Characterization of a Drosophila model for Chorea-Acanthocytosis

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Drosophila Vps13 mutants show overgrowth of larval neuromuscular junctions

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ABSTRACT

Chorea-Acanthocytosis is a rare neurodegenerative disorder caused by mutations in the VPS13A gene which lead to a severe reduction or absence of the VPS13A protein. How loss of this protein affects neuronal functioning is not known. In this chapter we use a Drosophila Vps13 mutant to study the consequences of Vps13 loss of function on neuronal development and plasticity in the larvae. Vps13 homozygous mutant larvae showed increased crawling speed, while their muscle size was not increased compared to wild type controls. This phenotype is associated with an overgrowth phenotype of neuromuscular junctions and the appearance of type 2 boutons. These characteristics point to an aberrant regulation of neuronal plasticity. The active zone marker, Bruchpilot, did not show increased staining intensity in Vps13 mutants compared to controls. However, the boutons of Vps13 mutant larval neuromuscular junctions contained more intense staining for the glutamate receptor, GluR2A. In conclusion, Vps13 mutants possess characteristics of a hyperkinetic phenotype which is associated with overgrowth of the larval NMJ.
INTRODUCTION

The neurodegenerative disorder Chorea-Acanthocytosis (ChAc) is characterized by chorea, orofacial dyskinesias, seizures and psychiatric symptoms [1]. It is caused by loss of function mutations in the VPS13A gene, which lead to reduced expression of the VPS13A protein [2-4]. The underlying mechanism of how VPS13A loss of function leads to neuronal dysfunction and neurodegeneration is not known.

Neurons differentiated from ChAc patient derived induced pluripotent stem (IPS) cells show increased spontaneous synaptic activity [5]. This increase is rescued when the cells are treated with an actin cytoskeleton stabilizing drug, suggesting that the increased activity is due to actin polymerization defects [5]. Additionally the cells also show increased neurite outgrowth [5]. In VPS13A knock down PC12 cells the neurite outgrowth after NGF stimulation is not altered, however the neurites display an abnormal morphology [6]. These results suggest a role for VPS13A in the regulation of neuronal activity and neurite formation.

Synaptic plasticity is the process in which neuronal connections change under changing conditions [7]. These conditions include learning and memory, growth, development and changing environmental conditions [7]. Under these conditions the synaptic connections with other neurons or muscles needs to be adjusted according to the altered circumstances. As an example, during learning and memory, new connections between neurons are created while specific existing connections are strengthened [7].

An established system in Drosophila to study synaptic plasticity is the larval neuromuscular junction (NMJ). NMJs are the structures where the termini of neurons innervate the muscles leading to muscle contraction and larval movement [8]. During the three larval stages in Drosophila development the larva grows several magnitudes in size. Accordingly, the muscles grow as well and in order to innervate these muscles the amount of neuronal synapses, called boutons, need to increase as well [9].

To understand the function of VPS13A we established, characterized and validated a Drosophila line harboring a mutation in the Drosophila ortholog of the human VPS13A gene, further referred to as Vps13. To investigate the effects of Vps13 dysfunction on neuronal function we studied larval NMJs. We found that Vps13 mutant larvae showed faster crawling behavior which did not coincide with an increase in muscle size. The NMJ of Vps13 mutant third instar larvae showed more boutons and the presence of type 2 boutons. The immunoreactivity of the glutamate receptor in the NMJ was increased suggesting the presence of more glutamate receptors. These results suggest that Vps13 is important in maintaining proper neuronal plasticity and postsynaptic glutamate receptor levels.
RESULTS

**Vps13 mutant third instar larvae show increased basal larval locomotor activity**

We previously showed that Vps13 loss of function resulted in a locomotor impairment in adult flies [10]. In order to investigate the locomotor behavior in larvae, we used a previously established assay to monitor larval mobility [11]. The Vps13 mutant larvae showed a higher basal larval locomotor activity compared to control larvae (Fig 1A and B).

![Fig 1. Vps13 mutant third instar larvae show increased basal larval locomotor activity.](image)

**Fig 1.** Vps13 mutant third instar larvae show increased basal larval locomotor activity.

(A) The crawling trajectory of a control and Vps13 mutant larva within 1 minute on an agar plate. (B) Quantification of the crawling distance of control and Vps13 mutant larvae within 1 minute. Mean and SEM of the crawling distance from at least 8 larvae is indicated.

Larval motility is mainly regulated by the proper coordination of neuronal innervation and muscle contraction [12]. In order to find the underlying mechanism for the locomotor phenotype in Vps13 mutant larvae we studied the larval NMJ. The *Drosophila* larva is compartmentalized in a number of segments with repeating patterns of muscles and neurons innervating these muscles (Fig 2A) [13]. All of the experiments were conducted on the NMJ’s of abdominal segment A2. Each hemisegment consists of a specific arrangement of 30 muscles of which muscles 3 to 8 are depicted in Fig 2B. The size of muscles 4, 6 and 7 of Vps13 mutants and controls were measured and were comparable (Fig 2C and D), demonstrating that increased larval crawling of VPS13 mutants cannot be explained by the presence of increased muscles size.

**Vps13 mutant larvae show NMJ overgrowth**

Vps13 mutant larvae showed faster crawling behavior which is not associated with the presence of larger muscles. The increase in basal larval locomotor function therefore may be caused by changes in neuronal activity. Increased neuronal activity is associated with an increase in the amount of NMJ boutons and thereby an increased larval crawling speed [8]. Environmental conditions like rearing temperature and nutrition also influence the bouton number of the NMJs and the basal larval locomotor activity [8,14].
The neurons of the larvae were visualized by staining with an antibody against HRP, which binds a neuronal antigen and stains presynaptic terminals, and by staining for Fas2, a neuronal marker for *Drosophila* NMJs. Fas2 is a protein involved in the growth and stabilization of neurons [9]. Fas2 mutants do not develop synapses at the NMJ and are not viable [9]. The *Vps13* mutant NMJs of muscle 6/7 and 4 showed an
increased amount of boutons compared to NMJs of control (Fig 3A and B, Fig 4A and B). Because the Vps13 Drosophila mutants were created by a Piggybac transposable element, a line in which this transposable element is precisely excised can serve as an additional control [10]. Indeed, NMJs of the excision line contained equal amounts of boutons compared to wild type NMJs (Figure 3). Most boutons present in control NMJs are type 1 boutons, however Vps13 mutant NMJs also contained type 2 boutons (Fig 3C and Fig 4). Type 2 boutons are smaller than type 1 boutons, often present in long strings and are mainly present under conditions of NMJ overgrowth [15].

**Bruchpilot staining intensity in the NMJ of Vps13 mutant larvae is comparable to wild type**

The active zones of the pre-synaptic neuron are the areas where vesicles with neurotransmitters fuse with the presynaptic membrane [16]. The assembly of the active zones is controlled by a protein called Bruchpilot [16]. It is a large cytoskeletal-like protein which oligomerizes and functions as a scaffold for synaptic vesicles to bind to and facilitates the release of their content in the synaptic cleft [16,17].
Drosophila Vps13 mutants show overgrowth of larval NMJ

The puncta of Bruchpilot present in the boutons are indicative of the active zones present in these boutons (Fig 5A). The pattern of Bruchpilot puncta in NMJs of Vps13 mutants was comparable to NMJs of control or excision line larva (Fig 5C). Additionally, the intensity of Bruchpilot staining was not changed (Fig 5B). The total level of Bruchpilot in samples from adult heads, enriched in neuronal tissue, was also not changed (Fig 5D). These results indicate that the amount of active zones was not changed in Vps13 mutants.

Glutamate receptor staining intensity is increased in Vps13 mutant larval boutons

The main excitatory neurotransmitter in the Drosophila central nervous system is acetylcholine. The Drosophila NMJ, however, uses glutamate as its neurotransmitter, comparably, glutamate is the main excitatory neurotransmitter of the human brain [7]. Postsynaptic glutamate receptors in the Drosophila NMJ are responsible for detecting the neurotransmitter in the synaptic cleft after which the muscle depolarizes [18]. Changes in the amount of glutamate receptor therefore have large impact on the efficiency of neuronal signaling in the Drosophila NMJ. GluR2A and GluR2B are the main ionotropic glutamate receptors expressed in the Drosophila larval muscles [18]. The two receptors have a redundant
function and knocking out both receptors, -and not only one-, leads to embryonic lethality [18]. Increased levels of GluR2A causes strengthening of the synaptic transmission and NMJ overgrowth [19,20].

GluR2A is present in the postsynaptic membrane of the muscle of both the controls and Vps13 mutants (Fig 6A). However, the GluR2A staining in the Vps13 mutant boutons was more intense compared to the boutons of control and excision line third instar larvae (Fig 6A and B). An increase in GluR2A presence in Vps13 mutant postsynaptic densities suggests an increase of glutamate receptor signaling and may indicate an increased synaptic communication.
Drosophila Vps13 mutants show overgrowth of larval NMJ

Fig 6. Glutamate receptor staining intensity is increased in the third instar larval NMJ of Vps13 mutants.

(A) NMJ’s of control, Vps13 and excision line third instar larvae stained for HRP (neurons, green) and glutamate receptor 2A (red). (B) Quantification of the intensity of GluR2A staining at the boutons of control, Vps13 and excision line NMJ’s. Depicted is the mean and SEM of the intensity of at least 70 boutons.
DISCUSSION

The Vps13 mutants showed an increase in basal larval locomotor activity. This was not correlated with an increase in the muscle size but with an increase in the amount of neuronal boutons of the NMJ. In contrast to controls the Vps13 mutants also displayed type 2 boutons, which is consistent with previous reports demonstrating that type 2 boutons are associated with faster crawling and NMJ overgrowth [8,15].

The NMJ size is controlled by several genetic and environmental factors, among which is the level of activity of the autophagy pathway [21]. The autophagy pathway is a catabolic pathway which degrades cellular constituents like proteins and organelles and can be influenced by both genetic and environmental changes. Increased autophagic flux leads to an increased amount of NMJ boutons while defects in autophagosome formation leads to a decreased amount of boutons [21]. Vps13 dysfunction has been shown to lead to impaired autophagic degradation [22], therefore we expected Vps13 dysfunction to lead to a decrease of NMJ bouton number. However in contrast to this we found an increased amount of boutons in Vps13 mutant NMJs. This discrepancy points to a potentially more complex regulation of NMJ size in Vps13 mutants than only by the autophagy pathway.

In a genome wide RNAi screen for genes involved in endocytosis, Vps13 was identified as one of the hits. Knock down of Vps13 in S2 cells, a Drosophila cultured cell line, leads to decreased endocytosis [23]. Therefore Drosophila Vps13 may affect the NMJ by its role in the endocytic pathway. Many endosomal mutants, like Dynamin, Endophilin and Synaptojanin mutants, lead to the increased development of boutons and especially lead to the appearance of many satellite boutons [24]. Satellite boutons are small protrusions emanating from a primary bouton [24]. However in Vps13 mutants no satellite boutons were observed. This suggests that although Vps13 knock down impairs endocytosis [23], the Vps13 mutant NMJ phenotype is clearly distinct from other endocytic mutants.

Vps13 mutant phenotypes are comparable to phenotypes of Drosophila Spinster mutants. The late endosomal protein Spinster plays a role in both endocytic trafficking and in autophagic degradation [25,26]. Spinster mutants have NMJ overgrowth and developmental defects which lead to a severely lowered viability just as Vps13 mutants [27,28]. Interestingly, the overgrowth of the Spinster mutant NMJs can be rescued by inhibiting autophagy [29]. Additionally, the developmental defects of a zebrafish Spinster mutant could also be partially rescued by inhibiting autophagy [26]. In line with this we showed that the decreased viability of Vps13 mutants could be partially rescued by inhibition of autophagy (Unpublished results). Taken all these data together Vps13 mutants have many similarities with Spinster mutants. These similarities may be related to a function of Spinster and Vps13 in both endo-lysosomal and autophagosomal degradation.

Increased GluR2A staining intensity was observed at NMJs of Vps13 mutants, which suggests an increase in the postsynaptic level of GluR2A. Overexpression of GluR2A leads to higher signal transmission at the NMJ and NMJ overgrowth [19]. Elevated GluR2A levels are also required for NMJ overgrowth and increased basal larval locomotor activity under certain conditions like higher rearing temperatures [14]. A higher level of GluR2A could therefore contribute to the NMJ overgrowth in Vps13 mutant condition.
This increase in NMJ size could subsequently lead to an increase in locomotor activity, consistent with previous reports [8].

Our results point to an effect of Vps13 dysfunction on NMJ development and synaptic plasticity. Therefore it may be interesting to investigate other processes in Drosophila which are influenced by synaptic plasticity, like learning and memory. It will also be of interest whether influencing the neuronal plasticity, either genetically or pharmacologically, has an effect on Vps13 mutant phenotypes like the NMJ overgrowth and larval crawling speed or possibly on viability, life span and neurodegeneration, phenotypes associated with Vps13 mutant adults as reported in Vonk et al. [10].

MATERIALS AND METHODS

Fly stocks and genetics
Fly stocks were maintained and experiments were performed at 25 °C on standard agar food. The Vps13(PB)c03628 stock was acquired from the Exelixis stock centre [30] and isogenized to the w1118 stock. The generation of the isogenic controls was performed as previously described. The excision fly lines were established previously by precise excision of the Piggybac element from the Vps13(PB)c03628 flies [10].

Larval crawling assay
The larval crawling assay was performed as published previously [11]. Crosses of the specific genotype were done at 25 degrees on Bloomington Nutrifly food. Third instar larvae were put on a petridish containing non-nutritive agar and the crawling distance within 1 minute was measured. This was repeated three times and the average distance per minute was calculated.

Western blot analysis
Flies were flash frozen in liquid nitrogen and heads were separated from bodies by using a vortexer. 30 µl freshly prepared Laemmli buffer (2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.004% bromophenol blue, 0.0625 M Tris HCl pH 6.8) was added per 10 heads and the samples were sonicated three times for 5 seconds on ice. 5% 2-mercaptoethanol (Sigma) was added and the samples were subsequently boiled for 5 minutes. Samples were run on 12% polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were incubated in 5% milk in PBS 0,1% Tween-20 and stained using the primary antibody in PBS 0,1% Tween-20 and stained using the primary antibody in PBS 0,1% Tween-20 over night at 4 °C. Staining with secondary antibodies (1:4000, GE Healthcare) was performed at room temperature in PBS 0,1% Tween-20. Signal on membranes was visualized using ECL or super-ECL solution (Thermo Scientific) in the ChemiDoc Touch (BioRad).
The following antibodies were used for Western blot analysis, alpha-tubulin (1:4000, Sigma, T5168), NC82 (1:100, Bruchpilot, DSHB)

**NMJ dissections, staining and imaging**

Size matched third instar larvae were dissected in PBS and fixed in 3.7% formaldehyde except in GLurIIA stainings where larvae were fixed in Bouin’s fixative. Larval fillets were permeabilized in 0.1% triton x-100 followed by blocking in Bovine Serum Albumin or Normal Goat Serum (Invitrogen). The following primary antibodies were used: anti-HRP (Jackson’s immunoresearch), anti BRP, FAS-2 and GluRIIA (DSHB). Z stack confocal images of muscle 6 and 7 or muscle 4 were taken on a zeiss 780 microscope. Images were maximally projected using Image J and the mean intensity values per bouton were calculated. Bouton numbers were counted manually by a person blinded for the experimental conditions.

**Quantifications and statistical analysis**

The statistical significance of the data was calculated using the Student’s t-test (2-tailed and where appropriate with welches correction). Plotted values show the average and error bars show the standard error of the mean. P-values below 0.05 were considered significant. In the figures P<0.05 is indicated by a *, P<0.01 by ** and P<0.001 by ***.
REFERENCES


