003]. As previously described, CRMP1 -/- mice show a decrease in PSD-95 and GAP43 levels and a disproportional distribution of MAP2 staining in the proximal parts of the dendrites in the hippocampus [Su et al., 2007]. We show

that mhtt induces a strong down-reguation of CRMP4 and CRMP1; this loss of protein expression may influence PSD-95 expression, linking plasticity and excitotoxicity. The idea that excitotoxicity plays a role in the degeneration observed in HD comes from early findings by Coyle and Schwarcz. In 1976, they observed that injection of kainic acid (a glutamate receptor agonist) into the rat brain induced death of striatal neurons with preservation of their axon [Coyle and Schwarcz, 1976; McGeer and McGeer, 1976]. Around 10 years later, Beal et al.; Ferrante et al. demonstrated that agonists of NMDA-type glutamate receptors induce a selective loss of GABAergic medium spiny projection neurons in the striatum [Beal et al., 1986; Ferrante et al., 1993]. This loss was characterized by the preservation of large spiny interneuron populations, like the ones containing acetylcholinesterase or nitric oxidesynthase (NOS), neuropeptide Y and somatostatin, which is also a neuropathological hallmark of HD [Goto et al., 1989; Dawbarn et al., 1985].Moreover, direct injection of NMDA receptor agonists into the striatum of rodents or non-human primates produced neurochemical, neuropathological, and behavioral changes characteristic of HD [Hantraye et al., 1990; Sanberg et al., 1989]. Hyperactivation of these types of glutamate receptors also leads to an increase in the production of reactive oxygen species (ROS). Neurons are very vulnerable to ROS damage due to their high basal metabolic rate and their relatively low levels of antioxidant defenses [Halliwell, 1992]. Interestingly, there is a link between ROS production and cytoskeletal impairments, since the release of ROS and the activation of the apoptotic pathway was observed after accumulation of F-actin in yeast models caused by dysregulation of actin turnover and polymerization [Gourlay et al., 2004]. Stabilizing F-actin resulted in the increase of ROS and vice et versa. During aging, there is a natural decrease in actin dynamics. Therefore, one could speculkate that actin and mitochondria-ROS coupling regulate cell death observed in aging. This coupling could also be involved in disease-related neurodegeneration. Indeed, accumulation of ROS is a feature of neurodegeneration, and was shown in brain tissue from Alzheimer's disease patients and in Drosophila models [Zhu et al., 2004; Fulga et al., 2007].

5.7 CRMP4 expression in the R6/2 transgenic mouse model of HD

Based on our *in vitro* findings, we decided to examine CRMP4 expression and mhtt aggregation *in vivo* . We used R6/2 transgenic mice of different ages. This mouse model expresses the exon1 of mhtt encoding for 153 polyQ, and shows normal growth and health parameters until 3 to 4 weeks of age. At this age, R6/2 mice present mhtt intranuclear inclusions (NIIs), mainly in the cortex. In accordance with the human disease, the first symptoms appear at a later stage, up to 6 weeks of age. In order to have a complete picture of the disease progression, we used 2, 4, 6, and 12 week-old R6/2 mice and WT littermates. The time course analysis of CRMP4 expression in the brain did not reveal any differences between R6/2 and wild-type mice. CRMP4 protein levels decreased over time in the brains of both genotypes, and dropped significantly at around 6 weeks of age. HD is a late onset disease (with the exception for the rare juvenile form); interestingly, the R6/2 mice develop the first symptoms at around 6 weeks of age, which corresponds to the significant and physiological drop of CRMP4 expression levels in the brain. Therefore, it is tempting to speculate that the normal down-regulation of CRMP4 may have dramatic effects in the context of HD. After all, we have shown that CRMP4 modulates the aggregation process and the toxicity induced by mhtt in vitro. Concerning the roles of CRMP4 in adulthood, we know that the protein has been linked to neuronal plasticity and neurogenesis. For instance, studies on the cat visual cortex revealed that CRMP4 stains far-reaching neurites in their immature stage, as well as somata and apical dendrites in the adult cortex [Cnops et al., 2006]. This suggests a role of CRMP4 in neuronal plasticity during development, and in the restricted capacity for cortical plasticity and axon growth following denervations in adult animals [Kaas et al., 1990; Gilbert and Wiesel, 1992; Darian-Smith and Gilbert, 1994; Chino, 1995; Arckens et al., 2000].The importance of CRMP4 in neuronal plasticity is also supported by the fact that CRMP4 expression in the adult brain is restricted to regions with adult neurogenesis, for instance dentate gyrus, hippocampus and the granular layer of the olfactory bulb [Pasterkamp et al., 1998b,a; Nacher et al., 2000; Ricard et al., 2000; Charrier et al., 2003]. These evidences suggest that CRMP4 remains important also at a later stage, although its expression is strongly reduced. Mhtt may impair the function of CRMP4 starting from an early age, leading to a complete loss of function when the physiological drop

occurs. It would be of great interest to generate a CRMP4 deficient mouse expressing mhtt and to analyze whether these animals show an earlier onset of disease onset. In this case, one might speculate that the juvenile form of HD may not only be due to a longer polyQ mutation in htt, but also to the impairment of functional proteins like CRMP4, which are important for the development and survival of neuronal cells.

6 Conclusions

In the present study I report collapsin response mediator 4 (CRMP4) as a modulator of mutant huntingtin. CRMP4 is a member of a family of proteins highly expressed during brain development and downregulated in adulthood.

Endogenous CRMP4 is down-regulated in PC12 cells expressing httexon1Q103, evenmore CRMP4 is recruited in the mhtt inclusions formed in these cells and in the cortex of R6/2 mice. Studies on CRMP4 showed that the protein may have a role in neurogenesis and neuronal plasticity. Indeed the expression of CRMP4 in the adult rat brain has been found restricted to the dentate gyrus of the hippocampus, the glomerular layer of the olfactory bulb and in the olfactory receptor neurons, which continue to be generated in the adult [Quinn et al., 1999; Pasterkamp et al., 1998b; Nacher et al., 2000; Ricard et al., 2000; Charrier et al., 2003; Parent et al., 1997; Scott et al., 1998]. A deregulation of CRMP4 in the presence of mhtt may contribute to the pathogenesis of the disease.

When we overexpressed CRMP4 in PC12 cells expressing httexon1Q103 we could see that the protein induces a decrease of 25 % in the number of aggregates formed in these cells. The overexpression of CRMP4 induces a decrease in the toxicity and in the percentage of mhtt inclusions overlaying with F-Actin. Furthermore I observed that in the presence of mhtt CRMP4 is cleaved by Calpain-1 and that the small CRMP4 subtype is directly binding to mhtt. CRMP4 might thence interfere with the tendency of elongated polyQ to self-assemble, thereby reducing the number of aggregates. The proteolytic cleavage of CRMP4 was already observed in neuronal cells under excitotoxicity condition preceding cell death. Further studies are needed to verify whether this proteolytic cleavage could be a marker for neuronal toxicity in the cell population degenerating in HD. CRMP4 has been shown to be crucial for cytoskeleton stability. It might be a key to understand the alterations and impairment occurring in the cells when htt is mutated.

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Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Eidesstattliche Erklärung

"Ich, Cecilia Nicoletti, erkläre, dass ich die vorgelegte Dissertationsschrift mit dem Thema: "Investigation of the Role of Collapsin Response Mediator 4 in Huntington’s Disease" selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (un- zulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe."

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