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Characterization of a *Drosophila* model for Chorea-Acanthocytosis

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CHAPTER **5**

Summarizing discussion and future perspectives

VPS13 family members and hereditary neurological disorders

In 2001 causative mutations for ChAc were identified in a large gene called *VPS13A* [1,2]. In order to gain more insight in the pathological mechanisms playing a role in ChAc, an understanding of the molecular functions of the VPS13A protein is required. Unfortunately, in spite of major efforts in the past few years, the molecular function of VPS13A is still not well understood. In this thesis we described the first well validated multicellular organism model for ChAc and determined the consequences of Vps13 loss of function to the central nervous system.

In humans, *VPS13A* belongs to a family of four *VPS13* genes, *VPS13A* to *D* [3] (Table 1). Mutations in three of the *VPS13* genes, *VPS13A*, *B* and *C*, lead to neurological disorders. Mutations in *VPS13B* cause a developmental disorder, called Cohen syndrome, characterized among others by microcephaly [4]. VPS13B is a peripheral membrane protein localized at the Golgi complex [5]. It interacts with Rab6 and regulates Golgi integrity [5,6]. The fact that mutations in VPS13B lead to a developmental disorder while mutations in VPS13A lead to neurodegenerative disorders may suggest a different role for the two proteins in human physiology.

VPS13A has more sequence similarity with VPS13C compared to VPS13B [3]. Mutations in VPS13C have been associated with Parkinson's disease [7]. VPS13C partially localizes to the outer mitochondrial membrane and knock down in cell culture cells leads to mitochondrial fragmentation and a decreased mitochondrial membrane potential [7]. Knock down of VPS13C also results in increased PINK/PARKIN mediated mitophagy [7]. VPS13C interacts with Galectin-12, a carbohydrate binding protein, and mediates its stabilization [8]. Depletion of VPS13C leads to degradation of Galectin-12 via a lysosomal pathway and a defect in adipocyte differentiation [8]. Whether and how defects in this pathway contribute to the development of Parkinson's disease is not clear.

The fact that mutations in *VPS13A*, *B* and *C* lead to neurological disorders points towards a function of at least these three VPS13 proteins in the central nervous system. To understand more about the underlying mechanisms of how loss of VPS13A, B and C lead to neurological diseases multicellular model organisms are crucial. Knock out mouse models have not been established for *VPS13B* and *VPS13C*. For *VPS13A* a knock out mouse has been established, however the phenotypes of this model are variable or even absent depending on the genetic background of the *VPS13A* knock out mice strains [9]. Therefore it is necessary to create other multicellular model organisms to study the role of VPS13A in neuronal tissue *in vivo*. We established a model in *Drosophila melanogaster* to study the function of the *Drosophila* ortholog of VPS13A. The *Drosophila* genome codes for three *VPS13* genes, orthologs to *VPS13A*, *B* and *D* [3]. The *Drosophila* ortholog of VPS13A referred to as Vps13, is ~29 percent identical to human VPS13A and contains the identified known domains of the human VPS13A protein. We have demonstrated that the *Drosophila* Vps13 mutant shows neurodegeneration and a shortened life span, resembling some features observed in ChAc patients as well [10]. Several phenotypes of the *Drosophila* Vps13 mutant were rescued by overexpression of the human VPS13A protein [10]. This further substantiates the suitability of the *Drosophila* model to examine the function of VPS13A and its role in maintenance of neuronal function.

	Associated with disease	# of amino acids	Localization	Function
VPS13A	Chorea-Acanthocytosis	3174	Unknown	Autophagic degradation, actin and tubulin cytoskeleton
VPS13B	Cohen syndrome	4022	Trans Golgi network	Maintenance of Golgi network integrity
VPS13C	Parkinson's disease	3753	Mitochondria	Mitochondrial health, adipogenesis
VPS13D	-	4388	Unknown	-

Table 1. Characteristics of *VPS13A*, *B*, *C* and *D*. Molecular characteristics and functions of the *VPS13A*, *B*, *C* and *D* proteins and the diseases associated with mutations in these genes.

Impaired protein homeostasis in neurodegenerative diseases

In *Drosophila* *Vps13* dysfunction leads to a defect in maintaining protein homeostasis in the central nervous system [10]. An association with an imbalance in protein homeostasis and neurodegeneration is known for a long time [11]. Protein aggregates can be found in postmortem brain tissue of several hereditary neurodegenerative diseases. PolyQ aggregates in Huntington's disease, plaques and tangles in Alzheimer's disease and Lewy bodies in Parkinson's disease [11]. The exact role of these protein aggregates in the development of the neurodegenerative disorders is still a matter of debate. Protein aggregation correlates with the diseases, however whether it contributes to the disease, whether it is "just" a consequence, or whether it is a protective mechanism for cells is not known [11].

We have demonstrated for the first time a link between *VPS13* dysfunction and impaired protein homeostasis in the central nervous system of a model organism [10]. One report showed the aggregation of PolyQ proteins and ubiquitylated proteins in a ChAc postmortem brain (n=1) [12]. This suggests that impaired protein homeostasis may also be present in ChAc patients. Another Neuro-acanthocytosis syndrome, Huntingtons disease like 2 (HDL2), has also been associated with defects in protein homeostasis. HDL2 is a disorder caused by an expanded CTG repeat in the *Junctophilin 3* (*JPH3*) gene [13]. Individuals with more than 40 repeats are at risk of developing the disease [14]. Recent reports show that the antisense strand of *JPH3* encodes for a polyQ repeat protein product [15]. This polyQ protein forms aggregates in the nuclei of neuronal cells [15]. Analysis of postmortem brain tissue of HDL2 patients confirmed the presence of intranuclear polyQ aggregates [14]. A *Drosophila* model for HDL2 shows that the accumulation of intranuclear polyQ aggregates is required for neuronal toxicity [16]. These data show that HDL2 is also associated with protein homeostasis defects and together with our results and the work of Walker et al. [12] this may suggest that that this is a common theme in Neuro-acanthocytosis syndromes.

Although an impairment of protein homeostasis may contribute to HDL2 and possibly ChAc pathology, the underlying mechanism of the imbalance in protein homeostasis is completely different. In HDL2 the protein aggregation is caused by an expanded polyQ tract which causes the expression of an aggregation prone protein [15]. However ChAc is a recessive disorder caused by loss of function mutations [1,2]. It is therefore more likely that the protein homeostasis imbalance observed in *Vps13* mutant flies is a

downstream effect of a defect in a protein degradation pathway, e.g. defects in the autophagy pathway.

VPS13A function in autophagic and lysosomal degradation

Human VPS13A has been shown to be involved in the autophagic degradation pathway [17]. The autophagy pathway is a catabolic pathway which degrades cellular constituents like proteins and organelles (Figure 1). VPS13A dysfunction leads to a defective autophagic degradation which therefore could contribute to the neurodegeneration observed in ChAc patients. Defects in neuronal autophagy lead to the accumulation of protein aggregates and neurodegeneration in mice [18,19]. Furthermore, a mutation in ATG5 has been found to lead to a familial form of ataxia [20]. The ATG5 mutation leads to an impaired autophagic flux in patient cells and yeast [20]. A *Drosophila* ATG5 mutant displays defects in autophagic degradation and neurodegeneration [20]. These phenotypes can be rescued by introducing the wild type ATG5 protein but not the mutant ATG5 [20]. These data show that autophagy plays a major role in maintaining protein homeostasis in the central nervous system and that defects in the autophagy pathway lead to neurodegeneration. VPS13A has been shown to play a role in the degradation of autophagosomes [17] therefore VPS13A dysfunction could lead to protein homeostasis defects and neurodegeneration due to impaired autophagic degradation.

Knock down of VPS13A leads to the accumulation of autophagosomes [17], however, the underlying cause of this autophagic defect has not been elucidated yet. Autophagosomes accumulate, among others, when fusion of autophagosomes with endo-lysosomal compartments is defective or in case degradation of autophagosomes by lysosomes is impaired. The fusion of autophagosomes with endosomes and lysosomes is facilitated by the microtubule cytoskeleton [21]. Autophagosomes seem to be formed randomly in the cytoplasm after which they are transported to the microtubule organizing center (MTOC) [22]. At the MTOC autophagosomes fuse with endosomes and lysosomes as the latter are also transported to the perinuclear region by the microtubule cytoskeleton [23,24]. VPS13A interacts with alpha-tubulin and knock down of VPS13A disrupts the microtubule cytoskeleton [25,26]. Therefore the accumulation of autophagosomes in VPS13A depleted cells could be caused by defects in transport of autophagosomes or endosomes via microtubules.

It may also be possible that VPS13A acts at the step of autophagosomes fusion with endosomal compartments. *Drosophila* Vps13 is associated with Rab7 positive membranes [10]. Rab7 is a Rab GTPase localized to late endosomes and lysosomes. Rab7 dysfunction leads to a defect in autophagosome to lysosome fusion and an accumulation of autophagosomes [27]. The autophagosomal and endosomal pathways are intertwined at several steps. Early endosomes and recycling endosomes are involved in the formation of autophagosomes while late endosomes and lysosomes are required for degradation of autophagosomes [28,29]. As a consequence of this close interaction between the two pathways, several proteins have shared functions in both autophagosomal and endo-lysosomal degradation.

The group of Vacuolar Protein Sorting (VPS) proteins, to which VPS13 belongs, is characterized in yeast by defects in transport of certain proteins to the vacuole [30]. Mutations in other VPS proteins have also been associated with neurodegenerative disorders. Mutations in VPS35 cause a dominantly inherited

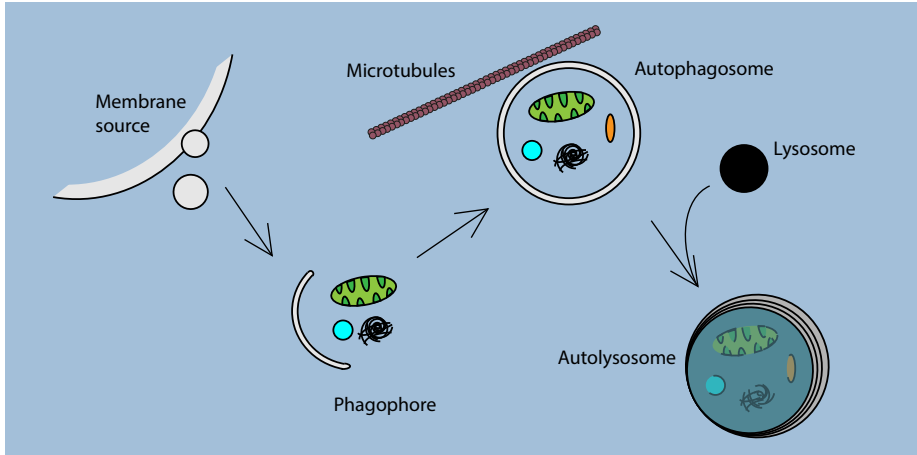


Figure 1. Schematic representation of the autophagy pathway. A phagophore is formed which enwraps cytosolic targets of the autophagy pathway like mitochondria, proteins and protein aggregates. The mature autophagosome is transported by the microtubule cytoskeleton to the perinuclear region. After fusion with a lysosome the autophagosomal contents are degraded.

form of Parkinson's disease and mutations in CHMP2B, an ortholog of VPS2 in yeast, cause frontotemporal dementia [31-33]. Interestingly, both VPS35 and CHMP2B mutations impair autophagic degradation and lead to accumulation of autophagosomes [34,35]. Even more, inhibition of autophagosome formation in CHMP2B depleted neurons suppresses the accumulation of autophagosomes and delays neuronal cell death [34]. This is similar to our results that inhibition of autophagosome formation suppresses the reduced viability of *Vps13* mutants (unpublished data). This concept was also shown in a zebrafish model in which depletion of Spinster leads to accumulating autophagosomes and developmental defects [36]. Inhibition of autophagosome formation in these zebrafish prevents the accumulation of autophagosomes and alleviates the developmental defects [36]. This indicates that a proper balance of autophagosome formation and degradation is crucial during development.

VPS13A at the crossroads of mitochondria and endosomes

Another factor which may contribute to the development of ChAc is mitochondrial dysfunction. Our results demonstrate that *Drosophila* Vps13 is a peripheral membrane protein associated with Rab7 positive membranes [10]. Interestingly, both yeast VPS13 and YPT7, the yeast Rab7 ortholog, localize to mitochondria-vacuole contact sites [37-39]. These contact sites have been shown to play a role in the exchange of phospholipids between the mitochondria and the vacuole [40]. When these mitochondria-vacuole contact sites are impaired other contact sites between the mitochondria and the ER, called ERMES, compensate for this loss of function [37,40]. Defects in both of these contact sites simultaneously leads to lethality. Additionally, stimulating mitochondria-vacuole contact sites prevents dysfunction in a ERMES compromised background [37]. VPS13 has been found in mitochondria-vacuole contact sites and is synthetic lethal with ERMES, therefore it is likely that it plays a role in establishing mitochondria to

vacuole contact sites and maintaining mitochondrial health in yeast [38,39].

Defective mitochondria are a hallmark of many neurodegenerative disorders, such as Alzheimer's and Parkinson's disease [41]. Candidate causative genes for Parkinson's disease, involved in mitochondrial quality control are *PARKIN* and *PINK1* [42,43]. Several groups established *Drosophila* models for Parkinson's disease in which *PINK1* and *PARKIN* were mutated [44-48]. These flies display a common phenotype: accumulation of damaged mitochondria, male sterility, impaired flying capability and neurodegeneration in the brain. Because of the similarities in phenotype between the *PINK1* and *PARKIN* mutant flies it was suspected that these two proteins function in related pathways. In 2006 three independent research groups showed that the *PINK1* mutant can be rescued by overexpression of *PARKIN* and that these proteins are in the same pathway controlling mitochondrial function [46-48]. *PINK1* localizes to the outer membrane of mitochondria with decreased mitochondrial membrane potential [49,50]. *PINK1* subsequently recruits *PARKIN* to the mitochondrial membrane [50]. Subsequently, at the mitochondrial outer membrane *PARKIN* ubiquitylates proteins, which is believed to aid in targeting of mitochondria for degradation via the autophagy pathway [49].

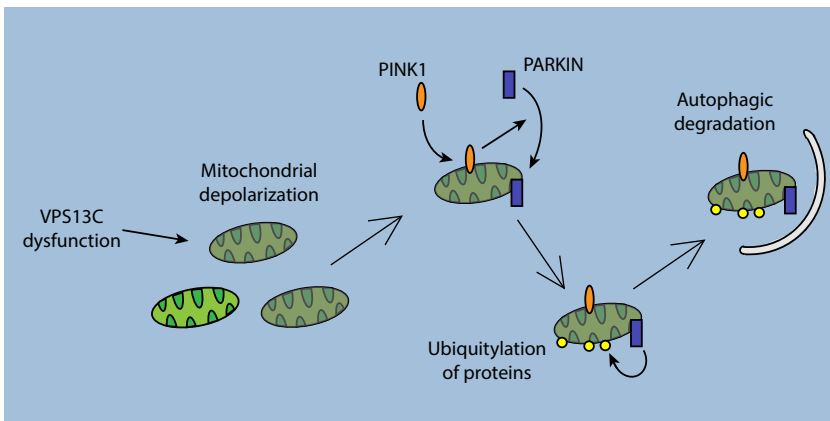


Figure 2. Schematic representation of PINK1 and PARKIN dependent mitophagy. VPS13C dysfunction leads to mitochondrial depolarization and fragmentation. PINK1 interacts with the outer membrane of depolarized mitochondria and recruits PARKIN to the mitochondrial membrane. PARKIN ubiquitylates proteins present on the mitochondria after which the mitochondria are taken up into an autophagosome and degraded.

This specific degradation of mitochondria via macroautophagy is called mitophagy (Figure 2). In *VPS13* knock out yeast mitochondrial markers accumulate inside autophagosomes, suggesting activation of the mitophagy pathway [39]. Mitophagy may be stimulated to clear the damaged mitochondria which are present under *VPS13* knock down conditions [39]. Interestingly *VPS13C* mutations are associated with a hereditary form of Parkinson's disease and knock down of *VPS13C* leads to decreased mitochondrial membrane potential and mitochondrial fragmentation [7]. Under this condition mitochondria are

degraded via a PINK1/PARKIN mediated mitophagy pathway [7]. This condition is very similar to the VPS13 knock out condition in yeast. Therefore it is interesting to investigate whether VPS13A also has a function in maintaining mitochondrial health or whether this is a VPS13C specific function.

Synaptic plasticity

A factor thought to contribute to many neurodegenerative diseases is excitotoxicity [51]. Excitotoxicity is the phenomenon in which excessive neuronal activity leads to neurological toxicity. One of the underlying factors of excitotoxicity is the excessive activation of postsynaptic glutamate receptors. Glutamate is the most important excitatory neurotransmitter in the mammalian brain. The excitation of neurons should be at an appropriate level. Overstimulation of glutamate signaling in experimental models causes neuronal dysfunction and death [51]. Excitotoxicity is suggested to contribute to the neurodegeneration in Huntington's, Alzheimer's and Parkinson's disease [51].

Excitotoxicity may also play an important role in ChAc pathology. Neurons derived from ChAc patient IPS cells have increased spontaneous activity and increased postsynaptic currents [52]. The neurons derived from ChAc patient IPS cells also show increased neurite outgrowth [52]. This is very comparable to the neuronal overgrowth that was observed in the *Drosophila Vps13* mutants (Chapter 4). This NMJ overgrowth was accompanied by increased presence of postsynaptic glutamate receptors (Chapter 4). These features of the *Drosophila* model suggest an increase in neuronal activity within the NMJ of the *Drosophila* model. Altogether, also in ChAc patients increased neuronal activity could cause excitotoxicity and contribute to the neuronal degeneration.

Concluding remarks and future perspectives

At the molecular and cellular level still little is known about VPS13A function. Due to the rare nature of ChAc and the limited availability of postmortem brain material the study of pathological mechanisms in ChAc patient brains is difficult. This underscores the importance of studying VPS13A orthologs in model organisms to find the possible molecular mechanisms of VPS13A. Most of what is known about the molecular function of VPS13 family members is provided by studies in yeast. However, yeast only has a single *VPS13* gene while humans have 4. The fact that mutations in the different VPS13 genes lead to different disorders indicates that the functions of the 4 VPS13 proteins may be different and not redundant. Yeast, with its single *VPS13* gene will provide valuable information, however studies in additional models have to be done to uncover the function of the specific VPS13 family members. The *Drosophila* model presented in this thesis will likely contribute to the knowledge about VPS13A function. It recapitulates many of the key features of described *Drosophila* neurodegenerative models and a number of the reported phenotypes can be rescued by overexpression of the human VPS13A protein. This shows that the *Drosophila Vps13* and human VPS13A share at least several cellular roles and it shows the relevance of this *Drosophila* model to study the function of VPS13A. Although findings in model organisms are informative the eventual concepts should be investigated in mammalian model systems as well.

Although progress in VPS13A research is slowly moving forward, the protein function at a molecular level is still far from understood. Also the impact of VPS13A dysfunction at the cellular level is not elucidated. Future research will need to take advantage of all the different models that are available to study its function. The *Vps13* mutant *Drosophila* model established in this thesis will contribute greatly to the understanding of the role of VPS13A in the central nervous system. Additionally, the screening for pathways or compounds that rescue features of *Vps13* mutants is straightforward in *Drosophila*. The compounds and pathways identified in this way may eventually lead to a potential drug to treat ChAc patients.

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