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Characterization of a *Drosophila* model for Chorea-Acanthocytosis

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2017

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):
Vonk, J. (2017). *Characterization of a Drosophila model for Chorea-Acanthocytosis*. [Thesis fully internal (DIV), University of Groningen]. University of Groningen.

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CHAPTER 1

Introduction and aim of the thesis

INTRODUCTION

Neuroacanthocytosis syndromes

The neurodegenerative disorder Chorea-Acanthocytosis belongs to the group of Neuroacanthocytosis (NA) syndromes [1]. The NA syndromes are rare diseases which are mainly characterized by neurodegeneration in the brain and the presence of acanthocytes [1,2]. Acanthocytes are red blood cells with irregularly spaced plasma membrane spikes. It is not known whether these acanthocytes contribute to the pathology of these diseases.

The NA syndromes are progressive disorders and lead to premature death [1]. Most patients develop chorea, which is the main hallmark of this group of diseases. Chorea is characterized by large involuntary movements and is most well-known from Huntington's disease. Other movement abnormalities, like dystonia, parkinsonism and tourettism have also been seen in NA patients [1]. Psychiatric disorders become more prominent during disease progression as well. These include bipolar disorder, obsessive-compulsive disorder, schizophrenia and depressions [1].

Chorea-Acanthocytosis (ChAc), McLeod Syndrome (MLS), Huntington's Disease-Like 2 (HDL2) and Pantothenate Kinase Associated Neurodegeneration (PKAN) are the core diseases of the group of NA syndromes (Table 1) [1]. The genetic cause of each of these diseases has been identified. ChAc, MLS and PKAN are recessive disorders caused by mutations in the *VPS13a*, *XK* and *Pank2* genes respectively [3-6]. HDL2 is a dominantly inherited disease caused by a CAG/CTG repeat expansion in the *Jph3* gene [7]. In this introductory chapter I will focus on Chorea-Acanthocytosis. For extended review of the other NA disorders see chapter 2.

Disease	Affected gene	Mutations	Mode of inheritance
McLeod Syndrome	XK	Deletions, nonsense, missense, splice site	Recessive
Chorea Acanthocytosis	VPS13A	Deletions, nonsense, missense, splice site	Recessive
Huntington's disease-like 2	Junctophilin 3	Expanded CAG/CTG repeat	Dominant
Pantothenate kinase associated neurodegeneration	Pantothenate kinase 2	Deletions, missense	Recessive

Table 1. Molecular basis of the four core NA syndromes.

Clinical features of Chorea-Acanthocytosis

The clinical features of ChAc include psychiatric disorders and movement disorders like chorea and dystonia [1]. One of the features that is characteristic for ChAc and is not present in the other NA

syndromes is orofacial dystonia. This includes biting of the lips and tongue and causes difficulties with eating. The lip and tongue biting is likely due to the dystonia these patients have, however this might also partly be a consequence of the obsessive compulsive nature of these patients. The patients also have difficulties with speech, which worsens during disease progression [8,9].

Initial diagnosis is mainly performed on the basis of the clinical features of the patient, the presence of acanthocytes and elevated CK levels in the serum. Patients on average start to show symptoms at 32 years of age [1]. Confirmation of the diagnosis of ChAc is done by Western blot for VPS13A protein levels in blood samples of the patient [1]. Current treatments for ChAc consist mainly of symptomatic drug treatment to control the chorea and dystonia. Deep brain stimulation has been performed to treat ChAc patients as well and leads to improvement of the chorea and dystonia [10].

Over the course of 15 to 30 years the disease progressively worsens and always ends in premature death. It is reported that ChAc patients, after a relatively slow decline in functioning, sometimes have a sudden death. This may be caused by the seizures that many of these patients have [11].

Loss of function mutations in the *Vps13A* gene cause ChAc

ChAc is a recessive disease caused by mutations in the *VPS13A* (Vacuolar protein sorting 13A) gene [3,4]. The VPS13A protein, also called Chorein, belongs to a family of four VPS13 proteins that have been identified in humans. The four VPS13 proteins are large proteins. VPS13A is the smallest member with a predicted length of 3174 amino acids. The four proteins share their highest homology in the C- and N-termini and all four contain a chorein domain of unknown function at their N-terminus [12].

A wide variety of mutations in *Vps13A* have been found to be associated with ChAc. The mutations are mostly nonsense, frameshift and splice-site mutations and deletions [13,14]. Only a small amount of missense mutations causing ChAc have been found. Therefore it is suspected that VPS13A is very tolerable to amino acid substitutions [13]. All of the *VPS13a* mutations known in ChAc patients lead to lower levels of VPS13A protein levels [15]. Heterozygous mutation carriers do not have lower levels of VPS13A protein and do not show any features of ChAc [15].

Mutations in *VPS13B* and *C* also lead to neurological disorders. VPS13C mutations lead to Parkinson's disease [16]. VPS13C localizes to the mitochondrial outer membrane and is required to maintain mitochondrial health [16]. Mutations in VPS13B are associated with a disease called Cohen syndrome, which is a developmental disorder causing mental retardation, facial dysmorphism, microcephaly and truncal obesity [17,18]. VPS13B is a protein localized to the cis-Golgi network and is involved in tubulation of the Golgi network [19]. Lower levels of VPS13B lead to loss of Golgi network integrity in cell culture and in fibroblasts from Cohen syndrome patients [19]. This loss of integrity probably leads to impairments in Golgi function, because Cohen syndrome patients have defects in glycosylation of proteins [20].

The fact that mutations in VPS13A, B and C lead to ChAc, Cohen syndrome and Parkinson's disease respectively suggests that the four VPS13 proteins are not completely redundant and cannot take over each other's function if one is mutated. Cohen syndrome is a multi-system developmental disorder

while ChAc and Parkinson's disease are progressive neurodegenerative diseases which start later in life. These differences in clinical manifestation of these diseases points towards different functions or tissue distribution of these three VPS13 proteins.

VPS13A is conserved among many different species. Homologous proteins are present in *M. musculus* [21], *D. melanogaster* [22], *C. elegans* [12], *S. cerevisiae* [23], *A. thaliana* [12] and *D. discoideum* [24] among others. The number of amino acids composing these proteins does not differ greatly. The areas of highest homology are in the N- and C-termini and all of these proteins contain a chorein domain at their N-terminus [12].

VPS13 function in cellular homeostasis

Vps13A and its orthologues have been studied in a variety of different organisms. Most of the knowledge about its function comes from studies using yeast as a model. Also a mouse model has been established and patient red blood cells and acanthocytes have been studied as well to find a function for Vps13A. Firstly, we will give a short overview concerning the function of VPS13 in different model organisms.

VPS13A in mice is primarily expressed in the testis, spleen, kidney and various regions of the brain [25], which is quite comparable to the distribution of VPS13A in humans [3]. A VPS13A knock-out mouse model for ChAc has been established which displays motor disturbances and acanthocytes at old age. However, in contrast to ChAc patients, the mouse does not have a shortened life span [21]. Genetic background has a large influence on these phenotypes as the knock-out mice show variable phenotypes dependent on the mouse strain under investigation [26]. This indicates that genetic modifiers of the ChAc phenotype are present in mice.

Most knowledge about the function of VPS13 comes from research in *S. cerevisiae*. Yeast VPS13 localizes to endosomes [27] and was initially identified in a screen for proteins involved in sorting of CPY, a peptidase, to the vacuole [28]. The yeast vacuole has a function comparable to the lysosomes in mammalian cells. In VPS13 deletion strains a portion of CPY is excreted instead of delivered to the vacuole. VPS13 has a function in the trafficking of proteins between the trans-Golgi network and the pre-vacuolar compartment, which has a similar function as the late endosome in mammalian cells [23,29]. It was shown that Vps13 in yeast is a membrane associated protein [29]. A slower delivery of misfolded protein cargo from the ER to lysosomes was also observed in VPS13 deletion yeast strains [30]. However they did not find any defects in Golgi to endosome trafficking, but an impairment was demonstrated in lysosomal delivery of an endocytosed dye called fm4-64 [30]. Despite the trafficking defects in VPS13 knock out yeast, the vacuoles do not have an aberrant morphology [31].

Under adverse circumstances *Saccharomyces cerevisiae* forms spores to survive. VPS13 localizes to the prospore membrane and knock out of VPS13 leads to a defect in prospore formation [32]. This prospore formation defect is caused by insufficient levels of PI(4)P and PI(4,5)P₂ at the prospore membrane [33]. Park et al. found that VPS13 interacts with Spo71 and together these proteins control prospore formation

and PI(4)P and PI(4,5)P₂ levels at the prospore membrane [34]. VPS13 localizes to mitochondria-vacuole contact sites and controls mitochondrial health [35,36].

A striking feature of nearly all ChAc patients is the presence of acanthocytes, however the percentage of acanthocytic red blood cells varies between patients [2]. Due to the relatively easy accessibility of blood samples, red blood cells of ChAc patients have been studied extensively. Increased Lyn kinase activity has been proposed as the cause of acanthocytes in ChAc patients. Lyn phosphorylates Band-3, a plasma membrane protein in red blood cells, which subsequently binds β -adducin a component of the cytoskeleton. It is suggested that the altered interaction of plasma membrane proteins and cytoskeletal components is causative for the presence of acanthocytes [37]. Another article shows that VPS13A interacts with cytoskeletal proteins β -adducin and β -actin and controls the actin cytoskeleton and cell shape [38].

Alterations of the signaling cascade regulating actin polymerization have also been suggested to cause acanthocytes [39]. Less polymerized actin was found in red blood cells from ChAc patients with acanthocytes. Also the knock down of VPS13A in K562 cells, a red blood cell progenitor cell line, is associated with a reduced level of polymerized actin and the reduction in phosphorylation of PAK1, Rac1 and PI3K, proteins known to affect actin polymerization [39]. These data combined point towards a function of VPS13 proteins in intracellular trafficking of membranes and proteins and a role in maintaining cytoskeletal integrity.

Using *Drosophila melanogaster* to study neurodegenerative disorders

Drosophila melanogaster has a central nervous system (CNS) which is located in the head and occupies part of the thorax. The *Drosophila* CNS regulates movement of the fly, memory, integrates sensory information and regulates complex behavioral outputs. *Drosophila* neurons possess comparable neurotransmitters as mammalian neurons. Due to the many similarities between mammals and flies at the cellular level and the ease of genetic and pharmaceutical manipulations *Drosophila* is a very suitable model to study the underlying molecular mechanisms of neurodegenerative diseases [40].

One of the first *Drosophila* models for a human neurodegenerative disease was published in 1998, which was a model to study the neurodegenerative disorder Sca3, caused by a CAG repeat expansion in the *ATXN3* gene [41]. It demonstrated that overexpression of Sca3-78Q, but not Sca3-27Q, caused nuclear inclusions and neurodegeneration [42]. The transgene was specifically expressed in the eye and led to a roughening and depigmentation of the eye [42]. Because of this clear phenotype the model was used extensively for modifier screens and several suppressors and enhancers of polyQ toxicity were identified [43]. The *Drosophila* eye phenotype proved to be a very straightforward and valuable way to study neuronal toxicity. Therefore the *Drosophila* eye was later used to study Huntingtin polyQ and Tau toxicity as well [44,45].

Besides studies in the *Drosophila* eye also the *Drosophila* brain has been studied extensively. By mutating PINK1 and PARKIN several groups established *Drosophila* models for Parkinson's disease [46-50]. These flies all have accumulation of damaged mitochondria, male sterility, impaired flying capability and neurodegeneration in the brain. Because of the similarities in phenotype it was suspected that these two proteins function in related pathways. In 2006 three independent research groups showed that the

PINK1 mutant could be rescued by overexpression of PARKIN and that these proteins are in the same pathway controlling mitochondrial function [48-50]. This pathway was later confirmed to be conserved in mammalian cells.

These studies show that mutations in genes leading to neurodegenerative diseases in humans can be studied by using *Drosophila*. About 77% percent of the genes known to cause a disease in humans have a close ortholog in *Drosophila* [51]. The major advantage which *Drosophila* has over other model organisms is that it combines a CNS and rather complex behavior with the availability of easy genetic and pharmacological tools for manipulations. This makes it possible to study the underlying pathological mechanisms of neurodegenerative diseases, like SCA3 and Parkinson's disease, and investigate potential therapeutic strategies.

AIM AND OUTLINE OF THIS THESIS

ChAc is a neurodegenerative disease, mainly presenting in patients in their mid-thirties. The pathophysiology is largely unknown and currently there is no treatment available. In order to understand how mutations in *VPS13A* lead to ChAc and how this can be prevented by a specific therapy a suitable model organism for this disease is required. Because fruitflies age relatively fast and possess a complex brain, the aim of this project was to develop and validate a *Drosophila melanogaster* model for ChAc. The *Drosophila melanogaster* ortholog of *VPS13A*, *Vps13*, contains all of the known domains of *VPS13A*. The strategy was to first characterize *Vps13* mutants and to examine the consequences on behavior, life span, locomotor function and cellular homeostasis of *Vps13* loss of function with a specific focus on neuronal tissue. To further show the relevance of this possible model to understand ChAc, our aim was to investigate whether expression of human *VPS13A* in the *Drosophila Vps13* mutant background rescues apparent phenotypes.

Chapter 2: Brain, blood, and iron: perspectives on the roles of erythrocytes and iron in neurodegeneration.

ChAc is part of the group of Neuroacanthocytosis (NA) syndromes. NA syndrome patients have neurodegeneration in the brain and the presence of acanthocytes in their blood. The four core NA syndromes are ChAc, McLeod Syndrome (MLS), Huntington's Disease-Like 2 (HDL2) and Pantothenate Kinase Associated Neurodegeneration (PKAN). PKAN is also part of another group of disorders called the Neurodegeneration with Brain Iron Accumulation (NBIA) disorders. This review investigates the different genes causing the neurodegeneration in both of these groups of diseases. Pathways affected in multiple of these diseases are discussed in order to find a link why these diseases present with similar features in patients.

Chapter 3: *Drosophila* Vps13 is required for protein homeostasis in the brain.

ChAc is caused by loss of function mutations in the *VPS13A* gene which lead to low levels of the VPS13A protein. The mechanism by which loss of VPS13A causes ChAc is not known. To gain more insight in the physiological role of VPS13A in maintaining neuronal health, we characterized a *Drosophila* *Vps13* mutant. We showed that the *Vps13* mutant has three main characteristics of *Drosophila* neurodegeneration models, a shortened life span, decreased climbing capability and the presence of vacuoles in the brain. Furthermore, the *Vps13* mutants showed a sensitivity to proteotoxic stress and an impaired protein homeostasis. Many of these phenotypes could be rescued by the overexpression of human VPS13A in the *Vps13* mutant background, underscoring the relevance of this *Drosophila* model for the understanding of VPS13A function.

Chapter 4: *Drosophila* Vps13 mutants show overgrowth of larval neuromuscular junctions.

ChAc patients present with neurological dysfunction and movement disorders like chorea. How VPS13A loss of function leads to neuronal dysfunction is not known. Therefore neuronal function and development was studied in *Vps13* mutant *Drosophila* larvae. The larval neuromuscular junction (NMJ) is an established model to investigate the function of glutamatergic excitatory neurons. The *Vps13* mutant larval NMJ showed neuronal overgrowth and the presence of type 2 boutons. This was associated with an increased basal larval locomotor function. Additionally, the NMJ muscles showed an increase in the level of postsynaptic ionotropic glutamate receptors. These results suggest an increase in neuronal activity at the *Drosophila* larval NMJ of *Vps13* mutants.

Chapter 5: Summarizing discussion and future perspectives.

Although it is already known since 2001 that *VPS13A* loss of function mutations lead to ChAc, the function of the VPS13A protein is not well understood. The *Vps13* mutant *Drosophila* model provides a multicellular organism model to study the underlying pathological mechanisms which may play a role in ChAc. It gives new insight into the pathways in which VPS13A may be involved. The data presented in this thesis will be discussed and set into the context of existing data. We discuss the data available on the function of VPS13A and the various cellular pathways it may be involved in and how these may contribute to the development of ChAc.

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