

Trafficking Regulation and Energetics

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par

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Trafficking regulation and energetics

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Table of Contents

ABSTRACT	1
ABBREVIATIONS	3
INTRODUCTION	
1 AXONAL TRANSPORT	9
1.1 Fast and Slow Axonal Transport	9
1.2 Basic Mechanisms of Axonal Transport	10
1.3 Molecular Motors	12
1.3.a Dynein	12
1.3.b Dynactin complex	13
1.3.c Kinesins	15
1.4 Motors' movement mechanism mechanisms	18
1.5 Models to study transport	21
2 AXONAL TRANSPORT IN NEURODEGENERATIVE DISEASES	25
Releasing the brake: restoring fast axonal transport in neurodegenerative disorders.	27
2.2 Fast axonal transport defects in neurodegenerative Disorders	27
2.2.a Engine trouble-when mutations in molecular motors cause neurodegenerative diseases	27
2.2.b Turbo ignition failure-a defect in an adaptor protein	28

2.2.c GPS failure-when transport directionality is altered in neurodegenerative disorders	28
2.2.d Problems on the road- when altered microtubules disrupt FAT	30
2.2.e Running out of gas-when energy supply is critical for FAT	30
2.3 Restoring Fat in neurodegenerative disorders	
2.3.a Rescuing the function of molecular motors	32
2.3.b Improving HTT function in FAT	32
2.3.c Microtubule acetylation	32
2.3.d Fueling transport	33
2.3.e FAT, the faster the better?	33
2.4 Concluding remarks	34
2.5 Acknowledgments	34
2.6 References	34
3 HUNTINGTON'S DISEASE	37
3.1 Symptoms	37
3.2 HD brain disease	38
3.3 <i>HTT</i> gene	39
3.4 HTT protein	41
3.5 HTT posttranslational modifications	43
3.6 PolyQ toxic mechanisms	45
3.6.a Proteolysis	46
3.6.b Aggregation	47
3.6.c Mitochondrial dysfunction	49
3.6.d Transcriptional deregulation	51

3.6.e <i>Excitotoxicity</i> 3.7 HTT function and dysfunction	
3.7.b HTT as an anti-apoptotic factor	59
3.7.c HTT, endocytosis and synapse	60
3.7.d HTT and ciliogenesis	61
3.7.e HTT and adult neurogenesis	62
3.7.f HTT and mammary gland morphogenesis	62
4 HUNTINGTIN IN INTRACELLULAR TRANSPORT	
4.1 HTT interacts with vesicles and the motor machinery	65
4.2 HTT is involved in intracellular transport	67
4.3 HTT regulates trafficking	68
4.4 BDNF transport in HD	71
4.5 General transport in HD	74

RESULTS

5 DROSOPHILA MELANOGASTER AS AN IN VIVO MODEL FOR STUDYING HUNTINGTIN'S FUNCTION IN TRANSPORT	81
5.1 Study presentation	81
5.2 Article I: Huntingtin's function in axonal transport is conserved in <i>Drosophila melanogaster</i>	82
6 VESICULAR GLYCOLYSIS SUSTAINS FAST AXONAL TRANSPORT	93
6.1 Study presentation	93
6.2 Article II: Vesicular glycolysis provides on-board energy for fast axonal transport	94
7 VESICLES ARE ENERGETICALLY AUTONOMOUS FOR TRANSPORT	117

7.1 Study presentation	
7.2 Article III: Molecular profiling of brain motile vesicles reveals their energetic autonomy for microtubule-based transport	118
DISCUSSION	
8 <i>D. melanogaster</i> as an <i>in vivo</i> model to study HTT function	162
9 Motile Vesicles energetic "carry-ons"	166
9.1 Local versus global	166
9.2 Glycolysis local ATP production and cellular functions	167
9.3 Glycolitic enzymes association to motile vesicles	169
9.4 Vesicular Glycolysis in Disease	170
BIBLIOGRAPHY	172

Abstract

Growing evidence support the idea that impairments in Fast Axonal Transport (FAT) play a crucial role in Neurodegenerative Diseases (NDs). Huntington's Disease is neurodegenerative disorder caused by an abnormal polyglutamine expansion in the N-terminal part of huntingtin (HTT), a large scaffold protein implicated in transport regulation. Both the presence of the mutated HTT as the loss of HTT leads to transport defects in mammals. In the fruit fly overexpression of the mutant HTT recapitulates the phenotype observed in mammals. However, it is still unclear whether HTT's function is conserved in *D. melanogaster*. Here, we show that *D. melanogaster* HTT (DmHTT) associates with vesicles, microtubules, and interacts with dynein. In rat cortical neurons, DmHTT partially replaces mammalian HTT in fast axonal transport, and DmHTT KO flies show axonal transport defects *in vivo*. These results suggest that HTT function in transport is conserved in *D. melanogaster*.

FAT is a process that requires a constant supply of energy. Mitochondria are the main producers of ATP in the cell. However, we have demonstrated that FAT does not depend on this source of energy, as previously thought, but it depends on glycolytic ATP produced on vesicles. Perturbing GAPDH or PK, the two ATP generating glycolytic enzymes, slows down vesicular transport. However, knocking down GAPDH does not affect mitochondrial transport. Furthermore, all of the glycolytic enzymes are associated with dynamic vesicles, and are capable of producing their own ATP. Finally, we show that this ATP production is sufficient to sustain their own transport, demonstrating the energetical autonomy of vesicles for transport.

Keywords: Fast axonal transport, glycolysis, Huntingtin, Neurodegenerative diseases, *Drosophila melanogaster*

1

ABBREVIATIONS

3-NP	3- Nitropropionic Acid		
ATP	Adenosine Triphosphate		
A2R	Adenosine 2 Receptor		
AD	Alzheimer's Disease		
ADP	Adenosine Diphosphate		
Akr1p	Ankrin Repeat Protein		
ALS	Amyotrophic Lateral Sclerosis		
ANF	Atrial Natriuretic Factor		
APLIP1	APP-Like-Interacting Protein 1		
APP	Amyloid Precursor Protein		
Arp1	Actin-related protein 1		
Arp11	Actin-related protein 11		
ATP	Adenosine Triphosphate		
BDNF	Brain Derived Neurotrophic Factor		
CAG	Cytosine-Adenine-Guanine		
CAP-Gly	Cytoskeleton Associated Protein-Glycine rich		
СВ	Cannabinoid		
СВР	CREB Binding Protein		
Cdk5	Cyclin-Dependent Kinase 5		
СМТ	Charcot-Marie-Tooth		
CNS	Central Nervous System		
CREB	c_AMP Responsive Element Binding Protein		
CSF	Cerebral Spinal Fluid		
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase		
GLT1	Glial Glutamate Transporter 1		
DG	Dentate Girus		
DRP1	Dynamin-Related Protein 1		
EGFR	Ependymal Growth Factor		
ER	Endoplasmic Reticulum		
GDNF	Glial Derived Neurotrophic Factor		

GPI	Phospho-Fructo Kinase
DHC	Dynein Heavy Chain
DIC	Dynein Intermediate Heavy Chain
FRAP	Fluorescence Recovery After Photobleaching
FTDP	Frontotemporal Dementia with Parkinsonism
GABA	Gamma Aminobutyric
HAP1	Huntingtin-Associeted Protein 1
НАТ	Histone Acetyl-Transferase
HD	Huntington's Disease
HDAC	Histone Deacetylase
HEAT	Huntingtin Elongation factor, protein phosphatase 2A,
	and TOR1
HIP1	Huntingtin Interacting Protein-1
HIP14	Huntingtin Interacting Protein-14
НТТ	Huntingtin
HMN7B	Hereditary motor neuropathy 7B
нк	Hexokinase
IGF-1	Insuline-like Growth Factor-1
IKK	IkB Kinase
JIP1	c-Jun NH2-terminal kinase-Interacting Protein 1
JNK	c-Jun-N-terminal Kinase
КАР	Kinesin Associated Protein
КНС	Kinesin Heavy Chain
KLC	Kinesin Light Chain
LIC	Dynein Intermediate Light Chain
LC	Dynein Light Chain
FAT	Fast Axonal Transport
FEZ1	Fasciculation and Elongation protein-ζ1
MAGUK	Membrane Associated Guanylate Kinase
Miro	Mitochondrial Rho
MKK7	Mitogen-activated protein Kinase Kinase 7
MRI	Magnetic Resonance Imaging
MSN	Medium-Spiny Neurons
МТ	Microtubule

NGF	Nerve Growth Factor		
NcoR	Nuclear receptor co-Receptor		
NES	Nuclear Export Sequence		
NF	Neurofilament		
NLS	Nuclear Localization Signal		
NMDA	N-methyl-D-aspartate		
NPY	Neuropeptide Y		
NRSE Neuron Restrictive Silencing Element-1			
OxPhos	Oxidative Phosphorylation		
p75 ^{NTR}	p75 Neurotrophic receeptor		
PACSIN 1	Protein kinase C and Casein kinase Substrate In Neurons		
PCM1	PeriCentriolar Material 1 Protein		
PD	Parkinson's Disease		
PGK	PhosphoGlycerte Kinase		
PGAM	Phospho-Glycerate Mutase		
PINK1	PTEN-Induced Putative Kinase 1		
PolyP	Polyproline		
PolyQ	Poly Glutamine		
PP2B	Protein Phosphatase 2B		
RANBP2	RAN-Binding Protein 2		
RE1	Repressor Element 1		
SMA	Spinal Muscular Atrophy		
Sp1	Specific Protein 1		
SPG	Spastic Paraplegia		
STR	Striatum		
TAF _{II} 130	TBP Associated Factor II 130		
ТВР	TATA Box Binding Protein		
ТСА	Tricarboxilic Acid Cycle		
TIRF	Total Internal Reflection		
Ti-VAMP	Tetanus neurotoxin insensitive Vesicle Associated		
	Membrane Protein		
TRAK	Trafficking protein Kinesin-binding		
TrkB	Tropomyosin-Related Kinase Receptor type B		
TRP	Tetratricopeptide		

 VDAC1
 Voltage-Dependent Anion-selective Channel 1

INTRODUCTION

1 Axonal Transport

Cells are dynamic systems in constant change that rely on intracellular transport to maintain their overall balance, preserving at the same time their structural and functional compartmentalization. Intracellular transport is responsible for the correct delivery, both in time and space, of newly synthesized molecules, is in charge of the clearance of misfolded proteins and protein degradation, and has an essential role in intra and intercellular communication.

Due to their unique architecture, neurons represent a special challenge for this active transport. A typical neuron consists of a cell body, numerous projections called dendrites, and a single elongated process that comprises over 99% of cell volume, the axon. While dendrites are in close proximity to the cell body, axons can extend up to one meter long. As a consequence, the distances covered by transported material are extremely long in axons. Therefore, the intracellular transport that occurs in axons is known as axonal transport and is essential for maintenance and function of a neuron.

1.1 Fast and Slow Axonal Transport

Axonal transport is divided classically in two distinct categories according to the bulk speed of their cargos (**Table 1**). Fast Axonal Transport (FAT) corresponds to the movement of cargos at speeds between 50-400 mm/day (0.5-5 μ m/sec) whereas slow axonal transport refers to those that move at rates that are several orders of magnitude slower, at around 0.3-8 mm/day (0.004 to 0.09 μ m/sec) (Brown, 2003). FAT and slow axonal transports differ not only in their bulk velocities, but also in the nature of the cargo transported. Membranous organelles like vesicles and mitochondria move in a FAT fashion, whereas slow axonal transport comprises mainly cytoskeletal components and cytosolic protein complexes (Tytell et al., 1981).

Table 1:

Cargo transport rates

Cargos	Overall Velocity (mm/day)	Instantaneous Velocity (µm/sec)	Directionality
Golgi derived vesicles	200-400	2-5	Anterograde
Endocytic vesicles, lysosomes, autophagosomes	100-250	1-3	Retrograde
Mitochondria	<70	0.3-0.8	Bidirectional
Microfilaments, cytosolic protein complexes	2-8	Unknown	Unknown
Microtubules, neurofilaments	0.1-1	0.3-1	Bidirectional

This classification of axonal transport is based on studies performed using radioisotopic pulse labeling. However, by direct observation of fluorescently tagged neurofilaments and microtubules (MTs) in axons, it was shown that they both move at instantaneous velocities similar to those of FAT. Nevertheless, this rapid movement is rare and separated by long periods of pause, leading to a low overall speed (Roy et al., 2000; Wang and Brown, 2001; Wang et al., 2000). The rapid movement of neurofilaments (NFs) and MTs indicates that they are transported by fast motors. It has recently been shown that both slow and fast transport share at least one motor, kinesin, which can switch from slow to fast cargos. This occurs through a mechanism involving Hsc70, which associates with kinesin for carrying slow cargos. When this association is disrupted, vesicular transport is favored (Terada et al., 2010).

1.2 Basic Mechanisms of Axonal Transport

Although the principles that govern axonal transport are not yet fully understood, the basic mechanism is well known. The major components of the trafficking machinery

are the molecular motors, which act as the "engines" of intracellular transport, and MTs, considered as the rails on which transport occurs. MTs are polarized structures with a very dynamic end known as the plus end, and a less dynamic one referred to as the minus end. In axons, MTs are oriented with the plus end towards the axon tip and the minus end facing the cell body. On the other hand, for dendrites the MTs polarity is mixed in the proximal segment close to the cell body whereas, in the distal part the polarity follows the same pattern as in axons (Kapitein and Hoogenraad, 2011) (Fig 1). Molecular motors walk along these MTs, being responsible for the active movement of the different cargos. In the synaptic regions, the actin filaments form the major cytoskeletal architecture, myosins being the motors involved in cargo delivery.



Figure 1: Cytoskeletal arrangements in neurons. Morphology of a neuron surrounded by closeups of specific compartments that show filament orientations. Microtubule plus-ends and actin filament plus-ends are marked with boxes. Graph shows the fraction of microtubules with distal minus-ends as a function of dendritic length, scaled to unit length. From Kapitein and Hoogenraad 2011.

Plus-end directed motors, kinesins, mediate anterograde transport from the cell body to the axon tip, while dynein move to the minus end of MTs, mediating the retrograde transport towards the cell body (Goldstein and Yang, 2000; Vale and Milligan, 2000). Molecular motors bind to a great variety of different cargos including both

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membranous organelles and non-membranous cargos such as cytoskeletal proteins and cytosolic protein complexes. Binding of molecular motors to their cargos can occur by direct interaction with transmembrane proteins or indirectly through the interaction with scaffolds or adaptor proteins. These adaptors are thought to play a crucial role in regulating specific motor-cargo binding and transport itself (Karcher et al., 2002).

1.3 Molecular Motors

Kinesins and dynein are the molecular motors known to be involved in long-range axonal transport. They both possess a catalytic domain referred to as the "head", characterized by the presence of a binding site for ATP and another one for MTs. Besides the motor head, kinesins and dynein are considerably different, suggesting functional differences outside this domain.

1.3.a Dynein

Dynein superfamily contains two major groups: cytoplasmic dynein and axonemal dynein, also known as cilliary or flagelar dynein (Hirokawa et al., 2010; Pfister et al., 2006). Axonemal dynein is involved in generating flagella and cilia beating by sliding the adjacent MTs one over the other. Cytoplasmic dynein's most known function is driving the movement of cargos retrogradely, but it also has been shown to be



Figure 2: Dynein structure. Schematic representation of dynein structure. It is constituted by several subunits. Dynein heavy chain (DHC), Dynein intermediate heavy chain (DIC), Dynein intermediate light chain (LIC) and dynein light chains (LC). involved in chromosome movement and mitotic spindle positioning for cell division (Roberts et al., 2013; Summers and Gibbons, 1971).

Cytoplasmic dynein (**Fig. 2**) is a multisubunit protein complex of approximately 1.5 megadaltons. It is composed of 12 polypeptide subunits: 2 heavy chains (520kDa), two intermediate heavy chains (74kDa), four intermediate light chains (33-59kDa) and several light chains (10-14 kDa) (Hirokawa et al., 2010; Roberts et al., 2013)

Dynein heavy chain (DHC) consists of three domains, each one carrying out an essential function. The stalk is located in the C-terminal region and is the MT binding domain; the head, which contains the ATP hydrolysis domain and therefore generates the energy for translocation; and the tail which is located in the N-terminal region and is responsible for cargo binding, dimerization and overlaps with dynein intermediate light chain and dynein intermediate heavy chain sites (Kikkawa, 2013).

Dynein intermediate heavy chain (DIC) binds directly to DHC, and are believed to be the ones to target the motor to its cargo (Paschal et al., 1992; Steffen et al., 1997).

Dynein intermediate light chain (LIC) interacts directly with DHC as well, and although its contribution to dynein function is not fully understood, available data suggests that its an ATPase that would probably be implicated in motility (Tynan et al., 2000).

Dynein light chains (LCs) are located in the base of the motor complex, and among others, LC is implicated in the recruitment of specific cargos (Chuang et al., 2001; King et al., 1998; Tai et al., 1998).

1.3.b Dynactin Complex

Although dynactin is not a motor *per se*, it is associated to dynein and regulates its activity. Dynein can bind some membranous cargos independently from dynactin, but the establishment of a functional dynein-cargo link depends on dynactin. The dynactin complex (**Fig. 3**) consists of two major structural domains, a rod which is thought to allow dynactin binding to different structures and a projecting arm that contains two globular termini with MTs binding sites (Schroer, 2004).

13

The rod domain looks like a short actin filament. It is composed of eight monomers of actin-related protein 1 (Arp1), which at one end is associated with the actin-capping protein CapZ (Schafer et al., 1994) and in the opposite end bears the actin-related protein 11 (Arp11) (Eckley et al., 1999) and the dynactin subunit p62 (Schafer et al., 1994). p62 in turn associates with p27 and p25 subunits of dynactin, forming a complex that sits at the end of the Arp1 rod (Eckley et al., 1999). Arp1 can directly bind to spectrins (Holleran et al., 1996), and this is probably the mechanism through which dynactin binds to different cellular structures.



Figure 3: Dynactin complex. Schematic representation of the multisubunit dynactin. MT and motor binding domains are indicated.

The projecting arm consists of four copies of dynamitin and two copies of p150^{*Glued*} and p24/22. The two p150^{*Glued*} form a dimer, generating a structure with two globular heads at the tip of the arm, which contains a CAP-Gly domain (cytoskeleton associated protein-glycine rich). This domain binds to MTs (Vaughan et al., 2002; Waterman-Storer et al., 1995), interaction which is necessary to increase dynein's processivity. p150^{*Glued*} interacts with dynein through the dynein intermediate chain, and also interacts with kinesin-2 and kinesin-5 (Deacon et al., 2003; Karki and Holzbaur, 1995; King et al., 2003; Vaughan and Vallee, 1995). Therefore one functions of dynactin could be to act as a coordinator for bidirectional transport by regulating both anterograde and retrograde motor activities along MTs and linking cargos to the MTs.

The dynein-dynactin complex is required for a broad range of cellular functions, including chromosome separation, nuclear migration, Golgi positioning as well as retrograde transport. Hence, the fact that inhibition of any of these are embryonic lethal does not come as a surprise (Gepner et al., 1996; Harada et al., 1998). The great importance this complex has in axonal transport is reflected in its implication in neurodegenerative diseases. This point will be further discussed in chapter two.

1.3.c Kinesins

Kinesins or KIFs are members of a broad superfamily of molecular motors, consisting of 15 different families: kinesin 1 to kinesin 14B. Morphologically, these motors can be classified according to the position of the motor domain in the molecule, into C, M or N-kinesins. Anterograde transport is powered by N-kinesins, in which the motor domain occupies the N-terminal region. C-kinesins contain the molecular motor domain in the C-terminal region of the molecule, and unlike most of the kinesins involved in transport, they move in the opposite direction to typical kinesins. M-kinesins play a role in MT depolymerization, and hold the motor domain in the middle of the molecule (Hirokawa et al., 2010).

Besides its role in transport, kinesins are implicated in other cellular functions including different stages of mitosis and MT depolymerization. We will next focus only on those kinesins implicated in axonal transport (**Fig. 4**).

Kinesin 1 family members were the first to be identified and are known as conventional kinesins. They are composed of two heavy chains (KHC) of 120 kDa and two light chains (KLC) of 64 kDa. The KHCs contain a globular head followed by a neck domain, a stalk and a tail (Hirokawa et al., 2010). The head located in the N-terminal part of the molecule contains the motor domain that binds to MTs and ATP.



Figure 4: Structure of kinesin families involved in intracellular transport. Kinesin-1 consists of two heavy chains and two light chains. Kinesin-2 is a heterotrimer constituted by Kif3A, Kif3B and KAP. Kinesin 3 is shown in its monomeric and dimeric form.

The stalk holds coiled-coil domains, and these domains of both KHCs interact between each other and are responsible for the dimerization. The tail located in the C-terminal region, associates with KLC and participates in regulation of motor activity and cargo binding. The KLC contains a region of interaction with KHC and a tetratricopeptide repeat (TPR) motifs that participates in cargo binding (Adio et al., 2006; Verhey and Hammond, 2009)(**Fig 4**).

Kinesin 1 family is represented by three isoforms: KIF5A, KIF5B and KIF5C, all of them expressed in neurons. Binding to different adaptor proteins allows them to transport distinct cargos. For example, amyloid precursor protein (APP) is transported by KIF5. One proposed model suggests that APP binds directly to KLC, functioning as an adaptor involved in axonal transport of tyrosine kinase receptors, β -secretase and presenilin-1 (Kamal et al., 2001; Lazarov et al., 2005). Another hypothesis suggests that APP is a cargo transported through the interaction of KIF5 with c-Jun NH2-terminal kinase-interacting protein 1 (JIP1) that in turn interacts with vesicular APOER2 or phosphorylated APP (Muresan and Muresan, 2005; Verhey et

al., 2001). Syntabulin associates with KIF5 and acts as an adaptor to recognize syntaxin vesicles (Cai et al., 2007b; Su et al., 2004). KIF5 is also involved in mitochondria transport through the Milton-Miro complex (Guo et al., 2005; Stowers et al., 2002), BDNF vesicles by the interaction with Huntingtin (HTT) and Huntingtin-associated protein 1 (HAP1)(Colin et al., 2008; Gauthier et al., 2004), and anterogradely transports retrograde motors by the interaction with LIS1-NDEL complex (Yamada et al., 2008).

Kinesin 2 family is heterotrimeric, composed of two motor domain-containing subunits from the KIF3 family and a non-motor accessory protein known as kinesin associated protein (KAP) (**Fig 4**). Similar to the KHC of kinesin-1, KIF3 polypeptides are constituted by a motor domain, a neck, a stalk and a tail. There is substantial evidence that KIF3A and KIF3B form heterodimers through binding by the stalk domains, while KAP subunit binds to it only after this heterodimer is formed (Brunnbauer et al., 2010; Doodhi et al., 2009; Yamazaki et al., 1996). Among the known cargos transported by kinesin 2 family motors there are fodrin-associated vesicles (Takeda et al., 2000), the N-cadherin containing vesicles (Teng et al., 2005) as well as the Kv1 channel (Gu et al., 2006).

Kinesin 3 family members or KIF1s initial characterization suggested that it is a globular protein with an N-terminal motor domain, whose behavior is consistent with a monomeric molecule (Okada et al., 1995). However, more recently, it has been demonstrated that it can exist in a dimeric state, and that this dimerization seem to be critical for processive motility at high velocities (Hammond et al., 2009) (**Fig 4**).

As Kinesin 1 family motors, adaptor proteins mediate transport of different cargos. Both KIF1A and KIF1B β are known to transport anterogradely Rab3 carrying synaptic vesicle precursors via the DENN/MADD or Liprin- α (Niwa et al., 2008; Okada et al., 1995; Wagner et al., 2009; Zhao et al., 2001). On the other hand KIF1B β transport mitochondria through the interaction with KBP (Wozniak et al., 2005).

17

1.4 Motors' movement mechanisms

Kinesins transport cargos anterogradely by walking processively on MTs tracks. This movement occurs by a mechanism where kinesin's heads step past one another, alternating the lead position and generating 8 nm steps. Kinesins contain two identical motor heads with catalytic domains in the N-terminal region opposite to where the cargos bind. In solution, both heads are attached to ADP, and move freely until one of these heads contacts a MT. Once this occurs, the head binds tightly to the MT causing ADP to be released and rapidly exchanged by ATP. This generates a conformational change that drives the detached rear head to move forward towards the plus end of the MT, where it will encounter and bind to the MT in front of its partner. The attached head hydrolyses ATP, and detaches from the MT, and the cycle repeats (Asbury, 2005; Asenjo et al., 2006; Gennerich and Vale, 2009; Rice et al., 1999).

Kinesin motors, when not bound to cargo, are kept in an inactive state by an autoinhibition mechanism (**Fig. 5**). In the absence of cargo, kinesin adopts a folded conformation that corresponds to an inactive state (Coy et al., 1999; Friedman and Vale, 1999; Hackney et al., 1992; Stock et al., 1999; Verhey et al., 1998). In this folded conformation, C-terminal regions of KHC interact with the motor domain, blocking ATPase activity and MT binding. KLC also takes part in this autoinhibition by physically separating the two motor domains (Cai et al., 2007a). This autoinhibition can be released by cargo binding (Coy et al., 1999; Imanishi et al., 2006). Basically, fasciculation and elongation protein- ζ 1 (FEZ1) binds to the C-terminal region of KHC and JIP1 to KLC, releasing the restraints of MT binding and processive motility (Blasius et al., 2007).



Figure 5: Autoinhibition and release of autoinhibition. Folded conformation of kinesin-1 in absence of cargo, blocks MT binding and processivity. The inhibition can be released by cargo-binding, as well as by the interaction with two binding partners, FEZ1 and JIP1.

In contrast to kinesin, dynein transports cargos to the minus end of MTs. It is a processive motor that moves along MTs taking frequently 8 nm steps, although longer as well as side and backward steps are also common (Reck-Peterson et al., 2006). Dynein binds to MTs through the stalk domain. During cargo transport, one stalk remains bound to MTs and due to a nucleotide-dependent conformational change, the linker of the DHC which is tightly bound to the MT, detaches and moves the other head towards the MT minus end. This head rebinds to the MT, allowing the cycle to continue and making the dynein complex to walk (Roberts et al., 2013).

A single cargo is transported throughout the cell by the combined action of different motors, which will determine the directionality of its movement. Moreover, a moving organelle can pause, and then either continue moving in the same direction or start moving in the opposite direction. How the motors share the load and how switching from plus end to minus end directed transport occurs, is poorly understood. Apparently, as shown by *in vitro* assays, kinesins are unable to coordinate their

movement collectively (Jamison et al., 2010) unlike dynein that has been suggested to be more efficient when cooperating with its partners (Soppina et al., 2009). Recent work using optical trap-based force measurements in living cells and *in vitro* confirmed this notion. Multiple dyneins are able to cooperate in force production under load. This cooperation is efficient as the leading dynein of a team can slow down by taking shorter step size, allowing the dyneins that are behind to catch up by taking larger steps. In this way, they cooperate and therefore share the load, becoming more efficient. Contrary to this situation, when multiple kinesins pull a cargo, they are unable to change the step size; therefore the trailing motors cannot catch up with the leading one and load is not distributed among the group. As a result, the leading kinesin bears most of the load and detaches from MTs more easily (Rai et al., 2013). However, this is in contradiction with studies performed in *D. melanogaster*, where APP vesicle velocity seem to depend on the amount of kinesin (Encalada et al., 2011; Reis et al., 2012).

The situation becomes even more complicated by the fact that motors of opposing polarities can attach to the same cargo (Hendricks et al., 2010). One main question in the field is how directionality is determined in this case, and how a cargo can change the direction of movement. The main hypothesis proposes that groups of opposing motors will pull in opposite directions, and as a tug-of-war, the stronger team will determine the directionality. It was described from experiments performed in purified vesicles from mouse brains, that a single vesicle contains approximately 2.8±1.6 dyneins, and 1.7±1.0 kinesins. Moreover, it seems that motors of opposite polarity can simultaneously functionally interact with the organelle, as *in vitro* motility studies performed with these vesicles show that they travelled for short distances and frequently changed direction (Hendricks et al., 2010). An in vitro study using DNA origami (see next section for full description) as a synthetic cargo enabled to further address this question. This system allows controlling the motor type, number, spacing, and orientation in vitro. Using this system it was described that altering the ratio of dynein to kinesin motors present influenced directionality of the cargo. Besides, when an immotile cargo bound to both kinds of motors is released from one type of motor, the cargo begins to move, further supporting the tug-of-war model (Derr et al., 2012).

20

1.5 Models to study transport

The complexity of studying intracellular transport has been previously discussed in this chapter. The problem lies mainly in dissecting the individual functions of the different components that take part in this process, and then further studying how all of the individual components work as a whole. To address the many questions concerning intracellular transport, a wide variety of approaches ranging from *in vitro* to *in vivo* techniques have been developed.

Motility of motor proteins can be studied by *in vitro* assays using purified motor proteins, thanks to the development of total internal reflection (TIRF) microscopy. One of the most commonly used strategies is the gliding assay (**Figure 6A**). This assay consists of immobilizing molecular motors to a glass coverslips and then adding fluorescently labeled *in vitro* polymerized MTs. In presence of ATP, when MTs encounter the motors, they are bound to and transported by the motors along the surface in a gliding movement. Using this strategy we can obtain information about sliding velocity, ATP dependence and some information about group dynamics.

Another very popular *in vitro* assay is the single motor assay (**Figure 6B**). In this case, the configuration is inverted in comparison to the previous assay. MTs are attached to the glass surface, then motors are perfused and the motility of single motors is monitored. Visualization is possible as motors may be either fused to a fluorescent tag or covalently labeled to an organic fluorophore or a quantum dot.

A variation of this method can be obtained by attaching small polymer beads to the motors (**Fig 6C**). In this case, the cargo can be observed by differential interference contrast microscopy. This has been thoroughly used to study the physical properties of motors, by optical trapping experiments.

A flexible *in vitro* strategy has been recently developed, which consists in a three dimensional structure of DNA called DNA-origami (**Figure 6D**) (Derr et al., 2012). This structure contains single stranded DNA at specific locations allowing the attachment of motor proteins via the complementary DNA. The number, identity and

21

the spatial localization of the motors can be tightly controlled. Furthermore, release of motors from the DNA-origami is as well feasible through photo-cleavable linkages. This is a powerful tool that will further allow studying motor interactions *in vitro*.



Figure 6: Models to study intracellular transport. Schematic representation of methods used to have a better understanding of the mechanisms that regulate intracellular transport. These methods go from pure *in vitro* analysis to tracking transported molecules *in vivo*.

In vitro reconstitution using an endogenous cargo is a more physiological implementation for *in vitro* assays (**Figure 6E**). This can be achieved by the purification of organelles from cells or tissues, but the purified cargos must either retain their capacities to interact with motors and/or contain their endogenous motors attached. For example, macrophages can phagocyte latex or magnetic beads that will be enclosed in a native vesicle and transported by endogenous motor proteins. These beads-containing organelles can be purified and used for *in vitro* experiments.

Similarly, preparations can be enriched for a specific organelle that is already labeled with a fluorophore. However, the nature of the cargo is generally unknown.

Additionally, intracellular transport can be studied directly in living cells by a combination of single molecule or fluorophore labeling of proteins with fluorescent videomicroscopy. In this way, a specific protein is tagged and expressed inside the cell. This allows its visualization as well as the tracking of its movement inside the cell (**Fig 6F**). This enables the study of the dynamic properties of a known cargo or motor in its native environment. Furthermore, study of physical properties of endogenous compartments has been achieved by internalization of latex beads and subsequent measurements with optical traps (**Fig 6G**) or quantum dots.

Studying axonal trasnport *In vivo* is more complex due to the difficulty to reconcile spatiotemporal resolution and tissue accessibility. The classical studies have been performed by radioactive pulse and chase experiments in mouse sciatic nerves. Radioactive amino acids are injected in the cell body, allowing monitoring newly synthesized proteins as they travel within the axon. The rate of transport of these proteins can be measured by studying, using western blot, the distance they covered at different time intervals. The squid giant axons have been useful to study axonal transport *in vivo*. Video microscopy recordings allow monitoring cargos in the intact structure. Finally, generation of transgenic worms and flies expressing motors and cargos tagged with fluorophores has enabled the study of intracellular transport of specific cargos directly in living animals.

2 Axonal transport in neurodegenerative diseases

In the previous chapter we described the working mechanism of axonal transport, the molecular complexes that take part in this, and how this process is regulated. We have highlighted as well the particular importance of FAT in neurons.

In this chapter we will discuss the link between axonal transport and neurodegenerative diseases, focusing mainly on evidence showing that in many neurodegenerative diseases, mutations that lead to the pathology occur in the genes coding for MTs, molecular motors or adaptor proteins. In addition, we will discuss possible strategies in which FAT can be restored and its possible implication in therapeutics of neurodegenerative diseases.

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Releasing the brake: restoring fast axonal transport in neurodegenerative disorders

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Emerging evidence suggests that the dysregulation of fast axonal transport (FAT) plays a crucial role in several neurodegenerative disorders. Some of these diseases are caused by mutations affecting the molecular motors or adaptors that mediate FAT, and transport defects in organelles such as mitochondria and vesicles are observed in most, if not all neurodegenerative disorders. The relationship between neurodegenerative disorders and FAT is probably due to the extreme polarization of neurons, which extend long processes such as axons and dendrites. These characteristics render neurons particularly sensitive to transport alterations. Here we review the impact of such alterations on neuronal survival. We also discuss various strategies that might restore FAT, potentially slowing disease progression.

Fast axonal transport defects in neurodegenerative disorders

Intracellular transport is responsible for the correct spatiotemporal delivery of newly synthesized molecules. It plays an important role in intra- and intercellular communication. Neurons are particularly sensitive to the impairment of this process due to the extremely long distances between the cell body and the tip of the axon.

FAT can be defined as the long-range directed movement of membranous organelles such as vesicles and mitochondria along microtubules (MTs). The rate of FAT is approximately 50–400 mm/day and is 10–50 times faster than the transport of soluble and cytoskeletal proteins, referred to as slow axonal transport. FAT is bidirectional and is driven by molecular motors (kinesins and dynein). Kinesins transport organelles anterogradely from the cell body to the axon terminals, whereas dynein mediates retrograde transport in the opposite direction (Box 1 and Figure 1). In addition to motors themselves, various

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protein complexes, referred to as adaptors (Table 1), are associated with the molecular motors. They play a crucial role in regulating FAT by linking cargoes to motors and/or integrating various intra- and extracellular signals.

The first evidence linking transport impairments to neurodegeneration came from studies identifying genetic mutations in the molecular motors of several neurodegenerative diseases (Table 1). Defects in transport have now been reported in many neurodegenerative disorders and have been discussed extensively in recent reviews [1,2]. Although the link between gene mutations and the alteration of FAT suggests a causal role in neurodegeneration, the relationship is less clear in some other diseases and alteration of FAT may be secondary to disturbances of cellular homeostasis such as energy failure. Nevertheless, axonal transport might be the Achilles heel in these diseases and whether the restoration of axonal transport is sufficient to cure neurodegenerative diseases is thus a central question in this field. Here we discuss some of the mutations identified in neurodegenerative diseases that directly affect transport machinery and focus on strategies that could be used to restore transport in neurons. Finally we discuss some of the issues that need to be solved for a comprehensive approach to the role of FAT in the etiology of neurodegenerative disorders.

Engine trouble – when mutations in molecular motors cause neurodegenerative diseases

In recent years, mutations of the genes encoding kinesin motors have been reported in hereditary spastic paraplegias (SPGs) and in axonal Charcot-Marie-Tooth disease type 2 (CMT2) (Table 1) [3,4]. In SPG, point mutations are found in the motor and stalk domains of the KIF5A gene encoding neuronal kinesin 1 (KIF5A) [4]. Some of these mutations alter FAT by decreasing transport velocity or MT binding affinity [5]. Until recently, neurofilaments were the only known cargo of this kinesin. However, KIF5A also transports the gamma aminobutyric acid (GABA)_A receptor to synapses via huntingtin-associated protein 1 [6] and may be involved in the correct functioning of the synapse. Mutations encoding KIF1A and KIF1B β , both of which belong to the kinesin-3 family, have also been reported in SPG and CMT2 patients, respectively [3,7,8].

Box 1. Molecular motors and FAT

Kinesins belong to a very large family of KIF motor proteins. This superfamily has 45 members, 38 of which are KIFs found specifically in the brain [1]. Kinesins contain a conserved Walker A ATP-binding motif and a MT-binding motif. Outside of these two domains, kinesins display a high level of diversity and they are involved in numerous functions in addition to axonal transport. Most kinesins (except kinesin-14) are anterograde motors, exerting forces on MTs that act in the opposite direction to those exerted by dynein. The diversity of the kinesins expressed, even within a single neuron, may reflect the nature of the cargoes transported. However, there has been no systematic analysis of the relationship to or specificity of kinesins for a particular transported cargo. Conventional kinesin-1 is crucial for axonal transport and is encoded by the *KIF5A*, *B*, and *C* genes in mammals. The B isoform is ubiquitous, whereas A and C are specific to neurons.

The cytoplasmic dynein family is much smaller, with just two known members: the axonemal dynein and the cytoplasmic dynein. The heavy chain of axonemal dynein 2 is involved specifically in intraflagellar transport and is not considered here. Cytoplasmic dynein is a large (1.5 MDa), multicomplex protein mostly comprising two homodimers of DYNC1H1, each of 530 kDa, forming the large motor domain, which has ATPase activity. It also contains two intermediate chains (ICs), four light ICs, and several light chains [78]. Specificity in the transport of cargoes is the result of the various combinations of the different isoforms of light and intermediate subunits. Dynein function is linked to the association of this protein with the dynactin complex, which contains several subunits, including p150^{Glued}, p62, P50, dynamitin, and actin-related protein 1 (Arp1). Dynactin is not a motor per se, but it enhances dynein activity and could serve as a platform linking vesicles to MTs. It may also be responsible for coordinating bidirectional transport in axons and dendrites as well as recruiting motor complexes on plus ends of MTs.

Defects in the retrograde motor dynein have also been found in patients with neurodegenerative diseases. Various mutations of the dynein, cytoplasmic 1, heavy chain 1 (*DYNC1H1*) gene were identified in patients with dominant spinal muscular atrophy (SMA) [9] and CMT2 [10]. The missense mutations found in SMA are located in the tail domain of dynein and affect its stability and function [9], whereas the *DYNC1H1* gene mutation identified in CMT2 patients affects the domain of the protein responsible for homodimerization [10]. This dimerization is essential for its binding to and movement along MTs. Together these studies highlight the crucial role of molecular motors on neuronal homeostasis.

Dynactin is a large multisubunit complex that associates with dynein and is required for transport (Box 1 and Figure 1). A mutation in the gene encoding the $p150^{Glued}$ subunit of dynactin (DCTN1) was first identified in a family with distal spinal and bulbar muscle atrophy, a lower motor neuron disease characterized by neuron loss and an accumulation of dynein/dynactin inclusions also known as distal hereditary motor neuropathy 7B(HMN7B) [11]. The mutation (G59S) affects the conserved cytoskeleton-associated protein glycine-rich (CAP-Gly) domain that links dynactin to tyrosinated tubulin, affecting its binding to MTs [11]. Five different mutations affecting the same CAP-Gly domain of DCTN1 have been found in families with Perry syndrome [12], a neurological disorder resembling Parkinson's disease (PD). The CAP-Gly domain is responsible for initiating retrograde transport from the distal region of the axons. However, different mutations may underlie the very different symptoms observed in Perry and HMN7B patients. In Perry syndrome, the mutations selectively impair retrograde initiation, disrupting the flux from the distal part of the neurites without interfering with other functions of dynactin. By contrast, the mutation causing HMN7B prevents the binding of $p150^{Glued}$ to dynein in a dominant-negative manner, thereby jeopardizing dynactin complex function entirely [13,14].

Collectively, the role of molecular motors in neuronal homeostasis is evident from the above discussion. However, a critical role for FAT in regulating neurodegenerative diseases requires further elucidation, because slow transport may also rely on FAT and the movement of organelles.

Turbo ignition failure – a defect in an adaptor protein

Huntingtin, the mutated protein in Huntington's disease (HD), is an adaptor protein that scaffolds the dynein/ dynactin complex. Reducing wild type huntingtin levels leads to a decrease in both anterograde and retrograde transport, whereas increasing the amount of wild type huntingtin accelerates transport in both directions [15– 19]. Consistent with a potential role in the regulation of bidirectional FAT, huntingtin binds directly to dynein and indirectly to the p150^{Glued} dynactin subunit and KIF5C, via the HAP1 protein [15,16,20,21]. In HD, pathogenic polyQ expansion causes a change in the conformation of the huntingtin protein, leading to the detachment of molecular motors and vesicles from MTs, resulting in a decrease in FAT efficiency, which is similar to that observed when huntingtin is silenced. This decrease in transport efficiency is sufficient to reduce trophic support to the striatum, the most affected region of the brain in HD (Box 2) [15].

Interestingly, the potential role of huntingtin in FAT regulation may also extend to other neurodegenerative diseases. Indeed, huntingtin regulates FAT of the amyloid protein precursor (APP), which might be critical for the pathogenesis of Alzheimer's disease (AD) [17]. In addition, the *MECP2* gene, which has been implicated in Rett syndrome, has been shown to regulate the transcription of a large array of genes including *Huntingtin* and *HAP1*. In mouse models of this disease, decreases in MECP2 expression led to decreases in the expression of these two genes. thereby decreasing axonal transport of brain-derived neurotrophic factor (BDNF) [22], whereas overexpression of Huntingtin or HAP1 restored axonal transport. Finally, although huntingtin plays a crucial role in FAT, it is also responsible for many other cellular functions including spindle orientation and ciliogenesis [23,24]; therefore, future studies should focus on determining whether FAT is the primary cause of neurodegenerative diseases such as HD.

GPS failure – when transport directionality is altered in neurodegenerative disorders

In addition to ensuring rapid transport of organelles via FAT, cells have to control their correct delivery within specific subcellular regions. This correct delivery is especially important in the case of neurons, in which secreted factors are anterogradely transported to and released from axon terminals whereas signaling endosomes undergo retrograde transport to cell bodies where they signal
Trends in Cell Biology December 2013, Vol. 23, No. 12



Figure 1. Restoring Fast Axonal Transport. Three strategies to restore axonal transport defects in neurodegenerative diseases. (A) c-Jun N-terminal kinase 3 (JNK3) hyperactivity has been reported in Huntington's disease (HD), Parkinson's disease (PD), and Alzheimer's disease (AD). The JNK3-induced phosphorylation of kinesin-1 inhibits its binding to microtubules (MTs). Pharmacological inhibition of JNK3, which reduces kinesin-1 phosphorylation, is neuroprotective *in vivo*. (B) The presence of an extended polyQ stretch in huntingtin leads to the detachment of molecular motors from MTs, inducing transport defects. Increased phosphorylation of huntingtin at S421 restores transport. This phosphorylation can be induced by the activation of the insulin-like growth factor 1 (IGF-1)/Akt pathway or by the inhibition of the phosphatase calcineurin. (C) Acetylated tubulin is decreased in HD and AD brains. Molecular motor interaction with deacetylated tubulin is reduced compared with the acetylated form, resulting in reduced vesicular and mitochondrial transport. Inhibition of the tubulin deacetylase histone deacetylase 6 (HDAC6) restores transport and is neuroprotective in mouse models of different neurodegenerative diseases (see text).

[25]. Adaptor proteins such as huntingtin could be important for such control mechanisms. Indeed, huntingtin regulates the directionality of transport, via its phosphorylation at residue S421. Upon huntingtin phosphorylation by the insulin-like growth factor 1 (IGF-1)/Akt pathway, kinesin-1 is recruited to the huntingtin/dynein/dynactin complex, promoting anterograde transport. By contrast, dephosphorylation by protein phosphatase 2B (PP2B)/ calcineurin triggers the detachment of kinesin, leading to retrograde transport [17,26,27]. Phosphorylation levels at this residue are lower in the pathological situation of HD than in healthy individuals, resulting in both lower transport efficiency and loss of regulation of the directionality of transport [18]. Another example of the importance of controlled directional transport comes from studies of the Rab7 gene mutations found in patients with CMT.

Protein	Function related to FAT	Mutation	Disorder	Refs
Molecular motors				
Kinesin KIF5A	Anterograde motor protein subunit	Missense mutations in the motor and stalk domain	SPG10	[4,5]
Kinesin KIF1A	Anterograde motor protein subunit	Missense mutations in the motor domain	SPG30, SPG10	[7,8]
Kinesin KIFIBβ	Anterograde motor protein subunit	Missense mutation in the motor domain	CMT2A	[3]
Cytoplasmic dynein 1 heavy chain 1	Retrograde motor protein subunit	Missense mutation in the stem domain that is important for dynein homodimerization	CMT2	[10]
Cytoplasmic dynein 1 heavy chain 1	Retrograde motor protein subunit	Missense mutation in the tail domain disturbs dynein stability and function	SMA	[9]
Adaptors/regulators				
Dynactin subunit 1	Dynein activator complex	Missense mutations in the CAP-Gly domain block dynein function	HMN7B	[11]
Dynactin subunit 1	Dynein activator complex	Missense mutations in the CAP-Gly domain retrograde transport initiation	Perry syndrome	[12]
Huntingtin	Scaffold protein for molecular motor proteins	Polyglutamine expansion interferes with motor protein interactions	HD	[15,79]
Small GTPase rab7	Essential regulator of endolysosomal trafficking	Missense mutations in a highly conserved region lead to an increase in anterograde transport of endo/lysosomes	CMT2B	[29]
Mitofusin 2	Adaptor protein for mitochondrial kinesin	Missense mutations lead to a defect in mitochondrial axonal transport	CMT	[80,81]
Presenilin 1	Controls kinesin and dynein function of APP transport	Missense mutations causing reduced anterograde transport	AD3	[82,83]
Parkin	E3 ubiquitin ligase involved in degradation of miro, a mitochondrial adaptor protein for kinesin	Missense mutations inhibit miro degradation and mitochondrial arrest	PD	[84]
PINK1	Ser/Thr kinase involved in phosphorylation of miro, a mitochondrial adaptor protein for kinesin	Missense mutations inhibit miro phosphorylation and mitochondrial arrest	PD	[84]
MT-related proteins				
Tau	MT-associated protein	Missense mutation in the second MT-binding domain	FTDP	[33]
Neurofilament light subunit	Component of intermediate filaments, involved in the formation of axonal networks	Mutations in the head and rod domain	СМТ	[85,86]
Spastin	MT-severing proteins	Missense, nonsense, and splice-site mutations	SPG4	[38]

Table 1. Mutations in transport-related proteins causing neurodegenerative diseases

Rab7 belongs to the Rab family of Ras-related GTPases that act as essential regulators of endolysosomal trafficking. Rab7-dependent organelles are crucial for the transport of retrograde signaling endosomes in response to induction by nerve growth factor (NGF) [28]. Missense mutations of the Rab7 gene have been identified in a few families with CMT2B [29]. These mutations lead to an increase in the anterograde transport of Rab7-dependent organelles, resulting in the mislocalization of endo/lysosomal organelles at the axon terminals. This results in premature degradation mediated by TrkA–NGF trophic signaling, which may contribute to CMT disease [30].

Problems on the road – when altered microtubules disrupt FAT

The second, if not the most important, components of efficient intracellular transport are MTs. Molecular motors proceed on MTs and their processivity depends on the quality of these tracks. MTs are subjected to modifications and bind to several MT-associated proteins that are likely to influence MT dynamics and MT-dependent transport [31]. As such, Tau acts as a MT stabilizer and regulates the activity of molecular motors [32]. Mutations of the *tau* gene have been associated with frontotemporal dementia with Parkinsonism (FTDP) [33]. Mouse models carrying the R406W mutation in tau display impaired binding of tau to MTs, leading to slowing of FAT and formation of tau protein aggregates in the cell body [34]. Tau modifications are also associated with AD, because one of its hallmarks is the presence of paired helical filaments containing hyperphosphorylated tau. Hyperphosphorylation of tau lowers its affinity for MTs, decreasing MT stability [35] and potentially impacting FAT. Consistent with this hypothesis, mutations of the tau gene have been shown to decrease FAT both in vitro and in vivo [36,37]. MT stability could also play a critical role in axonal transport and neurodegeneration. Indeed, most familial cases of SPG are caused by mutations of the gene encoding spastin [38], a protein involved in the severing of MTs. Spastin function is not fully understood, but mutant spastin might affect cargo transport by inducing abnormal bundling of MTs, thereby interfering with the 'road' function of MTs [39].

Running out of gas – when energy supply is critical for FAT

The transport of organelles such as mitochondria and vesicles requires ATP to provide dynein and kinesin with energy to propel the organelles along axons and dendrites. Bioenergetic defects are a common feature of neurodegenerative diseases such as HD and PD. In most

Trends in Cell Biology December 2013, Vol. 23, No. 12

Box 2. ALS and HD: neuromuscular versus corticostriatal connections, dying-back and dying-for mechanisms?

In both HD and ALS, degeneration is specific to a subset of neurons highly dependent on trophic support from a distant source (Figure I). The lower motor neurons are connected to muscles fibers by the neuromuscular junction, to control skeletal muscle contraction. The target muscles deliver several survival factors, such as NGF, glial cell-derived neurotrophic factor (GDNF), neurotrophin 3 (NT3), and BDNF, to the synaptic cleft and the neurons express the receptors for these factors. Ligand binding leads to the internalization of the receptor and its long journey towards the cell body mediated by the retrograde machinery, which transports survival signals to the nucleus. Any interference with the retrograde transport pathway may lead to a motor neuron disease [87]. The dynein machinery is affected in CMTT2 and several lower motor neuron diseases [10,11]. As a consequence, lower levels of trophic support result in greater susceptibility to neuronal stress.

In HD, the susceptibility of striatal neurons may reflect a lack of neurotrophic support from BDNF. Most of the BDNF required for maintenance and survival of striatal neurons comes from corticostriatal afferences. BDNF undergoes anterograde transport to the corticostriatal synapse, where it is released and binds to striatal TrkB receptors. Activated TrkB receptors are subjected to endocytosis and retrograde transport to the cell body, where they activate survival signals. In HD, several types of dysregulation occur along this route, decreasing the TrkB signal. First, mutant huntingtin interferes with BDNF gene transcription in the cortical neurons [88] and with BDNF anterograde transport [15]. BDNF delivery is thus decreased. In addition, the density, activation, and retrograde transport of striatal TrkB receptors are reduced in striatal dendrites [89–91]. Together, these various types of dysregulation are likely to result in the corticostriatal neurons being highly susceptible in HD.

These two examples illustrate how defects in long-range transport of trophic signals are likely to affect survival of neurons. These observations led to the development of alternative strategies, independent of restoring axonal transport, that would activate the survival pathways downstream from FAT. Indeed, receptor activation within the target tissues may be sufficient to ensure neuron survival. For example, chemical antagonists, antibodies, and small peptides binding and activating TrkB could potentially be used to activate TrkB receptors directly in striatal neurons, thus providing an effective treatment for HD. As a proof of concept, agonists of the TrkB receptor are neuroprotective in a genetic mouse model of HD [92]. So far, local activation of the survival pathways may be one effective strategy for treating neurodegenerative diseases linked to transport alteration.



TRENDS in Cell Biology

Figure I. Axonal transport and neurotrophic support. (A) Motoneurons of the ventral horn and corticostriatal projecting neurons in the brain depend on neurotrophic support from a distant source. Motoneurons in the spinal cord project their long axons to skeletal muscles and contact muscles at the neuromuscular junction. In this case neurotrophins (NTs) are delivered from muscles [93,94] and the corresponding receptors (Trks) are expressed presynaptically. On ligand binding, the neurotrophin receptor is internalized and transported along the axon to the cell body in endosomes. This long retrograde transport is critical for motoneuron survival. (B) The survival of the striatal neurons depends on the trophic support delivered by the cortical neurons. Brain-derived neurotrophic factor (BDNF) is produced by cortical neurons (and not the striatum) and is transported anterogradely along the axon and delivered at the corticostriatal synapse. TrkB is present on the striatal dendrites and on activation by BDNF is endocytosed and transported retrogradely towards the cell body. Both the long anterograde transport of BDNF in axons and the retrograde transport of BDNF-activated TrkB endosomes in striatal dendrites are critical for striatal neurons.

Review

neurodegenerative diseases, research has focused on the energetic defects linked to alterations of mitochondrial function [40]. In genetically determined cases of PD, mutations of genes involved in mitochondrial function such as PARK2 (Parkin), PARK6 (PINK1), and PARK7 (DJ-1) decrease the ATP/ADP ratio in neurons, further suggesting that a poor energy supply may contribute to the observed alterations of FAT in many neurodegenerative diseases. In support, transport of mitochondria is highly dependent on the energy provided by the mitochondria themselves [41]. Therefore, this impaired trafficking may result from mitochondrial dysfunction and the bioenergetic defects it causes. Two mutations directly linking energy production and neurodegeneration have been reported for CMT. One is a mutation of the gene encoding pyruvate dehydrogenase kinase isoenzyme 3 (PDK3), which, by decreasing pyruvate flux, links the glycolytic cascade to the Krebs cycle within mitochondria [42]. The second is a nonsense mutation in the DHTKD1 gene that encodes a mitochondrial protein shown to regulate energy production by the mitochondria [43]. Silencing DHTKD1 decreases energy production. The observation that axonal forms of CMT can be caused by mutations in either molecular motors (Table 1) or energyproducing enzymes from the mitochondria suggest that FAT is highly sensitive to alterations in energy homeostasis, because these mutations result in similar phenotypes.

In contrast to mitochondrial transport, which depends on mitochondrial ATP, the FAT of small vesicles, such as those containing neurotrophic factors (e.g., BDNF) or synaptic markers, depends on the glycolytic machinery [41]. Although mitochondria are the main source of energy for neurons, producing ATP more efficiently than glycolysis, FAT of vesicles is decreased after glycolytic enzymes are inhibited or silenced by RNAi and is not affected by the inhibition of mitochondrial ATP production. This apparent paradox can be accounted for by the specific location of the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphoglycerate kinase (PGK) on vesicles and the capacity of these vesicles to produce ATP independent of mitochondria. GAPDH was shown to be necessary and sufficient to propel vesicles along MTs. These findings suggest that energy sources are compartmentalized in cells and strategies should be developed for the selective provision of energy to specific organelles. Collectively these studies provide significant evidence for FAT being highly sensitive to energy dysregulation; however, the lack of energy, rather than FAT itself, could also be the cause of these neurodegenerative diseases.

Although components of axonal transport may also be involved in other cellular functions, the numerous mutations in genes that directly regulate FAT and the observed slowing of FAT in the discussed neurodegenerative disorders makes FAT a promising therapeutic target for such diseases.

Restoring FAT in neurodegenerative disorders

Rescuing the function of molecular motors

Kinesin phosphorylation by various kinases plays an important role in the regulation of motor activation and motor/cargo recruitment and release [44–49]. Alterations in the balance of kinase and phosphatase activities are

Trends in Cell Biology December 2013, Vol. 23, No. 12

often observed in neurodegenerative diseases, potentially resulting in the detachment or inactivation of motors. The efficacy of molecular motors also depends on the adaptor molecules, which not only specify the nature of the cargoes transported but also integrate signaling pathways and regulate FAT (Table 1). Studies in Drosophila have shown that changes in c-Jun N-terminal kinase (JNK) or mitogenactivated protein kinase kinase 7 (MKK7) levels lead to dissociation of the JNK-interacting protein (JIP)/kinesin complex and lower transport [50]. How do JIPs regulate FAT? One possible mechanism involves the regulation of JNK itself. JNK3, the neuron-specific isoform, phosphorylates kinesin-1, preventing its binding to MTs [51]. JIP3 may also be an interesting target for retrograde signaling. because it acts as an adaptor for dynein and mediates the dynein-dependent retrograde signaling of activated JNK3 and lysosomes in zebrafish [52]. The discovery that components of signaling pathways are transported together on vesicles provides a target for the modification of transport kinetics. As such, JNK3 inhibition effectively rescues FAT defects induced by pathogenic fragments of huntingtin in the squid model of axonal transport [51]. JNK hyperactivity has been reported in models of HD, AD, and PD and the inhibition of JNK activity in vivo is neuroprotective in animal models, further supporting this strategy as a potential therapeutic approach for improving FAT in neurodegenerative diseases [53].

Improving huntingtin function in FAT

Huntingtin acts as a scaffold for the molecular motors dynein and kinesin, through direct and indirect interactions. The polyQ expansion in mutant huntingtin decreases FAT, resulting in a weaker survival signal in response to BDNF/TrkB in striatal projection neurons (Box 2). In a search for pathways capable of modifying polyQhuntingtin-induced toxicity, huntingtin phosphorylation sites were found to control axonal transport. In pathological conditions, phosphorylation at S421 increases neuronal survival and restores transport, leading to the subsequent release of BDNF by cortical neurons [18]. Huntingtin phosphorylation at S421 can be induced by the IGF-1/ Akt pathway [26] or by the inhibition of calcineurin, which has been identified as the *bona fide* phosphatase of huntingtin [54]. Both pathways regulate FAT in HD neurons and FK506, a calcineurin inhibitor, restores FAT in cortical neurons from HD mice [27], suggesting that drugs selectively targeting calcineurin or the phosphorylation of the S421 residue of huntingtin may be useful for the treatment of HD. This strategy may also include other druggable phosphatases that act on S421 [55]. In addition to this site, huntingtin phosphorylation at positions S1181 and S1201 may also be targeted to promote axonal transport, because reduced phosphorylation of huntingtin at these sites increases, both in vitro and in vivo, the association of molecular motors and vesicles with MTs and increases both anterograde and retrograde transport of BDNF [56].

Microtubule acetylation

Histone deacetylases (HDACs) have emerged as potential treatment targets for neurodegenerative diseases. Unlike most HDACs, HDAC6 does not act on histone but deacetylates MTs in vitro and in vivo [57]. Acetylated MTs have a greater affinity for kinesin-1 and dynein [58,59]. The inhibition of HDAC6 activity, which increases MT acetylation, increases both the anterograde and retrograde FAT of JIP1, BDNF, and mitochondria [58–60], indicating that MT acetylation could be used to improve the speed of vesicles moving along them. Consistent with this, MT acetvlation is reduced in AD and HD [59.61] and HDAC6 inhibition rescues both anterograde and retrograde transport in HD neurons [59]. However, it remains unclear whether HDAC6 inhibition has a therapeutic effect in HD, because HDAC6 depletion in the R6/2 model of HD has no effect on mouse survival [62]. This lack of effect may be the result of phenotypic differences in the mouse model chosen. The R6/2 model overexpresses only exon 1 of huntingtin, which is encoded by 67 exons, resulting in the misfolding and nuclear aggregation of short pathogenic huntingtin fragments rather than defects in axonal transport. Further experiments with models expressing the fulllength huntingtin are therefore warranted.

HDAC6 inhibition has been shown to be useful in several neurodegenerative diseases in which FAT is altered. For example, HDAC6 deletion in the $SOD1^{G93A}$ mouse model of amyotrophic lateral sclerosis (ALS) has a significant effect on mouse survival by decreasing motor neuron degeneration while maintaining neuromuscular junction and muscle innervation [63]. However, it is unknown whether transport is restored in vivo in these mice. One study provided direct evidence for a link between HDAC6mediated rescue of FAT and neurodegeneration. A mouse model of CMT due to mutations in the 27-kDa small heatshock protein gene (HSPB1, also known as HSP27) [57] displayed a motor phenotype characterized by a significant loss of motor and/or sensorimotor axons, a decrease in the acetylation of α -tubulin, and a decrease in mitochondrial trafficking. HDAC6 inhibitors restored mitochondrial trafficking and reversed the CMT phenotype, as shown by the muscle reinnervation observed in $HSPB1^{S135F}$ mice [57]. HDAC6 inhibition may also be relevant for AD. Tubulin acetylation is reduced in the brains of patients with AD [61] and in the brains of various mouse models of AD [64,65]. HDAC6 inhibitors reverse the amyloid beta-induced changes in α -tubulin acetylation and mitochondrial trafficking in cultured hippocampal neurons [64] and HDAC6 knockout in a mouse model of AD restores α -tubulin acetylation in the brain and cognitive function in AD mice [65]. Also, in a fly genetic screen, mutations in HDAC6 were found to rescue defects in the muscles and neurons of a tauinduced model of AD [66]. Together these findings indicate that drugs improving MT acetylation may be useful for treating neurodegenerative diseases. For example, tubacin and tubastatin A are specific inhibitors of HDAC6 and are effective in vitro [67]. In addition, tubastatin A increases tubulin acetylation in peripheral nerves and improves the muscle phenotype of CMT mice [57]. However, it remains unknown whether these drugs are able to cross the bloodbrain barrier to exert therapeutic effects in the CNS.

Drugs stabilizing MTs may also be of interest for the treatment of neurodegenerative diseases. Increases in MT dynamics have been reported in PD and AD neurons, both *in vitro* and *in vivo*. The MPTP-induced model of PD

displays an increase in MT dynamics that could contribute to the FAT defects in PD neurons. Such defects can be restored by treatment with epothylone D, an agent that stabilizes MTs. Epothylone D and other MT-stabilizing drugs increase the density of MT tracks, decrease axonal dystrophy and neuronal loss, and improve cognitive functions in various tau-overproducing transgenic mouse models [68,69]. Whether MT-stabilization strategies could be useful for other neurodegenerative diseases, such as ALS, CMT, and HD, remain to be established.

Fuelling transport

Many laboratories have focused on restoring ATP levels in neurons. In particular, creatine has been reported to be of therapeutic interest for HD and PD, because it is involved in energy supply to muscle and brain cells under physiological conditions [40]. However, although creatine supplementation was shown to have an effect in various mouse models of PD and HD and be safe and tolerable for patients, no studies have demonstrated that creatine is neuroprotective in patients. Findings that 'on-board' energy is required to sustain FAT of vesicles in axons suggest that improving mitochondrial or bulk ATP in neurons might be insufficient to improve transport in the axons and dendrites of neurons [41]. Therefore, different strategies should be developed, depending on the type of transport - mitochondria or small vesicles - that we wish to ameliorate. Increasing mitochondrial ATP production would probably restore mitochondrial dynamics and function, including calcium buffering and antiapoptotic functions. With regard to FAT of vesicles, the mechanisms by which GAPDH is maintained and/or activated on vesicles will need to be elucidated and may open new possibilities for improving FAT, such as increasing energy supply.

FAT, the faster the better?

The idea that efficient FAT results in proper neuronal function is somewhat simplistic. This is especially the case for mitochondria, in which their improved function cannot simply translate to an increase in their velocity. Mitochondrial trafficking is tightly regulated because mitochondrial pausing is important for regulating calcium and energy homeostasis [70]. Mitochondrial arrest is also required to eliminate damaged mitochondria by a process called mitophagy [71]. Two genes implicated in this process (Table 1) are linked to genetic forms of PD and their mutations result in decreased mitochondrial pausing.

In addition, improving FAT does not always result in reduced neurodegeneration. For example, alterations in retrograde transport may promote neuroprotection in some situations. As discussed, mutations in Dync1h1, which encodes one subunit of dynein, impair FAT. In recent years, mice with allelic point mutations affecting the stalk domain of DYNC1H1 have been identified [72,73] and these mice show complex phenotypes including neuromuscular dysfunctions. Interestingly, among these strains, $Loa^{+/-}$ and $Cra^{+/-}$ mice display some neuroprotection in the $SOD1^{G93A}$ mouse model of ALS [74]. Expression of Bicaudal D2 N Terminus, which impairs dynein/dynactin function but has no particular neurodegenerative phenotype, also led to neuroprotection in transgenic $SOD1^{G93A}$ mice [75]. Intriguingly, these mice

Box 3. Outstanding questions

- Is restoring FAT sufficient to cure neurodegenerative diseases? As discussed in the text, this remains the key question. The unambiguous demonstration that FAT restoration is sufficient to slow neurodegeneration would certainly boost therapeutic studies focusing on FAT.
- Why do specific mutations in the same molecular motor affect different neurons and give different phenotypes?

It is worth noticing in Table 1, in the main text, that different mutations in the same gene (e.g., dynein or dynactin) lead to different neurodegenerative diseases. The observation that dynactin mutations have different effects on transport (blockade of dynein function versus retrograde transport initiation) and are responsible for different diseases (HMN versus Perry syndrome) illustrates the complexity of the underlying pathogenic mechanisms. The question of why some neurons and not others are specifically affected remains unanswered.

• Why do mutations in transport-related genes not necessarily affect neurons with the longest axons?

If slowing axonal transport is critical, one would assume that, for any transport-related mutations, neurons having long axons would be the first to degenerate. However, one has to consider for each mutation the nature of the cargoes that are transported and their requirement for the maintenance of specific neuronal connections.

• Can we follow FAT in humans?

If FAT is key for neurodegenerative disorders, assessing transport *in vivo* will be instrumental in understanding disease processes, following disease progression, and validating treatments. Such approaches have been recently validated in ALS mice which provided strong evidence of transport alteration *in vivo* [92]. Recently, heavy water pulses detected alterations in the transport and release of cargoes into the cerebrospinal fluid (CSF) of PD patients [95]. Because this technique remains indirect, *in vivo* imaging approaches such as Mn²⁺-enhanced magnetic resonance imaging (MRI) that allow measuring of axonal transport in live animals [96] may be of interest. However, such an approach cannot be used in humans given Mn²⁺ toxicity, highlighting the need for the development of new techniques.

display reduced retrograde transport and partial protection after axotomy. One hypothesis advanced to explain this apparent paradox is that decreases in retrograde transport may slow the dying-back signals, which are initiated at the synapse and propagate to the cell body. Supporting data from signaling phosphoproteome analysis of the $SOD1^{G93A}$ mouse model indicated an increase in retrograde death signals and a decrease in protective signals [76]. However, any interference with retrograde transport would probably decrease the transport of survival signals from the muscles back to the cell bodies (Box 2). Thus, although the possibility of slowing retrograde transport to decrease transport of retrograde death signals is attractive, the general impairment of retrograde transport might be deleterious in the long term.

Concluding remarks

Given the strong link between FAT and neurodegeneration and the potential therapeutic approaches that regulate FAT and display neuroprotection, it is tempting to speculate that FAT is 'the' crucial mechanism to target. However, the situation is not that simple, even for the molecular motors dynein and kinesin for which the link appears straightforward. Although dynein is the main retrograde molecular motor for FAT, it is also essential for the maintenance of intracellular structures such as the Golgi apparatus. It is also required for proper spindle orientation during mitosis and nuclear movement and through these mechanisms is critical for brain development. In support, mutations in dynein and dynein-associated proteins affect neuronal polarity and migration and cause brain malformations [77]. Therefore, in such cases, although FAT alterations are observed, developmental brain defects due to the presence of the genetic mutation in embryos may predispose to neurodegeneration.

Although these observations raise questions about the primary events in such diseases, it does not exclude the potential therapeutic effect of improving FAT. However, the strategies discussed above, such as JNK inhibition or MT acetylation, are likely to act on cellular mechanisms other than FAT. Therefore, it might be difficult to demonstrate unequivocally that restoring FAT (and only FAT) is sufficient to slow neurodegeneration (Box 3).

However, if the ultimate goal is to prevent neuronal death, is it critical to determine the role of FAT in neurodegeneration? Restoring FAT is likely to contribute, in concert with other cellular mechanisms, to protecting neurons. Also, because FAT is a highly regulated and sensitive system, it could serve as an effective and functional readout both to follow progression of the disease and to validate therapeutic strategies.

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3. Huntington's Disease

As discussed in chapter 2, Huntington's Disease (HD) is one of the many NDs known to be tightly related to intracellular transport. In this chapter we will discuss more in detail this pathology as well as the pathogenic mechanisms associated. HD is an autosomal dominant neurodegenerative disorder, caused by a CAG repeat expansion in the huntingtin (*HTT*) gene. This expansion results in a HTT protein containing an extended polyglutamine (PolyQ) stretch. The disease causes the atrophy mainly of the Striatum (STR) and the cerebral Cortex (CTX), having an impact on a person's motor abilities, and generating cognitive impairment and psychiatric disorders. It is a rare disorder, and its prevalence varies by ethnic origin. In the caucasian population of North America and Western Europe the prevalence is of 5-10 individuals per 100,000 (Agostinho et al., 2013). However, recent studies may suggest that this number could be underestimated (Fisher and Hayden, 2014).

HD is also known as Huntington's Chorea. "Chorea" comes from the Latin and Greek words meaning *chorus* or a *group of dances*. The term was given to many so-called "dancing disorders". Huntington's Disease was first accurately described in 1872 when a 22-year-old American doctor, George Huntington, wrote a paper called *On Chorea*, where he characterized HD as a hereditary condition and described the clinical presentation as a triad of motor, emotional and cognitive disturbances. His paper was later published in the *Medical and Surgical Reporter* of Philadelphia and the disorder he described became known as Huntington's Chorea. The knowledge of the disease's neuropathology increased during the 1900s, but the discovery of the gene in 1993 was the major milestone in the history of HD (The Huntington's Disease Collaborative Research Group, 1993; Zuccato et al., 2010). Nowadays, the original name Huntington's Chorea has been substituted by Huntington's Disease as the term "chorea" only accounts to the motor symptoms, whereas it is well known that people affected suffer from a triad of symptoms including cognitive and psychiatric disturbances.

3.1 Symptoms

HD is generally a late-onset disease, with the first symptoms appearing between the ages of 35-50. There is a 5% of the cases where symptoms appear earlier in life. This is a juvenile form of the disease that is accompanied by a faster progression and increased severity (Beighton and Hayden, 1981; Conneally, 1984). Symptoms of the disease can vary between individuals, but usually progress predictably. The earliest symptoms are often problems with mood or cognition. A general lack of coordination and an unsteady gait often follows. As the disease advances, uncoordinated body movements become more apparent, along with a decline in mental abilities and behavioral and psychiatric problems. Physical abilities are gradually impeded until coordinated movement becomes very difficult. Mental abilities generally decline into dementia. Complications such as pneumonia, heart disease, and physical injury from falls reduce life expectancy to around twenty years after symptoms begin. There is no cure for HD, and full-time care is required in the later stages of the disease. Existing treatments tend to relieve many of the symptoms, but no actual cure has been yet developed (Rosenblatt, 2007).

3.2 HD brain disease

Despite its ubiquitous expression, the presence of the polyQ stretch in HTT protein affects mainly the brain with a notable cell loss and atrophy of the Striatum (STR) (Reiner et al., 1988; Vonsattel and DiFiglia, 1998).

Prior to symptoms onset, a significant volume reduction in almost all brain structures is detected by magnetic resonance imaging (MRI)-based morphometric analysis (Rosas et al., 2003). The neurodegenerative process begins in the caudate and putamen, and as the disease progresses a significant loss of neurons is also observed in the cerebral CTX particularly in layers III, V and VI (Vonsattel and DiFiglia, 1998) (Fig 7). Besides these main areas affected, other regions are also disturbed in HD. Studies have suggested that globus pallidus, thalamus, subtalamic nucleus, substantia nigra, white matter and cerebellum could be markedly affected

(Reiner et al., 1988; Vonsattel and DiFiglia, 1998). There are also indications that the hypothalamus can be significantly atrophied in HD patients (Kassubek et al., 2004; Politis et al., 2008), although these changes are initially less obvious than those detected in the striatum.



Figure 7: HD brain lesion. Brain slices showing striatal atrophy caused by HD (left) compared to a normal brain (right). Cortical atrophy and an increased ventricular volume can also be observed

At the cellular level, HD is characterized by differential vulnerability of specific neuronal subpopulations within the STR and CTX (Ferrante et al., 1985). GABAergic medium-spiny neurons (MSN) preferentially degenerate in HD. Within the MSNs, those corresponding to the indirect pathway expressing enkephalin are affected at earlier stages of disease progression and to a larger extent (Albin et al., 1992; Reiner et al., 1988; Richfield et al., 1995), and have been associated with development of the chorea-like movement in HD. Degeneration of MSNs of the direct pathway late in the course of HD manifests as rigidity and bradykinesia (Berardelli et al., 1999).

3.3 HTT gene

HTT gene was first discovered in a small community around Venezuela's lake

Maracaibo, where a large concentration of HD patients was found. In 1983, *HTT* gene was the first disease-associated gene to be mapped to a human chromosome (Gusella et al., 1983). Ten years later, the Huntington's Disease Collaborative Research Group identified the DNA sequence and the precise nature of the HD-associated mutation (The Huntington's Disease Collaborative Research Group, 1993). They reported that the disease was linked to the IT15 gene (for interesting transcript 15), which contains a region with a polymorphic CAG (cytosine-adenine-guanine) triplet expansion. When researchers examined this polymorphic region in non-HD individuals they observed that the number of CAG repeats varied from 6 to 35 whereas HD individuals always contained over 40 repeats (The Huntington's Disease Collaborative Research Group, 1993). Therefore, HD disease was accounted to this trinucleotide expansion in the IT15 gene, nowadays known as *HTT* gene.

The gene coding for HTT is composed of 67 exons spanning over 180kb. The polymorphic (CAG)_n repeat is located in the first exon of the gene. Within the normal population the CAG repeat has a Gaussian distribution with a prevalence of 17 CAGs. HD patients contain an allele with an expanded stretch of CAG above the threshold of 36 repeats, although full penetrance occurs when the number of repeats exceeds 39 (McNeil et al., 1997; Rubinsztein et al., 1996). There is a gray zone between 32-35 repeats, in which individuals are asymptomatic, but are at risk of transmitting the disease to their offspring, due to a phenomenon known as "genetic anticipation". This phenomenon is defined by an increased severity of the symptoms upon transmission through successive generations. This is due to the fact that expanded CAG repeats are not stable, and tend to expand from generation to generation specially when it is of paternal transmission (Ranen et al., 1995).

Furthermore, there is a negative correlation between the number of repeats and the age of onset of the disease (**Fig 8**) (Andrew et al., 1997). Extremely large CAG repeats above 60 are often associated to juvenile HD, with disease onset during childhood or adolescence. This correlation is less evident in patients with shorter CAG repeats, where the length of the stretch accounts for about 60% of the variance in the age of onset (Andrew et al., 1993; Brinkman et al, 1997; Ranen et al., 1995; Wolf et al., 2007). This observation suggests the existence of genetic and environmental modifiers that could modulate age of onset and severity of disease.

Several studies revealed that a large set of genes distinct from the HD locus itself could contribute to modify disease onset and progression (Chattopadhyay et al., 2003; Li et al., 2003; Wexler et al., 2004). These modifiers relate to various mechanisms implicated in HD pathology as excitotoxicity, dopamine toxicity, metabolic impairment, transcriptional deregulation, protein misfolding and oxidative stress.



Figure 8: The correlation between CAG repeat length and age of symptom onset (From Andrew *et al.* 1993)

Most of HD patients contain only one copy of the mutated allele. However, homozygous cases of the disorder show no difference in the age of onset of the disease compared to heterozygous patients, but the rate of progression can be enhanced (Squitieri et al., 2003).

3.4 HTT protein

HTT is a large, 348 kDa protein, which is conserved from *Drosophila* to mammals. The primary structure of the protein consists of several domains that include a polyQ

stretch located in the NH2-terminus of the protein, which corresponds to the translated CAG repeat (**Fig 9**). It is a polymorphic region that begins in the 18th amino acid, and in unaffected individual reaches up to 35 residues (The Huntington's Disease Collaborative Research Group, 1993). The actual function of the polyQ tract is still not completely defined, though it is thought to be a regulator of HTT binding properties with its numerous interactors (Harjes and Wanker, 2003). The polyQ tract is capable of forming a polar zipper structure that would enable binding to transcription factors containing a polyQ region (Perutz et al., 1994). This region seems not to be essential for HTT's function but to function as a regulator of its activity. The absence of the polyQ stretch in fibroblasts culture contributes in modulating longevity and regulates energy homeostasis (Clabough and Zeitlin, 2006). Moreover, mice lacking of the polyQ tract live significantly longer than wild type mice (Zheng et al., 2010). The polyQ region is followed by a polyproline (PolyP) stretch that is thought to help its stabilization by maintaining the polyQ tract soluble (**Fig 9**) (Steffan, 2004).

Further downstream, HTT protein contains 36 putative HEAT (huntingtin elongation factor, protein phosphatase 2A, and TOR1) repeats (Takano and Gusella, 2002), which are thought to be organized in 4 domains (**Fig 9**) (Palidwor et al., 2009). These HEAT repeats are successive antiparallel alpha helices that are important for intramolecular interaction and for protein-protein interaction, suggesting that HTT may exert its function through its binding to different partners.

In the C-terminal region of the protein, there is both an active nuclear export signal (NES), and a less active nuclear localization signal (NLS) which suggests that HTT would be implicated as well in transporting proteins between the nucleus and the cytoplasm (**Fig 9**) (Cornett et al., 2005).

HTT also contains several consensus sites for proteolytic enzymes and posttranslational modifications (**Fig 9**). HTT is cleaved in several different fragments by many different proteolytic enzymes including caspases, calpain and aspartyl proteases (Hermel et al., 2004; Kim et al., 2001; Wellington et al., 2000, 2002). Many of the sites of cleavage are well characterized while others are not so well defined (Dyer and McMurray, 2001). The function of these cleavages in normal HTT's function is still unclear. On the other hand, it is well established that in the pathological situation protein cleavage generates short fragments that translocate to the nucleus deregulating transcription. Furthermore, reduction of huntingtin's

proteolysis diminishes toxicity of the mutant protein.



Figure 9: HTT protein. Schematic diagram of HTT protein, indicating the localization of the polyglutamine tract, followed by the polyproline sequence, and the four main groups of HEAT repeats. Additionally, the most studied sites of postranslational modifications have been added, as well as sites of proteolytic cleavage.

3.5 HTT posttranslational modifications

HTT is subjected to many posttranslational modifications including acetylation, phosphorylation, ubiquitination and sumoylation, which regulate many of its functions. In HTT, sumoylation and ubiquitination compete for the same target lysines at amino acids 6, 9 and 15 (**Fig. 9**). Sumoylation of HTT results in stabilization of N-terminal fragments, reduced aggregation, increased nuclear localization and increased neurodegeneration in a *D. Melanogaster* model of HD (Steffan, 2004). Ubiquitination of HTT are not effectively degraded by this pathway, leading to global defects in the ubiquitination-proteasome pathway that can result in increased levels of pro-apoptotic proteins (Jana et al., 2005).

Acetylation of HTT at lysine 444 (**Fig. 9**) directly influences HD by facilitating the autophagic clearance of mutant HTT. The acetyltransferase mediating the reaction is CBP (CREB-binding protein), and the de-acetylation process is catalyzed by histone deacetylase 1 (HDAC1). Interestingly, preferential acetylation of polyQ HTT over the wild-type protein was observed in cell culture as well as in brains from HD mouse

models and patients, and experiments with acetylation-resistant mutants showed that acetylation is correlated with protection from mutant HTT toxicity. Acetylated polyQ HTT is trafficked to autophagosomes for degradation, which most likely constitutes the detoxification mechanism (Jeong et al., 2009).

HTT is as well subjected to palmitoylation at C214 by the palmitoyl transferases HIP14 and HIP14L (Huang et al., 2009; Yanai et al., 2006). Palmytoylation of HTT seems to influence toxicity in neurons. Rendering HTT palmitoylation-resistant leads to increased inclusion formation, increased nuclear localization and increased toxicity in neurons. The interaction of HTT with HIP14 is polyglutamine length-dependent, and polyQ HTT exhibits less palmitoylation in an HD mouse model. As a consequence, reduced palmitoylation could be responsible for the axonal trafficking defects seen in HD which contributes to the formation of inclusion bodies and enhanced neuronal toxicity (Yanai et al., 2006). Interestingly, HIP14 knockout mice have some neuropathological phenotypes similar to HD mice (Sutton et al., 2013).

Phosphorylation can occur in numerous sites in HTT. HTT's residues S13 and S16 can be phosphorylated by the IKK (IkB kinase) complex (**Fig 9**). S13 seems to be a direct target of IKK, whereas the phosphorylation of S16 is facilitated by previous phosphorylation of S13. Phosphorylation of HTT at these sites leads to increased degradation of the protein by both proteasomal and lysosomal pathways. The presence of an expanded polyQ tract renders these phosphorylations less efficient and reduces mutant HTT clearance (Thompson et al., 2009). Furthermore, stress induced phosphorylation of residues S13 and S16 can modulate nuclear entry, subnuclear localization and toxicity (Atwal et al., 2011). Phosphorylation at S16 reduces the interaction of HTT with the nuclear pore, thus reducing nuclear entry (Havel et al., 2011).

HTT is phosphorylated at S421 upon IGF1/Akt pathway activation (**Fig. 9**) (Humbert et al., 2002). Akt is able to counteract the proapoptotic properties of mutant HTT *in vitro* and *in vivo* (Humbert et al., 2002; Pardo et al., 2006; Warby et al., 2005). S421 phosphorylation plays a crucial role in intracellular transport directionality regulation (Colin et al., 2008) and restores BDNF transport defects due to the presence of the polyQ stretch, thus leading to increased neuronal survival (Zala et al., 2008). Further studies have shown that phosphorylation of S421 results in reduced nuclear accumulation of caspase-6 cleavage fragments by reducing the activity of caspase-6

(Havel et al., 2011; Warby et al., 2009).

HTT can be phosphorylated by the cyclin-dependent kinase 5 (Cdk5) at S434, 1181 and 1201 (Fig 9). Interestingly, preventing phosphorylation at S1181 and S1201 in wild-type HTT renders the protein toxic, indicating the importance of these phosphorylations for HTT's function in pro-survival pathways (Anne et al., 2007). Phosphorylation at all three Cdk5 sites is decreased upon polyglutamine expansion, and constitutive phosphorylation protects against polyglutamine toxicity (Anne et al., 2007; Luo et al., 2005). Cdk5 activity is increased upon DNA damage in striatal neurons, and HTT phosphorylation by this kinase is neuroprotective after irradiation or oxidative stress-induced DNA damage (Anne et al., 2007). Further, the phosphorylation at S434 is in very close proximity to a cluster of proteolytic cleavage sites, which themselves may play a crucial role in the pathogenesis of HD. Phosphorylation at S434 has been shown to reduce HTT cleavage by caspase-3 at residue 513 (Luo et al., 2005). On the other hand, phospho-ablation at S1181 and 1201 in mouse reduces anxiety/depression-like behaviors associated to increased adult hippocampal neurogenesis. Indeed, HTT dephosphorylation at these sites increase BDNF dynamics, delivery and signaling in hippocampal neurons, a crucial factor for hippocampal neurogenesis (Ben M'Barek et al., 2013).

Many other sites of phosphorylation have been identified by mass spectrometry analysis of full-length HTT (Schilling et al., 2006). Further studies should be performed to explore the regulatory effect of these in HTT protein.

3.6 PolyQ toxic mechanisms

The presence of the polyQ expansion in HTT protein, leads to the specific death of neurons of the STR. One of the main questions in the field is why HTT, which is not particularly highly expressed in the STR with respect of other brain regions, leads to a differential neuronal degeneration. In this part of the chapter, the key cellular pathogenic mechanisms through which this death could occur will be discussed (**Fig 10**).

3.6.a Proteolysis

The presence of HTT polyQ containing fragments have been detected in HD mice and post mortem brains from HD patients (Landles et al., 2010; Mende-Mueller et al., 2001; Wellington et al., 2002). Indeed, both wild type and mutant HTT are known to be substrate of several proteolytic enzymes, including caspases, calpains, cathepsins and metalloproteases (**Fig. 9**) (Goldberg et al., 1996; Graham et al., 2006; Hermel et al., 2004; Lunkes et al., 2002; Miller et al., 2010; Tebbenkamp et al., 2012). However the mutant version of the protein is more susceptible to proteolysis, producing short N-terminal fragments that contain the polyQ stretch (Kim et al., 2001; Landles et al., 2010; Lunkes et al., 2002; Wellington et al., 1998). The generation of these short N-terminal fragments is toxic for the cell and is a crucial aspect in HD pathogenesis. Indeed, mutations rendering polyQ HTT less susceptible to proteolysis, have been shown to decrease toxicity *in vitro* and *in vivo* (Gafni et al., 2004; Graham et al., 2006).

Once HTT is cleaved, the N-terminal fragments translocate into the nucleus. This nuclear translocation of polyQ containing short N-terminal fragments, induces neuronal apoptosis (Saudou et al., 1998), probably due to detrimental transcriptional deregulation leading to neurodegeneration (Sugars and Rubinsztein, 2003).

The accumulation of small N-terminal fragments could be in part a result of a decreased interaction between HTT and the nuclear pore protein TRP (translocate promoter region) due to the presence of the elongated polyQ stretch (Cornett et al., 2005). This could lead to a reduction in the export towards the cytoplasm of mutant fragments, generation its accumulation in the nucleus.

Due to its importance in HD pathogenesis, major efforts have been made to study the negative effects of the small N-terminal fragments containing the polyQ stretch. However, no studies have yet focused on the function of the corresponding C-terminal fractions generated by these proteolytic cleavages.

3.6.b Aggregation

A common molecular event in polyQ diseases as well as in other neurodegenerative diseases as Alzheimer disease (AD), Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS), is the progressive accumulation of abnormal protein aggregates associated with neuronal loss.



Figure 10: Key pathogenic mechanisms of polyQ HTT. (A) Full-length mutant huntingtin is cleaved by proteases in the cytoplasm. Fragments are ubiquitinated and targeted to the proteasome for degradation. Induction of the proteasome activity and of autophagy protects against the toxic insults of mutant huntingtin proteins by enhancing its clearance. (B) HTT fragments accumulate in the cell cytoplasm and interact with several proteins causing impairment of calcium signaling and homeostasis (C) and mitochondrial dysfunction (D). (E) N-terminal mutant huntingtin fragments translocate to the nucleus where they impair gene transcription and form intranuclear inclusions. (F) The mutation in huntingtin alters vesicular transport and recycling. (From Zuccato et al, 2010).

In HD patients, it has been shown that N-terminal fragments of polyQ HTT accumulate to form aggregates in both the nucleus as in the neurites in the CTX and STR (DiFiglia et al., 1997; Li et al., 1999). These aggregates are also found in HD rodent models (Davies et al., 1997) and thus represent a major histopathological feature of HD. The composition of these aggregates seems to differ according to their location. Indeed, nuclear aggregates contain mostly N-terminal fragments of HTT whereas, the cytoplasmic inclusions contain both full-length HTT and the truncated forms (Cooper et al., 1998; Hackam et al., 1998; Martindale et al., 1998).

Although it is accepted that the presence of N-terminal fragments of HTT is essential for toxicity, the role of the aggregates has been extensively debated. HTT aggregates were considered to be toxic, as they could sequester other polyQ containing proteins that would therefore lose their physiological function, or aggregates could physically block other cellular functions. However, this notion of the toxicity of aggregates has changed. Evidence suggests that aggregates are not pathogenic, but rather sequester toxic soluble fragments as a mechanism to protect against HTT-induced cell death. No correlation was found between polyQ HTT aggregation and neuronal vulnerability (Saudou et al., 1998). Moreover, the presence of polyQ HTT aggregates in surviving neurons of advanced HD patients suggests that these structures might even promote neuronal survival (Gutekunst et al., 1999). This idea was further supported by experiments in primary neurons which showed that neurons bearing aggregates survived longer than those without them (Arrasate et al., 2004), and mice where in spite of the presence of inclusions, no neurodegeneration was detected (Slow et al., 2005). Finally, polyQ HTT aggregates have been reported in striatal interneurons (Kuemmerle et al., 1999), and in brain regions largely spared in HD such as the hippocampus (Becher et al., 1998; Kuemmerle et al., 1999).

Further evidence supporting this notion, argues that aggregates protect neurons by stimulating the autophagic process and clearance of mutant HTT (Ventruti and Cuervo, 2007). This activation occurs given that HTT aggregates sequester the negative regulator of the autophagic pathway, mTOR (Ravikumar et al., 2004). This reduction of free mTOR ultimately leads to the induction of autophagy and clearance of polyQ HTT fragments, thereby protecting from cell death.

Taken together, this provides further evidence of the protective role of polyQ HTT aggregates, as they would activate or be part of a pathway that promotes clearance

of mutant HTT.

3.6.c Mitochondrial dysfunction

Mitochondria have been extensively studied in many neurodegenerative diseases including HD. These organelles are the main source of ATP in the cell needed to sustain cellular homeostasis. Neurons have a high-energy demand that are met by mitochondria, they buffer intracellular calcium levels, sequester apoptotic factor, playing a vital role in neuronal function and survival.

There is extensive evidence indicating that mitochondrial dysfunction is involved in HD pathogenesis. HD patients suffer from weight loss early in the disease progression, which gives a hint of probable metabolic defects in HD. Early studies showed that in HD brain, mitochondria present an abnormal ultrastructure (Goebel et al., 1978). Cellular metabolism dysfunctions were also observed in HD patients, suggesting that mitochondrial dysfunction could be a critical pathogenic component (Jenkins et al., 1993; Sánchez-Pernaute et al., 1999). These studies found that bioenergetics defects are present even in the asymptomatic HD patients, suggesting they may initiate disease onset. Besides, studies on HD brain and peripheral tissue showed decreased activity in complex II, III and IV of the mitochondria respiratory chain (Gu et al., 1996). This was further supported by studies performed in HD cells and animal models (Browne et al., 1997; Seong et al., 2005) showing deficits in mitochondria respiration in association with the polyQ expression.

Consistent with these observations, mitochondrial inhibition generates striatal pathology reminiscent of HD (Browne, 2008). A clear example is the effect of the mitochondrial inhibitor 3-NP (3-nitropropionic acid). Systemic administration of 3-NP in rodents and primates induced striatal cell loss along with motor and cognitive deficits (Beal et al., 1993; Brouillet et al., 1993, 1995; Palfi et al., 1996).

Mitochondria defects include problems with calcium handling. Isolated mitochondria from animal HD models have shown to have less resistance to calcium challenge (Brustovetsky et al., 2005; Gizatullina et al., 2006; Oliveira et al., 2006; Panov et al., 2002), even before any pathological changes appear (Panov et al., 2002). This was

further validated, as similar results were observed in mitochondria isolated from lymphoblast of HD-patients. Moreover, adult-onset HD mitochondria were proved to be less sensitive to calcium than the ones from juvenile-onset HD individuals (Panov et al., 2002). Mitochondrial respiratory function was as well studied in cells, confirming that the presence of polyQ HTT alters mitochondria calcium homeostasis (Oliveira et al., 2006). It was suggested that these defects are due to the interaction of mutant HTT with the outer mitochondrial membrane, as isolated normal mitochondria reproduce the same defects when incubated with the mutant HTT (Panov et al., 2002).

An additional deleterious effect of mutant HTT is that it affects mitochondria biogenesis. Mutant HTT interacts with PGC-1 α , a coactivator of several transcription factors, and a key regulator of mitochondrial biogenesis, energy homeostasis, adaptive thermogenesis, and glucose metabolism (Chaturvedi and Beal, 2008). Mutant HTT directly impairs the ability of PGC-1 α to activate downstream target genes involved in processes including mitochondrial biogenesis and respiration (Weydt et al., 2006). Interesting to note is the finding that the expression of PGC-1 α is reduced several fold in medium spiny neurons but increased almost 50-fold in interneurons from knock-in HD mice (Cui et al., 2006). This suggests that the selective vulnerability of medium spiny neurons and the resistance of interneurons, which are spared in HD, may be a consequence of altered PGC-1 α expression and mitochondrial dysfunction.

Recently, it was demonstrated that polyQ HTT triggers mitochondrial fragmentation, which is linked to an arrest in mitochondrial transport and neuronal death. Cycles of mitochondrial fission and fusion are necessary for neuronal function, and there is strong evidence that an imbalance in these opposing processes initiates neurodegeneration (Knott and Bossy-Wetzel, 2008). HTT impairs the mitochondrial fission/fusion balance and thereby causes neuronal injury. This was shown both in cells from HD patients and in HD mouse models. In this case, the fragmentation of mitochondria occurs before the presence of neurological deficits and HTT aggregates, suggesting it is an early event in HD pathogenesis. Interestingly, mutant HTT abnormally interacts with the mitochondrial fission GTPase dynamin-related protein 1 (DRP1) which in turn stimulates its enzymatic activity. Importantly, mutant

HTT-mediated mitochondrial fragmentation, defects in anterograde and retrograde mitochondrial transport, and neuronal cell death are all rescued by reducing DRP1 GTPase activity. Thus, DRP1 might represent a new therapeutic target to combat neurodegeneration in HD (Song et al., 2011).

3.6.d Transcriptional deregulation

DNA transcription is a highly regulated process that is altered in HD, resulting in abnormal levels of expression of a number of genes. Both wild-type and mutant HTT interact with several transcription factors, suggesting that abnormal interactions may lead to transcriptional deregulations (Sugars and Rubinsztein, 2003). Microarray studies have shown changes in the expression profile of brain tissue from HD patients and HD models that appear before neurological symptoms. (Fossale et al., 2002; Gafni et al., 2004; Hodges et al., 2006; Sipione et al., 2002). Several mechanisms through which this deregulation occurs have been described.

One of the mechanisms by which HTT regulates transcription, is through its interaction with Sp1 (Specific protein 1) and with the co-activator TAF_{II}130 (TBP (TATA Box binding protein) associated factor II 130) (Dunah et al., 2002). Sp1 normally binds to promoter regions and with TAF_{II}130, interaction needed for the recruitment of the transcriptional machinery. The direct protein-protein interaction between HTT and Sp1 activate the transcription of Sp1 target genes (**Fig 11A**). The presence of the polyQ expansion impairs the soluble association between Sp1 and TAF_{II}130, and directly interferes with the binding of Sp1 and DNA (**Fig. 11B**) (Dunah et al., 2002). A role for Sp1 is supported by the downregulation of two relevant genes in HD: the dopamine D2 receptor and the nerve growth factor receptor. Sp1 disruption appears to occur early in human HD pathogenesis, as it is already detectable in presymptomatic postmortem tissue (Dunah et al., 2002).



Figure 11: examples of three transcriptional defects induced by the mutant HTT. (A) HTT facilitates transcription of Sp1 target genes. (B) Expanded polyQ in HTT represses transcription by abnormal interaction with Sp1 and TFII130. (C) REST is sequestered in the cytoplasm by HTT, promoting gene transcription. (D) This interaction is lost in HD, increasing the availiability of REST in the nucleus which binds to RE1 sites, suppressing transcription. (E) CBP is able to acetylate histones at different lysine sites, establishing the conformation of DNA-chromatin suitable for transcriptional gene expression. (F) Mutant HTT interacts with the acetyl-transferase domain of CBP, leading to general transcriptional repression. (Z)

HTT is known to play a role as a general facilitator of neuronal gene transcription for genes containing the regulatory region called repressor element-1/ neuron restrictive silencing element-1 (RE1/NRSE) sequence in their promoter. RE1/NRSE is recognized by REST/NRSF, which acts as a transcriptional repressor. Wild-type HTT inhibits REST/NRSF silencing, as it sequesters and recruits it to the cytoplasm, therefore impeding its binding to and activation of the nuclear RE1/NRSE sequence

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(Fig 11C) (Zuccato et al., 2003a, 2003b). REST/NRSF target genes expression has been shown to be downregulated in HD. PolyQ HTT impairs HTT's ability to retain REST/NRSF in the cytoplasm leading to pathological levels of REST/NRSF in the nucleus and loss of wild-type HTT-mediated regulatory activity on gene transcription (Zuccato et al., 2003a) (Fig 11D). In particular, BDNF exon II promoter contains a RE1/NRSE regulatory region, which is of particular importance for striatal neurons, given that this factor is produced in the CTX and delivered to the STR to promote survival.

CBP (CREB Binding Protein) is known to interact with wild-type HTT, and it is involved in chromatin remodeling through its histone acetyl-transferase (HAT) activity (Fig 11E). This favors transcriptional factors recruitment as TAFII130, in this way activating survival genes (Lonze and Ginty, 2002). In HD, CRE-dependent transcription is altered (McCampbell et al., 2000; Nucifora et al., 2001). A decrease in the expression of genes regulated by CRE is one of the early events that occur in HD mice and HD patients (Glass et al., 2000; Wyttenbach et al., 2001). CBP interacts with other polyQ proteins through its polyQ domain (McCampbell et al., 2000; Nucifora et al., 2001). Nonetheless, the presence of CBP as being a part of the aggregates has not yet been described (Yu et al., 2002). Soluble polyQ HTT directly binds to the acetyl-transferase domain of CBP blocking its acetyl-transferase activity (Steffan et al., 2001). This results in the condensation of the chromatin, which leads to a decrease in gene transcription due to a restricted accessibility (Fig 11F). The defect in acetyl-transferase activity as a pathological mechanism, led to think about histone deacetylase (HDAC) inhibitors as a potential therapeutic approach to restore gene transcription in HD. (Ferrante et al., 2003; Hockly et al., 2003; Sadri-Vakili et al., 2007; Steffan et al., 2001), however, until now, non of the drugs proved useful in patients.

3.6.e Excitotoxicity

Excitotoxicity occurs due to an over-activation of N-methyl-D-aspartate (NMDA) receptors that leads to an increased calcium release from the ER and increased intracellular concentration. With time, mitochondria calcium storage is exceeded

resulting in mitochondria swelling, and release of calcium, cytochrome c apoptosisinducing factor (AIF) and other pro-apoptotic factors into the cytoplasm (Zeron et al., 2004). Excitotoxicity occurs from either overactive glutamate release, reduced glutamate uptake by glial cells, or glutamate hypersensitivity of NMDAR.

As MSNs depend on glutamate activation from cortical cells (Kawaguchi et al., 1995), excitotoxicity is an appealing mechanism to explain mutant HTT-mediated toxicity in striatal cells. Indeed, intrastriatal injections of NMDAR agonists showed a pattern of striatal cell death similar to the one observed in HD brain (Coyle and Schwarcz, 1976; McGeer and McGeer, 1976), perhaps providing some explanation to the increased susceptibility of these neurons in HD. Early evidence directly supporting the hypothesis that excitotoxicity is involved in striatal cell loss, came from human patient brain slices demonstrating reduced NMDAR binding, specifically in the STR (Young et al., 1988).



Figure 12: dysfunctional neurotransmitter systems and growth factors in corticostriatal pathway. An increased glutamate release an elevated activity of glutamate receptor NMDA can result in excitotoxicity-induced cell death. A decreased glutamate uptake by glial cells can contribute to increased neuronal vulnerability. Other neurotransmitter systems can also influence glutamate release. Reduced BDNF release from cortical afferents contribute to neuronal death. (From Zuccato, 2010)

An increase in glutamate release due to the presence of polyQ HTT could be a reason explaining this preferential cell loss. Several studies using different HD mouse models and cells, reported an increase in glutamate release by cortical afferents with increased excitotoxicity, while others showed a complete resistance (Fan and Raymond, 2007; Hansson et al., 1999; Morton and Leavens, 2000) (Fig 12). This discrepancy seems to be related to the different transgenic models employed in the different studies, particularly in the size of the transgene expressed. Mutant HTT induced excitotoxicity has been confirmed in models expressing full length mutant HTT (YAC), but not in transgenic mice bearing only a N-terminal fragment of the protein (R6/2 and N171-82Q), that rather show striatal resistance to excitotoxicity. The development of resistance to excitotoxicity in these models may represent a compensatory mechanism tending to reduce the toxic effects of the polyQ containing fragment.

Possible mechanisms for increased excitotoxicity include changes in NMDARs protein levels or post-translational modifications. Indeed, there is a higher expression of NR1A subunit of the receptor is in striatal neurons from HD mice, and this is associated to an increase NMDAR activity (Fan et al., 2007). In addition, polyQ HTT's interaction with PSD95, a scaffolding protein of the postsynapse, is reduced leading to sensitization of NMDAR (Sun et al., 2001). NMDA function is modulated as well by posttranslational modifications such as phosphorylation, and the mutant HTT has been shown to increase NMDAR phosphorylation promoting overactivation of the receptor (Song et al., 2003). Finally, NMDAR's endocytosis is inhibited by the presence of the mutant HTT, stabilizing the receptors to the membrane (Fan et al., 2007; Metzler et al., 2007)

On the other hand, it was proposed that impaired clearance of glutamate in the synaptic cleft by glial cells could enhance excitotoxicity leading to MSNs death. The glial transporter GLT1, responsible for the removal of extracellular glutamate, is downregulated in HD patients and mouse models (Arzberger et al., 1997; Behrens et al., 2002; Estrada-Sánchez et al., 2009; Faideau et al., 2010; Hassel et al., 2008; Liévens et al., 2001), increasing the levels of glutamine in the synaptic cleft.

Furthermore, in a mouse model expressing polyQ HTT specifically in astrocytes, a decrease in the expression of glutamate transporters and glutamate uptake, was associated with neuronal dysfunction through a non cell-autonomous mechanism (Faideau et al., 2010).

In addition to glutamate, other neurotransmitter systems that control the activity of corticostriatal synapses can contribute to render striatal neurons more sensitive to excitotoxic stimuli. Adenosine (A), A2 receptors (A2R) and cannabinoids (CB) receptors, enhance glutamate release upon activation. Indeed, in HD there is an increase of A2A receptor density and adenylate cyclase activity in the STR, possibly contributing to excitotoxicity (Tarditi et al., 2006). On the other hand, CB receptors are particularly abundant in corticostriatal terminals, and regulate glutamate transmission and BDNF release, possibly participating in pathogenesis (Marsicano et al., 2003).

A crucial input of the STR comes from the Substantia Nigra pars compacta, which represents the main striatal source of dopamine. Dopamine can influence on glutamate release by acting on D2 receptors (D2R) located in the cortical afferents. In line with this hypothesis, dopamine transporters and D2R are downregulated in HD brains (Augood et al., 1997; Cha et al., 1998, 1999; Ginovart et al., 1997), maybe by this means altering glutamate release.

3.7 HTT function and dysfunction

With the discovery of the HTT gene, many animal models of the disease have been generated in species as diverse as *C. elegans, D. melanogaster,* zebrafish, rodents, and large animal models including monkeys, miniature pigs and sheep. Most of the studies performed in these models have been focused on the toxic effect of the mutant protein.

However, nowadays it is believed that the pathological effect of PolyQ HTT is due to

both a gain of new toxic function and a loss of the normal function of the protein therefore; understanding the normal function of HTT becomes extremely important. Normal HTT has been shown to play a role in a wide variety of cellular processes, through its interaction with many other proteins.

One of the best-described functions of HTT, which is altered in pathological situation, is its involvement in intracellular transport. This will be described in detail in Chapter 4.

3.7.a Huntingtin in embryonic development

Huntingtin is widely expressed during embryogenesis, and it is associated with a number of interacting proteins. These interactions suggest that HTT may be involved in several processes including morphogenesis, neurogenesis and neuronal survival. HTT's essential role in embryonic development has been clearly demonstrated, as knock-out mice (*Htt-/-*), where HTT is completely inactivated, are embryonic lethal before day 8.5 (Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995). This defect during development in the absence of HTT lies in extraembryonic tissues, likely due to the impairments in the nutritive functions of the visceral endoderm (Dragatsis et al., 1998). Apart from its role in early extraembryonic function, HTT is important for Central Nervous System (CNS) formation, as cortical and striatal architectural anomalies are generated by a reduction of HTT level below 50% (Auerbach et al., 2001; White et al., 1997). Moreover, conditional inactivation of the *Htt* gene in the midbrain and hindbrain results in congenital hydrocephaly implicating HTT in the regulation of cerebral spinal fluid (CSF) homeostasis (Dietrich et al., 2009). Analyses of chimeras Htt -/- showed that HTT is crucial for establishing and maintaining especially cortical and striatal neuronal identity (Reiner et al. 2003). Furthermore, HTT downregulation starting at E 14.5, leads to spindle misorientation in neuronal progenitors and affects cell fate of cortical progenitors of the ventricular zone (Fig. 13). The correct orientation of spindle poles depends of the dynein/dynactin complex, which also generates the pulling forces essential for proper cell division. HTT interacts with dynein and regulates spindle orientation by ensuring the proper localization of dynein and p150Glued subunit of dynactin at the spindle pole (Godin et al., 2010).

Fig 13: Loss of huntingtin in cortical progenitors favors neuronal differentiation. During cortical development, progenitor cells located in the ventricular zone (VZ, light yellow) undergo divisions to ultimately generate post-mitotic neurons (green) that migrate radially to form successive cell layers. Apical progenitors (blue) divide to give rise to apical progenitors and another type of progenitors, the basal progenitors (pink) that divide away from the VZ and ultimately generate neurons (green). In control cells (left panel), most cleavages of progenitor cells are vertical, promoting progenitor cell renewal. However, removing huntingtin (Htt, right panel) changes the nature of the division cleavages (horizontal), decreasing the pool of cycling progenitors and increasing neuronal differentiation. (From Godin et al 2011).



Changes during development might not have phenotypical manifestations until later in life. These findings suggest that HD could be described as a developmental disease, where abnormal development predisposes neurons for a later stressor, resulting in cell death.

3.7.b HTT as an anti-apoptotic factor

Various data highlight the importance of HTT function in post-mitotic brain cells. HTT has been shown to have a prosurvival role, as *in vitro* overexpression of wild-type HTT protects cells from toxic stimuli, and from toxicity due to the expression of its mutant counterpart (Ho et al., 2001; Rigamonti et al., 2000). In line with this, depletion of wild-type HTT in cells render them more sensitive to cell death due to an increase in caspase-3 activity (Zhang et al., 2006).

The way HTT exerts cellular protection has been suggested to occur through various mechanisms. One of the mechanisms proposed, is through the physical interaction of HTT with caspase-3, which could thereby inhibit its proteolytic activity (Zhang et al., 2006). On the other hand, HTT can as well act by blocking the apoptosome complex, thereby inhibiting caspase-3 and caspase-9 activity (Rigamonti et al., 2000). Wild-type HTT also interacts with huntingtin interacting protein-1 (HIP-1), inhibiting the formation of the HIP1/Hippi complex that activates caspase-8 dependent apoptosis (Gervais et al., 2002).

In vivo work also demonstrated the role of wild-type HTT as an anti-apoptotic molecule. Heterozygous adult knockout mouse present morphological alterations in the basal ganglia with apoptotic cell death (Nasir et al., 1995; O'Kusky et al., 1999). Moreover, specific neuronal inactivation of HTT in adult mice results in a progressive neuronal degeneration, with apoptotic cells in the CTX, STR and hippocampus (Dragatsis et al., 2000). In addition, overexpression of wild-type HTT confers in vivo protection against neurodegeneration after ischemia (Zhang et al., 2003), and protects against apoptosis triggered by excitotoxicity (Leavitt et al., 2006).

More recently, the antiapoptotic role of huntingtin has been highlighted also in nonmammalian models. In fact, apoptotic cell death has been found in zebrafish embryos in which HTT has been knocked down by morpholino technology. HTT morpholino injected zebrafish showed a massively increased cell death as indicated by caspase-3 activity especially in the mid- brain/hindbrain region of the developing zebrafish embryo. This increased apoptosis was accompanied by a severe underdevelopment of the CNS (Diekmann et al., 2009).

3.7.c HTT, endocytosis and synapse

Normal communication between neurons is regulated by a number of proteins in the synapse. Wild-type HTT is highly expressed in the presynaptic terminals of nerve cells (DiFiglia et al., 1995), and interacts with various cytoskeletal and synaptic vesicle proteins that are essential for exocytosis and endocytosis, thereby taking part in controlling synaptic activity (Smith et al., 2005). Exocytic trafficking by dendritic recycling endosomes provides critical components for post-synaptic membrane formation and maintenance Synaptic components are also retrieved from the plasma membrane by endocytosis and can be either reinserted or degraded.

An important regulator of membrane trafficking from recycling endosomes to the plasma membrane is the small GTPase Rab11. HTT is important for the localization and function of Rab11. In the absence of HTT, the level of Rab11 on membranes is reduced. Furthermore, the nucleotide exchange activity of Rab11 is altered in cells depleted for HTT suggesting that HTT interacts with a complex containing Rab11 and is involved in Rab11 activation (Li et al., 2008). In line with this, in the HD context, mutant HTT interferes with Rab11 activity impairing vesicles formation from recycling endosomes (Li et al., 2009, 2010; Richards et al., 2011; Steinert et al., 2012). This suggests that mutant HTT could inhibit Rab11 activity by interfering with normal HTT function. This defect in the trafficking of material to the membrane leads to spine loss (Richards et al., 2011) and reduced neurotransmitters release and synaptic dysfunction (Steinert et al., 2012)

Furthermore, HTT interacts with PACSIN 1 (Protein kinase C and Casein kinase Substrate In Neurons)/syndapin, syntaxin and endophylin A, which collectively play a role in synaptic transmission as well as in synaptic vesicle and receptor recycling. (Smith et al., 2005). These interactions depend on the length of the polyglutamine repeat and are enhanced by the presence of an expanded CAG, leading to impairment of synaptic transmition in HD (Smith et al., 2005). Moreover, the proteins SH3GI3 (Sittler et al., 1998) and endophilin B1b (Modregger et al., 2003) bind to HTT. They belong to the endophilin family of proteins and have a role in vesicle endocytosis, and through this association HTT could regulate dynamin (Sittler et al.,

1998)

Early findings show that HTT directly binds the SH3 domains of PSD95 (Sun et al., 2001), a key molecule in synaptic transition and a component of the membrane associated guanylate kinase (MAGUK) protein family that binds to NMDA and kainate receptors in the postsynaptic densities (Li et al., 2003). In HD, the interaction between polyQ HTT and PSD95 is decreased. This suggests that in disease, more PSD95 is released affecting in this way NMDA receptors activity, and probably leading to their overactivation (Sun et al., 2001).

HTT interacts as well with Huntingtin Interacting Protein-14 (HIP14), whose involvement in endocytosis is suggested as it contains high sequence similarity with Ankrin repeat protein (Akr1p), a yeast protein known to play a role in endocytosis (Singaraja et al., 2002).

3.7.d HTT and ciliogenesis

Cilia are cellular organelles with a sensory role, and are involved in motility, and flow generation. These structures have as well been related to a number of complex syndromes termed ciliopathies, shown to have defects in cilia biogenesis and function (Saudou, 2012).

HTT's involvement in ciliogenesis has been shown as *in vitro* and *in vivo* depletion of HTT leads to reduced ciliogenesis. Wild-type HTT regulates ciliogenesis by interacting through HAP1 with pericentriolar material 1 protein (PCM1). PCM1 localization and trafficking is essential for ciliogenesis regulation. Loss of HTT in mouse cells impairs the retrograde trafficking of PCM1 and thereby reduces primary cilia formation (Keryer et al., 2011).

Ciliogenesis is altered by the presence of the pathological polyQ expansion. Defects in cilia length and organization have been described in HD mouse model and HD patients. Contrary to what is expected, the presence of an abnormal polyQ expansion leads to abnormally long cilia with asynchronous beating. In consequence, CSF flow is perturbed leading to an impaired migration of neuroblasts along the rostral migratory stream (RMS), which could explain the olfactory abnormalities reported in

HD patients (Keryer et al., 2011).

3.7.e HTT and adult neurogenesis

The role of HTT in adult neurogenesis has been assessed by depleting HTT in mature neurons of the CTX and hippocampus. This targeted depletion has been shown to impact on dendritic arborization, and long-term survival of newborn Dentate Girus (DG) neurons. Moreover, this effect seems to be non-cell autonomous as newborns are affected despite the fact that HTT is still expressed in these neurons. These defects seem to be linked to brain derived neurotrophic factor (BDNF) transport, release and signaling (Pla et al., 2013).

Besides, the phosphorylation state of wild-type HTT has shown to regulate hippocampal adult neurogenesis through a mechanism associated to BDNF transport and signaling as well. The absence of phosphorylation of HTT at serine 1181 and 1201 promotes maturation of newborn neurons in the DG of the hippocampus due to an increased delivery and signaling of hippocampal BDNF (Ben M'Barek et al., 2013).

3.7.f HTT and mammary gland morphogenesis

HTT expression is not restricted to the brain. Among many other tissues, it is also detected in mammary tissue (Moreira Sousa et al., 2013). The role of HTT in mammary gland development has been explored, showing that it is a key regulator of mammary stem cell division and differentiation. Depletion of HTT results in mitotic spindle misorientation of basal progenitors in mammary glands. As a consequence, there is a decrease of basal and luminal progenitors and altered mammary morphogenesis. The mechanism underlying this defect involves the misslocalisation of NUMA, LGN dynein and dynactin. The proper localization of these proteins in the spindle poles and cell cortex depends on HTT, and is essential to achieve a correct positioning of spindle poles during cell division (Elias et al., 2014).

Taken together, there is evidence that HTT, due to its size and structure, associates to numerous others proteins. These multiple interactions result in HTT's involvement in various cellular functions, many of them yet to be discovered. HTT has been found in different cellular locations, where it may act as a scaffold protein for the activation and correct assembly in time and space, of specific molecular partners.

The presence of an extended polyQ stretch may alter several of these interactions, therefore modifying cellular processes. On the other hand, mutant HTT can in addition gain new toxic functions.

HTT is ubiquitously expressed. So, why is it that the presence of an extended polyQ stretch affects particularly striatal neurons? For most of the described abnormalities, their relevance in this specific cell death is still uncertain. Impairments of the cortico-striatal circuit due to excitotoxicity and to the loss of BDNF support from the CTX may predominantly contribute to this differential loss of striatal neurons in HD. These dysfunctional cell-cell interactions could affect other neurons, therefore generating a positive-feedback loop that will finally lead to neuronal death.

Currently, most of the therapeutics used in HD tends to ameliorate the primary symptomatology of the disease. One of the main problems of finding an effective therapy to HD lies in the fact that HD could occur through to a combination of interrelated processes. Furthermore, some of altered mechanisms discussed could be primary events, but many of them could represent secondary events that would be less interesting for therapeutic intervention. Therefore a compound that targets only one pathological mechanism may not be effective. Moreover, these mechanisms should be the earliest dysfunctions that occur in HD

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4. HTT in intracellular transport

As discussed in the previous chapter, Huntingtin's (HTT) structure suggests it could function as a scaffolding protein due to its large number of interactors, and the presence of around 36 HEAT repeats that are important in mediating protein-protein interaction. In this chapter we will discuss the interactions that confer HTT its role as a regulator of intracellular transport, and how the presence of the polyQ mutation in HTT alters this function. This trafficking dysfunction has been shown to be a key pathogenic event in HD.

4.1 HTT interacts with vesicles and the motor machinery

HTT is a protein that is predominantly found in the cytoplasm of cells. The first hints of HTT having a role in vesicular transport came from biochemical analysis of subcellular fractions from human cortical extracts that showed the presence of HTT associated to vesicles and synaptosomes. This localization was further examined by immunohistochemical analysis in human and rat brains, confirming the presence of HTT around vesicles and associated to microtubules (MT) (DiFiglia et al., 1995; Gutekunst et al., 1995; Trottier et al., 1995). Indeed, the structure of HTT protein supports this idea as it contains functional domains consistent with association to membranes. The first 18 N-terminal amino-acids of HTT, immediately adjacent to the polyQ region (**Fig 14**), constitute a membrane-binding domain that controls the association of HTT to membrane bound structures as the Golgi, Endoplasmic Reticulum (ER), mitochondria and vesicles (Atwal et al., 2007; Rockabrand et al., 2007). It was also described that HTT can be palmitoylated at cysteine-214 by its copartners HIP14 and HIP14L (**Fig 14**) (Sutton et al., 2013; Yanai et al., 2006). Palmitoylation of HTT is consistent with a role in vesicular trafficking regulation.



Figure 14: Huntingtin's domains related to transport. Schematic representation, showing the relevant features relating huntingtin and transport. Huntingtin directly interacts with HAP1, the retrograde molecular motor dynein, HAP40 and optineurin, an adaptor of Myosin. Furthermore, it contains a palmytolation site at cysteine 214, and a phosphorylation site related to trafficking at serine 421. In the N-terminus there is a membrane-binding domain (gray), and between the region between amino acids 172 and 372 have been shown to associate to phospholipids from the plasma membrane.

Much of the information gathered from analysis of HTT interacting proteins, suggested a role of HTT in intracellular transport. HTT associated protein 1 (HAP1) was the first protein to be identified to interact with HTT and has been proposed to regulate the interaction of HTT with molecular motors (Engelender et al., 1997; Li et al., 1995). HAP1 is involved in axonal transport via its interaction with kinesin light chain, a subunit of the kinesin motor complex that drives anterograde transport along microtubules (Colin et al., 2008; McGuire et al., 2006). HTT interacts as well with the retrograde motor dynein, as seen by mass spectrometry analysis of purified HTT (Kaltenbach et al., 2007; Ratovitski et al., 2012). This association is both direct and indirect. On one hand, HAP1 interacts with amino acids 879-1150 of p150^{Glued} subunit of dynactin (Engelender et al., 1997; Li et al., 1998). p150^{Glued}, in turn interacts with dynein and is essential for dynein mediated retrograde transport. On the other hand, yeast two hybrid experiments demonstrated that HTT interacts directly with dynein intermediate chain. Mapping experiments identified the region of

interaction of HTT with dynein intermediate chain (DIC) as being located in the 601-698 residues of HTT (Caviston et al., 2007).

Besides its association with components of long-range intracellular transport, HTT's interaction with actin-based motors through optineurin (Faber et al., 1998; Sahlender et al., 2005) as its interaction with HAP40 (Pal et al., 2006) suggests a possible role of HTT in regulating transport along both MTs and actin cytoskeletons.

4.2 HTT is involved in intracellular transport

Knocking down and knocking out experiments showed that HTT is implicated in axonal transport. The first evidence of a role of HTT in intracellular transport came from a study performed in *D. melanogaster* that provided direct evidence of HTT's is implication in axonal transport (Gunawardena et al., 2003).

The reduction of HTT protein expression caused an axonal blockage phenotype consisting of organelle accumulation within larval nerves. This phenotype is characteristic of mutations in molecular motors required for axonal transport (Bowman et al., 2000; Gunawardena and Goldstein, 2001). Loss of HTT also caused degeneration in the adult eye of flies, a phenotype similar to that observed in mutations in dynein and p150^{Glued}. Further insight about the role of HTT in axonal transport came from the direct observation of BDNF vesicles dynamics in cells. Over expression of HTT enhanced the velocity of BDNF containing vesicles while reducing the time they spend pausing. In support of a positive role of HTT in stimulating transport of vesicles that contain BDNF, it was demonstrated that downregulation of HTT leads to a decrease in velocity of moving vesicle and an increase in the number of stationary vesicles as well as in the time they spend pausing (Gauthier et al., 2004). This reduction in BDNF dynamics due to the absence of HTT was observed in cortical neurites (Colin et al., 2008) as well as in hippocampal axons (Pla et al., 2013). The involvement of HTT in BDNF transport along MTs is bidirectional, as it stimulates both anterograde and retrograde transport (Dompierre et al., 2007; Gauthier et al., 2004). HTT stimulates BDNF transport through the direct interaction with dynein and HAP1, which in turn interacts with p150^{Glued} and kinesin. HAP1 is essential for this mechanism, as expression of HTT lacking the HAP1 interacting region is not able to stimulate transport (Pardo et al., 2010). This is also shown as the downregulation of HAP1 reduce BDNF transport, and in this conditions HTT cannot enhance transport (Gauthier et al., 2004). In addition, depletion of HTT in cells, leads to a disruption of the Golgi, consistent with defective dynein/dynactin function (Caviston et al., 2007; Pardo et al., 2010; del Toro et al., 2009), and suggesting that HTT might facilitate dynein mediated transport. Mapping experiments identified a binding site for DIC to residues 601-698 of HTT. Re expression of HTT constructs lacking for either the interaction domain with HAP1 or dynein failed to resemble the Golgi, further supporting HTT function in the maintenance of the Golgi apparatus via dynein (Pardo et al., 2010).

The role of HTT in transport is not restricted to BDNF-containing vesicles. Tropomyosin-related kinase receptor type B (TrkB) is the BDNF receptor, and its vesicular transport depends on HTT as knocking down HTT reduced vesicular transport of the receptor (Liot et al., 2013). Similar observations to those of BDNF were made for APP and Ti-VAMP (tetanus neurotoxin insensitive vesicle associated membrane protein) containing vesicles. Upon depletion of HTT, vesicles show reduced anterograde and retrograde velocities in cortical neurons (Colin et al., 2008; Her and Goldstein, 2008). Analysis of trafficking in *D. melanogaster* larvae showed that a reduction of HTT influences Rab11 transport in vivo (Power et al., 2012). Furthermore, incubation of anti-HTT antibodies, led to reduced vesicular association to MTs and to reduced bidirectional motility in an in vitro motility assay with native purified vesicles (Caviston et al., 2007). Mitochondrial dynamics was also examined in cells with decreased levels of HTT, and an increase in pausing along with a decrease in bidirectional motility was shown (Trushina et al., 2004), demonstrating that HTT is not only involved in vesicular transport, but also regulates transport of other organelles.

4.3 HTT regulates trafficking

It is broadly accepted that HTT acts as a transport facilitator, as silencing HTT decreases both anterograde as retrograde transport. However, HTT involvement in

intracellular transport is also supported by the role of HTT phosphorylation as a regulatory mechanism for intracellular transport.

Indeed, the phosphorylation state of HTT at specific sites can regulate the efficiency of transport. Two cyclin-dependent kinase 5 (Cdk5) phosphorylation sites have been identified in HTT, serine-1181 and serine 1201 (Anne et al., 2007). In physiological conditions, the absence of phosphorylation of both sites leads to an increase in the processivity of BDNF containing vesicles. Both anterograde and retrograde transport velocities are higher, due to an increased recruitment of molecular motors and association of vesicles to MTs. Therefore HTT is able to control and enhance BDNF transport, through specific phosphorylation sites that are regulated in response to Cdk5 signaling (Ben M'Barek et al., 2013).



Figure 15: huntingtin regulates transport directionality. Phosphorylation of huntingtin at serine 421 leads to kinesin recruitment to vesicles, favoring plus-end directed transport (right). On the other hand, the absence of phosphorylation at this site, results in a release of kinesin from the molecular motor complex, resulting in retrograde movement.

HTT has also been suggested to act as a molecular switch in controlling anterograde and retrograde transport, highlighting the role of HTT as a regulator of axonal transport (Colin et al., 2008). Vesicle motility is generally not unidirectional, but is characterized by frequent directional switches and pauses. Wild type HTT can also be phosphorylated at position S421 by the IGF-1 (insulin growth factor 1)/ Akt pathway (Humbert et al., 2002) and dephosphorylated by calcineurin (Pardo et al., 2006). When comparing the effect of expressing the phosphomimetic construct S421D to the unphosphorylatable construct S421A, major differences were shown in BDNF, Ti-VAMP and amyloid precursor protein (APP) vesicles dynamics. Phosphorylation at this site resulted in faster velocity and longer runs in anterograde transport, whereas expression of S421A favored faster velocity and longer runs in the retrograde direction. This suggests that HTT acts as a switch that regulates transport directionality. The directionality of a cargo, is thought to be the result of a "tug of war" mechanism, where the number of oppositely directed motors associated to a cargo at a certain moment will determine the directionality of its movement (Hendricks et al., 2010). Indeed, HTT regulates transport directionality, by controlling molecular motor attachment. When phosphorylated at serine 421, HTT recruits kinesin-1 to the dynactin complex, favoring transport in the anterograde direction. On the other hand, when HTT is not phosphorylated at this site, kinesin-1 detaches and vesicles are more likely to undergo retrograde transport (Fig 15) (Colin et al., 2008). Thereby, these studies linking signaling pathways such as Akt and calcineurin to a specific movement suggest that HTT could have a broader role as a scaffold protein that integrates extracellular and intracellular signals to deliver cargos to other subcellular compartments of neurons. Through this mechanism, a postsynaptic neuron that is the target of neurons in which HTT is phosphorylated will receive a higher amount of BDNF compared to a situation where HTT is not phosphorylated in the presynaptic neuron. The postsynaptic neuron will in turn show high activation of its TrkB receptor and downstream signaling pathways such as PI3K-Akt, Ras-MAPK and CREB phosphorylation and therefore increased survival (Zweifel et al., 2005).

Finally, HTT may be involved in the ability of vesicles to switch from short-range transport on actin cytoskeleton, to the long-distance transport on MTs and *vice versa*. Rab5, known to specifically associate to early endosomes, forms a complex by associating to HAP40 and HTT. The HTT–HAP40 complex on Rab5-associated

endosomes is a switch for cytoskeletal affinity between actin and microtubules. HAP40 mediates the recruitment of HTT to Rab5 onto early endosomes. An increase in cytoplasmic levels of HAP40, the early endosome positive for Rab5, leads to association with actin, possibly through a linker, such as optineurin, that binds to myosin VI. (**Fig 14**) When HAP40 levels are decreased, Rab5-positive early endosomes bind to MTs (Pal et al., 2006).

4.4 BDNF Transport in HD

The involvement of HTT in intracellular transport raises the question of whether the presence of the polyQ stretch has a deleterious effect in this process. Evidence suggesting this hypothesis, comes from the fact that the levels of BDNF in striatum (STR) of HD brains is reduced (Ferrer et al., 2000; Gauthier et al., 2004). In the corticostriatal neuronal circuit, BDNF is produced by cortical neurons, transported anterogradely to the axon tip where it is secreted. This BDNF is endocytosed by striatal neurons which express the TrkB receptor, and acts as a prosurvival factor (**Fig 16**) (Altar et al., 1997). This is essential for striatal neurons, as they are unable to produce BDNF, therefore requiring cortical BDNF support for survival (Baquet et al., 2004). Hence, trafficking of BDNF in cortical neurons is essential to maintain BDNF levels in the STR.

The assumption that mutant HTT renders intracellular transport less efficient was verified by direct visualization of BDNF vesicles in neuronal cell lines derived from knock in mice, and cortical neurons transfected with HTT constructs. These experiments demonstrated that anterograde and retrograde BDNF transport is altered in presence of the mutant protein. Furthermore, the mechanism through which this defect occurs was uncovered. As described previously, HTT is part of the motor complex that drives BDNF transport (**Fig 15** and **Fig 16**). The presence of the polyQ HTT alters the interaction of this complex, as the association between HTT and HAP1/p150^{*Glued*} is increased in the presence of an expanded polyQ stretch. This leads to a detachment of this complex from MT, demonstrating the mechanism underlying retrograde transport defects in the presence of the polyQ expansion. However, delivery of BDNF from cortex (CTX) to the STR depends on anterograde

transport. Keeping in mind that HAP1 and kinesin interact, the effect of mutant HTT in kinesin's association to MTs was studied. Indeed, this interaction is reduced due to the presence of the polyQ expansion, leading to impaired anterograde transport of BDNF vesicles (Gauthier et al., 2004). This reduced BDNF delivery from cortical neurons to the striatum due to transport defects, is believed to be an important mechanism involved in the specific vulnerability of the STR in HD.

Phosphorylation of mutant HTT has been shown to be involved in the pathogenic BDNF transport defect that occurs in HD. As discussed before, phosphorylation of wild-type HTT at serine-421 by Akt kinase regulates BDNF transport (Colin et al., 2008). Phosphorylation favors anterograde transport whereas the absence of phosphorylation promotes retrograde movement (**Fig 15**). In the pathological context, reduced phosphorylation at serine-421 was detected in the endogenous mutant HTT, in cell cultures as in human and transgenic mouse brains (Warby et al., 2005).

In addition, Akt pathway has also been shown to be deregulated in the brain tissue of HD animal model (Colin et al., 2005; Gines et al., 2003), and phosphorylation of mutant HTT at serine-421 was shown to abolish mutant HTT toxicity (Humbert et al., 2002; Pardo et al., 2006). Taken together, all of this suggested that the phosphorylation at this position is a key process participating in HD pathogenesis. Actually, phosphorylation of polyQ HTT at serine 421 induced by prosurvival pathways such as IGF/Akt pathway restores BDNF transport defects, as it reinstates the correct interaction of HTT with p150^{Glued} subunit of dynactin and their association with MTs in vitro and in cells (Zala et al., 2008). The transport-dependent release of BDNF is known to be reduced in the presence of polyQ HTT (Dompierre et al., 2007; Gauthier et al., 2004). Recovery of transport deficit by phosphorylation is reflected as well in a recovery of BDNF release. Furthermore, both IGF-1 treatment and Akt overexpression, as inhibition of HTT's phosphatase calcineurin, are able to compensate for the transport defects observed in HD cells, and restore trophic support in disease situation (Pineda et al., 2009; Zala et al., 2008). Therefore, compounds or signaling pathway that rescue the altered transport may be of therapeutic interest



Figure 16: BDNF transport in Huntington's Disease. BDNF is normally produced in the Cortex and transported anterogradelly to support survival in the Striatum (Bottom left). Huntingtin works as an enhancer of this transport through its interaction with the molecular motors (upper left). When Huntingtin is mutated, a modification of these interactions leads to detachment of the complex associated with vesicles from microtubules (upper right), altering BDNF delivery to the Striaum. This results of degeneration first of the Striatum end then of the Cortex (bottom right)

4.5 General transport in HD

Although defects in BDNF transport seems to play a pivotal role in HD pathogenesis, the implication of HTT in transport is not exclusive for this cargo, as the dynamics of other proteins has been shown to be affected by the presence of mutant HTT.

Some early studies performed in models including *D. melanogaster*, squid axoplasm and mice, have focused their attention on the general transport of organelles, suggesting that HTT mutation does not alter transport of specific cargos, but blocks transport of organelles in general. Indeed, overexpression of human HTT exon 1 containing the expanded polyQ stretch in D. melanogaster, revealed defects in axonal transport as shown by accumulation of cellular organelles and synaptic vesicle in nerves (Gunawardena et al., 2003; Lee et al., 2004). It was suggested that the expression of mutant HTT caused a depletion of active motors by sequestering dynein, dynactin and kinesin into cytoplasmic aggregates, as the soluble pool of motor proteins was decreased in these flies (Gunawardena et al., 2003). Similar observations were made in neurons from a mouse model expressing full-length HTT: general transport was shown to be altered and there is a titration of motor proteins from the soluble pool (Trushina et al., 2004). However, this is in contradiction with the observations made in squid axoplasm, in cells and other mouse models of HD (Gauthier et al., 2004; Her and Goldstein, 2008; Szebenyi et al., 2003; Wu et al., 2010). In these conditions the transport defect was shown to occur independently from aggregation, as these structures were not detected. Hence, although aggregates could sequester molecular motors from the soluble pool, the defect in transport occurs through an independent mechanism concerning the loss of normal function of HTT, and not involving HTT aggregates. Experiments performed in isolated axoplasm extruded from squid giant axons, demonstrated that the perfusion of HTT fragments containing the polyQ expansion decreased bidirectional motility of general vesicular population. An interesting point that comes out from these experiments, is that the only presence of HTT containing expanded polyQ tract in the cytoplasm is sufficient to affect transport, as isolated axoplasm contains neither nuclei nor protein synthesis. Therefore, transport defects occur by the influence of cytoplasmic mutant HTT(Morfini et al., 2009; Szebenyi et al., 2003).

Although it is well accepted that transport defects occur in HD, the identity of the cargos and transported vesicles altered by the mutation remains unclear. Therefore, many studies have focused on analyzing whether dynamics of specific cargos that could have direct relevance in the disease was affected. Tightly related with BDNF transport defects, and BDNF signaling pathway, defects in TrkB transport were described. After release by cortical neurons, BDNF binds to and activate TrkB receptors in striatal dendrites and promote survival. Indeed, the polyQ expansion alters the dynamics of TrkB containing vesicles. Moreover, specifically within isolated striatal dendrites, retrograde transport of ligand-bound TrkB and BDNF/TrkB-induced signaling through ERK phosphorylation and c-fos induction are decreased in neurons from an HD mouse model (Liot et al., 2013). Striatal neurons are highly dependent on BDNF signaling for survival, and this pathway is affected in two independent ways, both relying on intracellular transport. Firstly, BDNF delivery and release by cortical neurons is affected. In addition, striatal neurons show defective transport of BDNFbound TrkB vesicles, resulting in altered signaling. These two deficiencies could have a synergistic deleterious effect that renders striatal neurons more sensitive to cell death due to mutant HTT. It was also demonstrated that when HTT is mutated GABA_A receptors transport is reduced. The kinesin motor Kif5, along with HAP1, are responsible of GABA_A receptors trafficking. These receptors at synapses regulate brain excitability, altering inhibitory synaptic currents, which may explain some of the defects of brain information processing occurring in HD (Twelvetrees et al., 2010).

Transport defects due to the presence of mutant HTT might as well influence autophagy. As discussed in Chapter 2 autophagy defects have been implicated in HD. In neurons, autophagosomes formation is a polarized process, where autophagosomes are generated and engulf cargos in the axon and then undergo retrograde transport towards the cell body. As in other cases, HAP1/HTT complex regulates autophagosomes dynamics, promoting processive transport. The presence of mutant HTT disrupts this process in primary neurons, without altering autophagosome formation or cargo loading, resulting in accumulation of charged autophagosomes, probably due to inefficient clearance (Wong and Holzbaur, 2014).

These observations seem to suggest that HTT alters global intracellular transport. Furthermore, a variety of cargos that are not clearly related to HD pathogenesis,

have been shown to be affected by the presence of the expanded polyQ HTT. Mutant HTT affects the dynamics of vesicles containing neuropeptide-Y (NPY) Ependymal growth factor receptor (EGFR) and atrial natriuretic factor (ANF) (Sinadinos et al., 2009; del Toro et al., 2006). However, contrary to previous analyzed cargos, the dynamics of p75 neurotrophic receptor (p75^{NTR}), a neurotrophin receptor, is not affected by the presence of polyQ HTT, demonstrating that although polyQ HTT alters the dynamic of many cargos, their effect is specific to certain but not all of them (del Toro et al., 2006). Interestingly, in HD condition, the dynamics of a single cargo can be affected in a cell specific manner. Motility of a fluorescently labeled APP, a well-characterized cargo for anterograde axonal transport was studied in cultured neurons from mutant mice expressing HTT with expanded polyglutamine repeats. This study showed that APP dynamics was affected by the expression of mutant HTT in hippocampal and striatal neurons, but not in cortical neurons (Her and Goldstein, 2008). Therefore, the identity of the cargos affected by the mutation could also depend on the nature of the neurons in which transport occurs. This specificity could be due to the presence of some neuron-specific adaptor proteins. Therefore, the protein complexes associated with HTT-dynamic vesicles needs to be precisely established to understand better the impact of transport defects in disease initiation and development. For example, HTT has been shown to interact with Kif5, Kif3A and Kif1A (Kaltenbach et al., 2007; Ratovitski et al., 2012; Shirasaki et al., 2012). Trafficking of synaptic vesicle precursors depends on HTT interacting kinesins Kif5 and Kif1A; but they rely as well on Kif1B_β (Hirokawa et al., 2010). So are all synaptic vesicles precursors affected to the same extent by mutant HTT? Similarly, mitochondria are transported by kinesin-1, but also by Kif1C and Kif1Ba. Indeed, some studies do not agree on the extent to which, some neurons but not others, show altered transport of mitochondria (Gauthier et al., 2004; Trushina et al., 2004). Besides the obvious differences in models and analysis approaches used in the different studies, the dependence of mitochondria on different kinesins, not all of them known to associate with HTT, could be underlying these differences.

Taken together, all of these studies show that HTT is a key regulator of MT based intracellular transport. HTT acts as a scaffold protein that coordinates attachment of

different types of motors to vesicular cargos. Both the depletion as the expression of mutant HTT, have shown to affect a wide range of aspects of intracellular transport. In HD, trafficking defects induced by the mutant HTT, has a major role in disease pathogenesis through the regulation of BDNF and TrkB transport, which are maybe potentiated by the defect in other cargos.

Furthermore, HTT has a more global role in intracellular transport as it coordinates vesicular transport by integrating signals from different regulatory pathways; HTT can coordinate binding to the appropriate molecular motors and control motility.

Many aspects of HTT-dependent trafficking remain less clear. For instance, what are the molecular cues that specify the targeting of huntingtin to distinct organelles, such as the ER, Golgi and transport vesicles? One possibility could involve the many posttranslational modifications of HTT such as phosphorylation or palmitoylation. Indeed, many sites have been identified in HTT, and some have shown to regulate toxicity in HD (Aiken et al., 2009; Gu et al., 2009; Luo et al., 2005; Schilling et al., 2006; Thompson et al., 2009). However, whether these posttranslational modifications are functionally related with HTT's role in trafficking is still unknown. A more profound knowledge of HTT's role in intracellular transport is essential to shape future studies of the molecular mechanisms underlying HD.

Results

RESULTS

5 Drosophila melanogaster as an *in vivo* model for studying huntingtin's function in transport.

5.1 Study presentation

Huntingtin (HTT) is the protein that when mutated gives rise to Huntington's disease, a late onset fatal neurodegenerative disease. Major efforts have been made to understand what are the pathogenic mechanisms leading to the differential cell death of striatal neurons in HD. It was suggested that the presence of an elongated polyQ stretch leads to a gain of new toxic functions of HTT. However, many studies have also demonstrated that the normal function of HTT is altered in the disease, and these disruptions have been implicated in the pathogenesis.

HTT is a scaffold protein known to interact with many partners and through these interactions is probably involved in numerous cellular processes. Particularly, via its interaction with molecular motors and adaptor proteins HTT regulates intracellular transport. The presence of the elongated polyQ stretch alters transport of several different vesicular cargos, including BDNF, which was shown to be a key factor in HD pathogenesis.

Overexpression of N-terminal fragments of human HTT has been used as an approach to generate models of HD in *D. melanogaster*. However, studies using these flies were based on the notion that HD occurs due to the gain of new toxic functions of HTT protein. Therefore, to elucidate whether the role of HTT in transport is conserved in the fruitfly, studies have been made by knocking out and knocking down the endogenous protein. However, these studies were not concluding, as major discrepancies were found among them.

In this study we show that *D. melanogaster* HTT (DmHTT) interacts with the molecular motor dynein, and associates with microtubules as well as with vesicles. In mammalian neurons, DmHTT co-localizes with BDNF-containing vesicles. In these cells we could assess that DmHTT is dynamic, and co-transports with BDNF vesicles. Importantly, DmHTT is able to rescue the transport deficit due to the downregulation of the endogenous HTT. Finally, in larvae from DmHTT-KO flies, we

could prove that the absence of HTT leads to a reduced fast axonal transport of synaptotagmin vesicles.

Our results indicate that HTT function in transport is conserved in D. melanogaster, and that flies are an excellent model to study axonal transport *in vivo*.

5.2 Article I

Zala D, <u>Hinckelmann MV</u>, Saudou F. **Huntingtin's function in axonal transport is conserved in** *Drosophila melanogaster.* PLoS One, 2013.

Huntingtin's Function in Axonal Transport Is Conserved in *Drosophila melanogaster*

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Abstract

Huntington's disease (HD) is a devastating dominantly inherited neurodegenerative disorder caused by an abnormal polyglutamine expansion in the N-terminal part of the huntingtin (HTT) protein. HTT is a large scaffold protein that interacts with more than a hundred proteins and is probably involved in several cellular functions. The mutation is dominant, and is thought to confer new and toxic functions to the protein. However, there is emerging evidence that the mutation also alters HTT's normal functions. Therefore, HD models need to recapitulate this duality if they are to be relevant. *Drosophila melanogaster* is a useful *in vivo* model, widely used to study HD through the overexpression of full-length or N-terminal fragments of mutant human HTT. However, it is unclear whether *Drosophila* huntingtin (DmHTT) shares functions similar to the mammalian HTT. Here, we used various complementary approaches to analyze the function of DmHTT in fast axonal transport. We show that DmHTT interacts with the molecular motor dynein, associates with vesicles and co-sediments with microtubules. DmHTT co-localizes with Brain-derived neurotrophic factor (BDNF)-containing vesicles in rat cortical neurons and partially replaces mammalian HTT in a fast axonal transport assay. DmHTT-KO flies show a reduced fast axonal transport of synaptotagmin vesicles in motoneurons *in vivo*. These results suggest that the function of HTT in axonal transport is conserved between flies and mammals. Our study therefore validates *Drosophila melanogaster* as a model to study HTT function, and its dysfunction associated with HD.

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Introduction

The fruit fly *Drosophila melanogaster* (Dm) has various characteristics that make it a useful model for medical and fundamental research. For example, more than 75% of human disease genes have a homologue in flies [1], and the bipartite UAS-GAL4 system developed by Perrimon and collegues offers an extremely flexible tool to control, in space and time, the expression of transgenes [2]. Also, the numerous mutant and transgenic flies available, and the associated databases, are powerful tools for both large output screening and basic research.

For these reasons and despite obvious morphological, size and neuronal circuitry differences between human and fly brains, *Drosophila melanogaster* is widely used in neurodegenerative diseases research. In particular, several fly strains have been generated to model Huntington's disease (HD), a dominantly inherited neurodegenerative disorder caused by an abnormal polyglutamine (polyQ) expansion in the huntingtin (HTT) protein [3–7]. These models are based on the overexpression of the full length, or Nterminal fragments of, human HTT. Flies expressing mutant HTT show neuronal dysfunction, such as defects in synaptic transmission and axonal transport, neuronal degeneration, locomotor deficits and shorter lifespans [4–8]. HD fly models have been extensively used to validate candidate approaches and to search for gene modifiers that rescue neurodegeneration [9–15]. Importantly, these studies are based on the notion that the mutation in HTT results in a gain of new toxic functions that are unrelated to wild-type HTT function. Indeed, the HD mutation is dominant and the overexpression of the HTT fragments containing the polyQ expansion is sufficient to induce phenotypes in flies. However, recent evidence in mammals suggests that alteration of the wild-type HTT functions also contributes to HD [16–19]. This duality, both gain and loss of function, of the pathogenic mechanisms raises the issues of whether the overexpression of polyQ HTT in flies faithfully recapitulates mammalian HD, and the degree to which HTT function is conserved between flies and mammals.

HTT is a large scaffold protein of 350 kDa in human and of a predicted 400 kDa in fly. HTT interacts with hundreds of proteins and regulates several cellular functions [14,16,18,20,21]. For example, laboratories have reported that HTT is a positive regulator of microtubule-(MT)-based transport [22–25]. This function is altered upon polyQ expansion [22,24] and vesicular transport is slowed down as a result.

Three studies have investigated the function of *Drosophila* HTT (DmHTT) in axonal transport [24,26,27] but there are discrepancies between their findings. Silencing DmHTT by sh-RNA resulted in accumulation of axonal organelles, characteristic of

severe transport defects [24]. This phenotype was more evident in kinesin heavy chain heterozygous flies. By contrast, the second study reported that HTT knock-out flies are viable with no obvious developmental defects and normal axonal transport [26]: no synaptotagmin accumulation was observed in axons. However, neither study directly assessed the dynamic nature of vesicles in axons through the observation of fluorescent cargo by videomicroscopy [28]. Importantly, in mammals, in contrast to the silencing or depletion of molecular motors, HTT silencing reduces but does not totally block axonal transport of cargo and does not result in the accumulation of axonal organelles. Finally, a recent study reported a defect in the dynamics of Rab11 but not Rab5 vesicles in *Drosophila* larvae in which DmHTT was silenced by RNAi [27]. These results suggest that DmHTT could play an important role in flies.

Here, we report the study, by various complementary approaches, of the role of HTT in fast axonal transport. Our results indicate that the function of HTT in axonal transport is evolutionarily conserved between flies and mammals.

Materials and Methods

Statistical Analyses

Statview 4.5 software (SAS Institute Inc.) was used for statistical analysis. Groups were compared by ANOVA followed by Fisher's PLSD post hocs analyses. The criterion for statistical significance was set at P<0.05. Error bars, S.E.M.

Flies

D. melanogaster flies were stocked at 18° C on a fly standard medium. Recombinant *ok6-Gal4:UAS-Syt-GFP* (X) flies were a kind gift from ML Parmentier, control w118 flies and DmHTT-Knock out flies are a kind gift from S. Zhang and N. Perrimon [26]. Flies were maintained at 25° C for the experiments. Offspring L3 larvae were collected for video-microscopy. 3-5 larvae from the same genotype were laid on an 18 mm round coverslip. A second coverslip was added to form a sandwich and the system was mounted in a modified homemade Ludin chamber with a gentle compression of the larvae. Larvae were then observed by fast-video-microscopy.

Plasmids and Antibodies

Dm620HTT-GFP was previously described [29]; GFP was obtained from Invitrogen (pCDNA-N1-GFP). *Drosophila* antibody (DmHTT-S50) was generated by immunizing rabbits with two peptide sequences: H2N-RHRRDRNKAKGPAPQC-CONH2 and H2N-CVASDEDKQGQGHRQQ-CONH2 (Eurogentec, Rabbit DX Speedy-2 peptides program). Antisera analysis by indirect ELISA was performed to quantify the response of different bleeds. The following antibodies and dilutions were used for immunoblotting experiments: rabbit anti DmHTT (DmHTT-S50, 1:1:500) rabbit anti GFP (1:1000, Institut Curie), mouse anti DIC (1:2000, Millipore), mouse anti huntingtin (4C8, 1:3000, Euromedex), mouse anti α -tubulin (1:3000, Sigma-Aldrich), mouse anti β -actin (1:5000, Sigma-Aldrich).

Immunoprecipitation Experiments

HEK 293 cells were transfected with GFP or Dm620HTT-GFP using calcium phosphate-based method. Two days post-transfection, cells were washed once with ice-cold PBS and pelleted. Cells were lyzed in the following buffer; 50 mM Tris pH8.0, 150 mM NaCl, 1% NP40, 1:100, containing a cocktail of proteinase inhibitor (Sigma-Aldrich) and PMSF 1 mM (Sigma-Aldrich).

Immunoprecipitation was performed with μ MACS GFP-Tagged Protein Isolation Kit according to the provided protocol (MACS molecular). Anti-HA beads were used as negative controls (MACS molecular).

Microtubule Depolymerization-Repolymerization Assay

HEK 293 cells where lyzed by passing the samples 10 times in a 15 G syringe in ice cold BRB80 buffer (Pipes 80 mM, MgCl₂ 1 mM and EGTA 1 mM) containing a cocktail of protease inhibitor (Sigma-Aldrich) and PMSF 1 mM (Sigma-Aldrich). Samples were clarified at 10,000×g for 30 min at 4°C. Supernatant (Total) was incubated at 37°C with 1 mM GTP for 15 min, then Taxol 50 μ M was added to stabilized microtubules for 15 min at 37°C. For negative control, Taxol was replaced by nocodazole 50 μ M. Samples were centrifuged at 10,000×g for 30 min at 30°C and supernatant and pellet containing microtubules and associated proteins were collected. Pellet was resuspended in one fifth of the supernatant volume.

Subcellular Fractionation

HEK 293 cells were lyzed by passing the samples 10 times in a 15 G syringe in ice cold Hepes 4 mM and sucrose 320 mM buffer containing a cocktail of proteinase inhibitor (Sigma-Aldrich) and PMSF 1 mM (Sigma-Aldrich). Samples (Total) were centrifuged at 3000 g for 10 min at 4°C to recover pellet (P1) and supernatant (S1). S1 was centrifuged at 10,000×g for 30 min at 4°C to recover pellet (P2) and supernatant (S2). S2 was centrifuged at 90,000×g for 90 min at 4°C to recover pellet (P3) and supernatant (S3). P1 and P2 were re-suspended respectively in the same volumes of S1 and S2 samples. P3 was re-suspended in one fifth of the S3 volume.

Neuronal Cultures

Primary striatal neurons were prepared from E17 rat embryos as previously described [30]. Briefly, cortices were dissected in cold DM buffer pH 7.4 (Na₂SO₄ 82 mM K₂SO₄ 30 mM, MgCl₂ 5.8 mM, CaCl₂ 250 nM, Hepes 1 mM, Glucose 20 mM and Phenol red) supplemented with kynurenic acid 1 mM and MgCl₂ 8 nM. Tissue digestion was performed with 2 incubations of 10 min in a solution of papain and cysteine followed by 2 incubations of 7 min and 30 sec in a solution of trypsin inhibitor. Tissues were then dissociated mechanically in optiMEM-1 (Gibco) supplemented with glucose. Neurons in suspension (5 millions) were electroporated with Amaxa Nucleofector kit for rat neurons (Lonza) according to the supplier's manual. Neurons were plated on glass coverslips coated with poly-L-lysine (1 mg/ml). After 3 hours, medium was replaced with fresh Neurobasal medium supplemented with 2% B27, 2 mM glutamax, 1% penicillin/ streptomycin, 10 µM forskolin and 100 µM IBMX (Sigma-Aldrich).

Microchambers and Neurons Plating

Microchambers and neuronal plating were previously described [31,32]. Briefly, silicon wafer obtained by photolithography was used to create the master with SU8 resin. The channels are 3 μ m high, 5 μ m wide and 450 μ m long. Silicon elastomer with its curing agent was used (PDMS, Sylgard 184, Dow corning) to create the devices. The microfluidic devices were then coated with poly-D-lysine (0.5 mg/ml) and laminin (10 mg/ml) overnight at 4°C and then washed three times with neuronal medium. One million neurons were plated in the cell body/proximal compartment of the chamber.

Videomicroscopy

The microscopes and the chambers were kept at 37°C. Images were recorded every 200 ms with a 100 X PlanApo N.A. 1.4 oil immersion objective. For the gene replacement studies on neuronal rat culture, images were collected in stream mode using a Micromax camera (Roper Scientific) set at 2×2 binning. For DmHTT dynamics, images were collected using a spinning disk (Yokogawa CSU-X1 head) and a EM-CCD camera (Evolve) set at 1×1 binning. Projections, animations and analyses were generated using ImageJ software (http://rsb.info.nih.gov/ij/, NIH, USA). Maximal projection was performed to identify the vesicles paths, which in our system corresponds to vesicle movements in axons. Kymographs and velocity analyses were generated with the KymoToolBox, a homemade plug-in previously described [31].

Results

Drosophila N-terminal Huntingtin Fragment Interacts with Dynein Intermediate Chain

The N-terminal part of mammalian HTT interacts with proteins belonging to or associating with the molecular motor complex: there is a direct interaction with Dynein Intermediate Chain (DIC) [23] and an indirect interaction, via the Huntingtin Associated Protein 1 (HAP1) [33], with the p150^{Glued} dynactin subunit [33,34] and with Kinesin light Chain (KLC) [35]. Ectopic expression of terminal fragments of HTT containing these interacting domains allows fast axonal transport of vesicles

[22,25,36,37]. Conversely, full length HTT constructs lacking either the HAP1 or the dynein interacting domains alter or impair vesicular transport [38]. These results suggest that N-terminal HTT is necessary and sufficient to support transport of vesicles.

To test whether DmHTT functions in the transport of vesicles in flies, we first used an N-ter 620 amino-acid fragment of DmHTT. We previously showed that this fragment restores the spindle orientation defect of neuroblasts in HTT knock-out flies [29]. This function of HTT is associated with the recruitment of molecular motors at spindle poles and requires the motor proteins involved in vesicular trafficking. These findings suggested that this N-ter-620 amino-acid fragment of DmHTT may carry the domains allowing HTT binding to DIC. To investigate this possibility, we performed clustal analyses using various fragments of mammalian HTT and DmHTT. We limited the lengths of the mammalian HTT sequences analyzed on the basis of various findings: 1) Caviston and colleagues showed that human HTT fragment including amino-acids 600-1483 binds directly to dynein intermediate chain (DIC) and that a GST-HTT [536-698] fragment co-elutes with DIC of mouse brain cytosol [23]; this indicates that the minimal relevant region is [600-698]; 2) that partial deletion of this region from the full-length HTT Δ [633– 672] abolished HTT transport function [38]. Clustal analyses showed that human HTT [600-698] partially aligns with the first 620 amino acids of DmHTT. In particular, the human HTT[600-649] and DmHTT[380-430] amino-acid sequences share an identity of 30% and a similarity of 62% (Fig. 1A).

Α

В





Figure 1. The dynein interacting domain of human HTT is conserved in *Drosophila melanogaster*. (A) Alignment of hHTT[600–698] and DmHTT[381–549]. The red box highlights the high homology region. (B) Western blot analyses of the co-immunoprecipitation of Dm620HTT-GFP with endogenous DIC on transfected HEK 293 cells: Dm620HTT-GFP but not control GFP co-precipitates DIC using an anti-GFP antibody. Anti-HA antibody was used as negative control and showed no Dm620HTT-GFP or DIC signal. doi:10.1371/journal.pone.0060162.q001

We next investigated whether the N-Terminal 620 amino acid fragment of DmHTT interacts with DIC. HEK 293 cells were transfected either with the Dm620HTT-GFP or GFP constructs, and anti-GFP and control anti-HA antibodies were used for immunoprecipitation experiments. GFP and DmHTT-GFP were efficiently immunoprecipitated. DIC was only co-immunoprecipitated with Dm620HTT-GFP. Thus, fly HTT, like human HTT, interacts with human DIC (Fig. 1B).

N-terminal Fragment of *Drosophila* Huntingtin Associates with Mammalian Vesicles

To investigate whether DmHTT acts in axonal transport of vesicles, we transfected HEK 293 cells with a cytosolic control protein (GFP) or with Dm620HTT-GFP and determined the distribution of the proteins in subcellular fractions.

Western blotting analyses indicated that cytosolic proteins (α tubulin and β actin) were abundant in the S3 fraction (the cytosol), and were scarce in the small membrane fraction P3 (vesicles and small membranes). As expected, endogenous mammalian HTT and DIC were present in both S3 and P3 fractions, confirming that a significant proportion of these proteins were associated with vesicles. Transfected GFP was mostly found in the S3 fraction and thus displayed a characteristic cytosolic profile. By contrast, Dm620HTT-GFP was mostly in the vesicular fraction, P3 (Fig. 2).

Thus, DmHTT associated with vesicles in mammalian cells, consistent with a putative function of fly HTT in dyneindependent transport of vesicles along microtubules.

Drosophila N-terminal Huntingtin Colocalizes with BDNF Vesicles in Axons

Mammalian HTT is involved in the vesicular transport of several types of cargos, including *Brain-Derived Neurotrophic Factor* (BDNF) [22,25,39]. Cortical neurons produce BDNF that is transported along the axons to the striatum where it is released [40]. The binding of BDNF to striatal postsynaptic TrkB receptors mediates survival of the striatal neurons. We tested whether DmHTT associates with BDNF vesicles by transfecting cortical rat neurons with Dm620HTT-GFP and BDNF-mCherry constructs. As expected, BDNF-mCherry showed a typical punctate staining in axons and dendrites, characteristic of vesicles (Fig. 3A). To assess possible co-localization of the two proteins, we re-sliced the stacks of images along neurites and analyzed their distribution intensities (Fig. 3B). BDNF-mCherry staining displayed characteristic vesicular pattern as shown by the presence of punctae within axons (Fig. 3B) and of marked peaks of intensity (Fig. 3B). In contrast, control GFP staining was more diffuse (Fig. 3B). Linescan analysis revealed that the GFP channel fluctuations did not coincide with BDNF peak intensities. In contrast, the Dm620HTT-GFP signal was punctate and co-localized strongly with BDNF vesicles (Fig. 3B). Quantification of the line-scan further indicated that a significant percentage of BDNF vesicles (69.1% + / -2.5%, n = 343) were also positive for Dm620HTT-GFP. The number of peaks of DmHTT was greater than the number of BDNF vesicles, suggesting that, consistent with the wider role of mammalian HTT in transport, DmHTT was involved in the transport of cargo other than BDNF. These findings further implicate DmHTT in the axonal transport of vesicles.

Drosophila Huntingtin Associates with Microtubules

We next investigated whether DmHTT, like its mammalian homologue, associates with microtubules. We performed a microtubule depolymerization-repolymerization assay with transfected HEK 293 cells to analyze whether Dm620HTT-GFP cosedimented with microtubules (MT) (Fig. 4A). We found α -tubulin to be enriched in the MT fraction and massively depleted from the soluble fraction (S) indicating that the assay efficiently purified MT-associated proteins. β -actin, the p50 dynamitin subunit of dynactin and GFP, that have no MT binding motifs, were found mostly in the soluble fraction (S). By contrast, dynein (DIC) and dynactin (p150^{Glued}) were highly enriched in the MT fraction. Similarly, endogenous HTT and ectopically expressed Dm620HTT-GFP were recruited to microtubules. To investigate



Figure 2. *Drosophila* **Huntingtin N-terminal fragment associates to small vesicles.** HEK 293 were transfected with GFP or Dm620HTT-GFP and cells were subjected to subcellular fractionation (T: total, P3: vesicles, S3: cytosol, P2: larges membranes, S2: cytosol plus light membranes, P1: nuclei and S1: post-nuclear supernatant) followed by Western blotting analyses to reveal the distribution of the different proteins (GFP, Dm620HTT-GFP, endogenous HTT, DIC, α -tubulin and β -actin). Immunoblot shows that GFP is mostly in the soluble fraction (S3) whereas Dm620HTT-GFP is enriched in the vesicles fraction (P3). doi:10.1371/journal.pone.0060162.g002



Figure 3. *Drosophila* **Huntingtin N-terminal fragment colocalizes with neuronal BDNF vesicles.** Rat cortical neurons were electroporated to express BDNF-mCherry and GFP or Dm620HTT-GFP, fixed and immunostained. Fluorescence was analyzed by confocal microscopy. (A) Confocal images showing the localization of both BDNF-mCherry and Dm620HTT-GFP in double-transfected neurons. (B) Resliced z-stack along an axon with channel intensity values (top) and immunostaining (bottom) with GFP channel (green) showing the localization of GFP (left) or Dm620HTT-GFP (right) and mCherry channel (red) showing the localization of BDNF-mCherry-containing vesicles. Whereas GFP intensity is diffuse and does not correlate with BDNF, Dm620HTT-GFP shows a punctate signal that partially co-localizes with BDNF. Stars show when the two peaks of intensity co-distribute in the line-scan and co-localize in the z-resliced images. doi:10.1371/journal.pone.0060162.q003

whether Dm620HTT-GFP was non-specifically associated with the MT fraction, we treated cell extracts with nocodazole to depolymerize microtubules. In these conditions, Dm620HTT-GFP was not precipitated, suggesting a specific association of DmHTT with MTs (Fig. 4B).

These various results in mammalian cells show that the Nterminal part of *Drosophila* HTT interacts with dynein, associates with the vesicular fraction, co-localizes with BDNF-containing vesicles in axons and associates with microtubules. Therefore, DmHTT, like HTT, appears to function in MT-based transport of vesicles.

Drosophila Huntingtin Displays Fast Axonal Transport Properties and is Co-transported with BDNF Vesicles

We then investigated whether DmHTT could have a dynamic behavior in axons. We used a homemade microfluidic device derived from a previously described microfluidic culture platform to analyze transport in axons [31,32,41]. The device allows central nervous system axons to grow into fluidically isolated microchannels. Cortical neurons were electroporated with Dm620HTT-GFP construct and plated in the proximal compartment of the microfluidic chamber. Three to five days after plating, axons reached the distal chamber that is 450 μ m away from the proximal compartment that contains the cell bodies and most of the dendrites [30,39]. To analyze whether DmHTT displays fast



Figure 4. *Drosophila* **Huntingtin N-terminal fragment associates with microtubules.** HEK 293 were transfected with GFP or Dm620HTT-GFP and cell extracts were subjected to a microtubule depolymerization – repolymerization assay to pellet microtubules and the microtubule-associated proteins which were analyzed by Western blotting. (A) Whereas GFP does not pellet with the microtubules fraction (MT) but is soluble (S), Dm620HTT-GFP co-sediments with microtubules. (B) MT binding assay in presence of Taxol or Nocodazole. Dm620HTT-GFP is not pelleted in Nocodazole-treated cells when microtubules are depolymerized. doi:10.1371/journal.pone.0060162.g004

axonal transport, we used spinning-disk confocal videomicroscopy to record the movement of GFP particles in the axons of cortical neurons expressing Dm620HTT-GFP. We observed diffuse fluorescence along the axons with some spots of greater fluorescence intensity, similar to the pattern observed in fixed and immunostained axons (Fig. 5A and Fig. 3B). Analyses of the movies and the associated kymographs revealed rapid anterograde and retrograde movements (Fig. 5A and Movie S1). The velocities of these movements were determined: Dm620HTT moved with a mean anterograde velocity of $2.7\pm0.2 \ \mu m/s \ (n=34)$ and a mean retrograde velocity of $1.3 \pm 0.2 \ \mu m/s$ (n = 13). These velocities are typical of fast axonal transport. Having demonstrated that DmHTT is dynamic (Fig. 5A), we assessed whether DmHTT was co-transported with BDNF-containing vesicles. We electroporated embryonic rat cortical neurons with Dm620HTT-GFP and a construct encoding BDNF-mCherry. BDNF-mCherry is transported within axons with dynamics characteristic of fast axonal transport [31,39,42]. Analyses of BDNF vesicle dynamics revealed fast and highly processive movements (Fig. 5B) at velocities similar to those previously reported [31,39,42]. Twocolor analysis of the dynamic behavior of Dm620HTT-GFP and BDNF-mCherry within an axon revealed the trafficking of both proteins (Movie S2). Kymograph analysis of the dynamics of the two proteins indicated that they were co-transported (Fig. 5B). We conclude that Drosophila HTT is dynamic and is associated with BDNF vesicles during their transport within axons.

Drosophila Huntingtin Partially Rescues Loss of Mammalian HTT in Fast Axonal Transport

We then tested if endogenous mammalian HTT could be replaced by *Drosophila* HTT. We silenced rat HTT by treatment with a siRNA that was previously validated and does not have any off-target effects [25,31]. This siRNA effectively decreased levels of endogenous HTT (Fig. 5C). We analyzed BDNF dynamics by videomicroscopy and observed, as previously demonstrated [22,25,36], that the siRNA caused a marked decrease in both anterograde and retrograde transport of BDNF (see the characteristic kymographs with colored trajectories and graphs of mean velocities, Fig. 5D and 5E). Next, we expressed the Dm620HTT-GFP construct in the rat HTT-silenced neurons; this construct is insensitive to the rat siHTT RNA (Fig. 5C). Videomicroscopy analyses of BDNF transport in these samples revealed a significant recovery of BDNF transport in both anterograde and retrograde (see kymograph analysis and BDNF velocities, Fig. 5D and 5E). Although the recovery to control values was not complete, this experiment demonstrates that *Drosophila* HTT function.

Drosophila melanogaster Huntingtin is Present in Fly Brains

Investigations of HTT function in flies have been impeded by the lack of antibodies recognizing the endogenous protein. The expression of the HTT gene in flies has been investigated by in situ hybridization: expression is ubiquitous but weak during development [26]. To analyze whether the HTT protein is produced in Drosophila brains, we raised antibodies, in rabbit, against two antigenic regions of the D. melanogaster protein sequence. The polyclonal antibody obtained recognized Dm620HTT-GFP but not GFP produced in HEK 293 cells (Fig. 6A). We tested whether this DmHTT antibody recognizes endogenous fly HTT, by testing third instar larval brains from wild-type and HTT-KO flies [26]. The antibody recognized the endogenous DmHTT, although the signal intensity was weaker than that for ectopically expressed Dm620HTT. Nevertheless, the antibody gave a specific band corresponding to the predicted size of DmHTT of 400 kDa (Fig. 6B); importantly, this band was absent from HTT-KO fly brain samples. DmHTT is therefore expressed in the brain of third instar larvae.



Figure 5. *Drosophila* **Huntingtin is dynamic and rescues loss of mammalian huntingtin in axonal transport.** (A) Dm620HTT-GFP is dynamic. Image within a microchannel, of an axon from a rat cortical neuron expressing Dm620HTT-GFP. The generated kymograph below shows the vesicular trajectories and the paths that were analyzed (green for anterograde moving vesicles, red for retrograde moving vesicles and blue for stationary vesicles). (B) Two-colors kymograph obtained from neurons co-transfected with BDNF-mCherry and Dm620HTT-GFP shows co-transport of DmHTT and BDNF-mCherry in axons. (C) Western blotting shows silencing of endogenous rat *Htt* gene and its replacement by fly *htt* in cortical neurons. (D) The generated kymograph shows the vesicular trajectory in the three conditions and the paths that were analyzed. (E) The mean velocity of anterograde and retrograde moving vesicles was calculated. (Mean+S.E.M, p* <0.05, p**<0.01 and p***<0.001). doi:10.1371/journal.pone.0060162.g005



Figure 6. Fast axonal transport is impaired in *Drosophila* **larvae knock out for HTT.** (A) Anti DmHTT-S50 antibody recognizes Dm620HTT-GFP but not GFP when expressed in HEK 293 cells. (B) DmHTT-S50 recognizes a band of about 400 kDa in *Drosophila* L3 larvae brain extracts (see star). This band is absent in HTT-KO fly extracts. (C) Typical kymographs of wild-type and HTT-KO larvae with the analyzed trajectories for synaptotagmin-GFP vesicles. (D) Quantification of the mean velocity of axonal synaptotagmin-GFP vesicles in wild-type and HTT-KO larvae. (E) Distribution of synaptotagmin-GFP vesicles velocities shows a reduced number of fast-moving vesicles in HTT-KO larvae compared with wild-type flies.

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Fast Axonal Transport is Impaired in Drosophila Huntingtin Knockout Mutants

To confirm that DmHTT is involved in axonal transport, we crossed *ok6-Gal4:UAS-Syt-GFP* flies, expressing the vesicular

synaptic marker synaptotagmin-GFP in motoneurons, with DmHTT-KO flies [26]. Larvae at stage L3 obtained from these crosses were collected and placed between two coverslips. The dynamics of synaptotagmin vesicles were recorded by confocal

videomicroscopy (Movie S3 and 4) [28,31,43], and kymographs were generated from the data collected (Fig. 6C). They revealed lower mean velocity of synaptotagmin in DmHTT-ko flies than in wild-type flies (Fig. 6D). Analysis of the velocity distribution revealed that there were fewer fast-moving vesicles in the HTT-KO than wild-type flies (Fig. 6E). These results demonstrate that DmHTT promotes axonal transport in flies and thus that this function is conserved between mammals and flies.

Discussion

Almost two decades have passed since the identification of the CAG/polyglutamine expansion in the HTT gene/protein which leads to Huntington's disease [3]. Despite intense research, HD remains incurable and has a devastating impact on patients' lives. Various studies describe HTT as a scaffold or platform protein interacting with several different protein complexes that are involved in diverse cellular functions [14,16–18,20]. For example, HTT associates with transcription factors to regulate the expression of genes that, in turn, regulate neuronal survival [44–46]. HTT also regulates endo-exocytosis by interacting with components of the endo-exocytic and recycling machinery [47,48]. As a result of HTT being part of these various complexes, it acts as a pro-survival and anti-apoptotic factor, regulates synaptic transmission and brain homeostasis, and is also involved in the control of cell maturation and differentiation [16,18,29,49].

HTT also serves as a platform for the molecular motor complexes, dynein and kinesin [25,50]. The interactions between HTT and these complexes have been extensively described [33-35]. Silencing or reducing HTT activity affects the capacity of the dynein/dynactin complex to function properly in various models: reducing wild-type HTT levels reduces axonal transport [22,23,25]; and it affects spindle orientation in neuronal progenitors, a mechanism that depends on the dynein/dynactin complex [29]. It also reduces ciliogenesis by altering the MT and dyneindependent trafficking of cilia and basal body components to the base of the cilia [49]. Thus, the absence of HTT impairs these processes and as a consequence cells, especially neurons, accumulate several, albeit mild, cellular dysfunctions that lead to neurodegeneration as observed in HD [51]. The polyglutamine expansion also interferes with these functions. The polyQ expansion in HTT leads to defects in axonal transport [22] that are of similar severity as those associated with the loss of function phenotype induced by HTT silencing. Importantly, this effect is similar in $\text{ST}Hdh^{\text{Q109/+}}$ and $\text{ST}Hdh^{\text{Q109/Q109}}$ cells generated from heterozygotes and homozygotes knock-in mice [22,52] and therefore appear to result from a dominant negative effect of the mutant protein over the wild-type protein. In addition to this mechanism, polyQ HTT expression in cells or mice also affects axonal transport due to the formation of neuritic aggregates that physically perturb intracellular trafficking [7,24,53]. The cellular phenotype (reduced intracellular transport of organelles and of protein complexes) observed in HD neurons may therefore result from the gain of new toxic functions as well as the loss of normal function(s).

Drosophila melanogaster has been widely used in HD research particularly for the overexpression of short N-ter fragments or fulllength human HTT proteins with the polyQ expansion [4–7] this approach being based on a pure gain of function paradigm. The recent development of DmHTT knock-out flies facilitates the analysis of HTT functions in flies [26]. HTT inactivation in flies does not lead to the early embryonic lethality described in mice [54]. This difference is presumably the result of the essential role of mammalian HTT in extraembryonic tissues [51]. Therefore, progressive defects in HTT-KO flies may be analogous to the effects of inactivation of HTT in adult mice [19]. The phenotype of flies expressing the exon1 of HTT with 93Q worsens in the fly HTT-KO background, consistent with wild-type HTT having a role in adult flies [26]. This observation is very similar to the finding that the HD phenotype in mouse is modified by levels of wild-type HTT in heterozygotes [55]. The absence of HTT in flies may therefore result in mild dysfunctions similar to those described in mammalian models. Our findings that Drosophila HTT regulates axonal transport is in agreement with the previous study demonstrating that loss of HTT induces a spindle orientation defect of neuronal progenitors both in mammalian brain and in fly neuroblasts; spindle orientation depends on appropriate functioning of the dynein/dynactin complex [29]. The findings that HTT-KO flies recapitulate subtle phenotypes observed in mammals confirm that Drosophila melanogaster is a highly relevant model for studying HTT function and dysfunction in the context of HD.

Supporting Information

Movie S1 Movie shows Dm620HTT-GFP dynamics in rat axons located in the distal part of a microchannel. The right part corresponds to the tracked vesicles within the channel. Green dots are for anterograde movements, red for retrograde movements and blue for stationary vesicles. Arrows indicate the position of vesicles within the axon. (MOV)

Movie S2 Movie shows from left to the right the dynamic behaviour in the distal part of a microchannel of the following proteins: Dm620HTT-GFP, BDNFmCherry and overlay. Arrows indicate the position of vesicles within the axon.

(MOV)

Movie S3 The top movie shows the dynamic behaviour of synaptotagmin-GFP vesicles in motoneuron axons from control larvae and the bottom movie shows the vesicles that were subsequently tracked. Green dots are for anterograde movements, red for retrograde movements and blue for stationary vesicles.

(MOV)

Movie S4 The top movie shows the dynamic behaviour of synaptotagmin-GFP vesicles in motoneuron axons from DmHTT-KO flies and the bottom movie shows the vesicles that were tracked. Green dots are for anterograde movements, red for retrograde movements and blue for stationary vesicles.

(MOV)

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Author Contributions

Conceived and designed the experiments: DZ FS. Performed the experiments: DZ MVH. Analyzed the data: DZ MVH. Wrote the paper: DZ FS.

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6 Vesicular glycolysis sustains fast axonal transport

6.1 Study presentation

Neurons are unique cells based on their high degree of polarity. These cells contain one axon, which can be over a meter long in humans. Neurons depend on a highly energy-dependent fast axonal transport (FAT) to translocate vesicles that contain cargo proteins such as trophic factors and neurotransmitter receptors to the axonal periphery. One of the most challenging problems facing mammalian neurons is represented by axonal transport. Axons form synapses very distant from the cell body, therefore requiring of an efficient FAT of vesicles and other organelles along the axon. This rapid form of transport over long distances is mediated by microtubule-based kinesin and dynein motor proteins, which are highly demanding in energy.

In a cell the energy is mainly produced by two mechanisms. Firstly, glucose is broken down in the cytosol through the glycolytic pathway that converts glucose into pyruvate and generates only 2 molecules of ATP. In contrast to glycolysis, mitochondria produce 34 ATP molecules and provide most of the energy in the cell. Paradoxically, mitochondria are not evenly distributed along axons and tend to accumulate at specific sites such as synapses or nodes of Ranvier, thus raising the question of how vesicular transport is fueled along potentially long stretches of axons devoid of mitochondria.

In our study we demonstrate that the ATP needed for the FAT of vesicular cargoes does not come from mitochondria but from glycolysis. Inhibiting mitochondrial ATP production significantly reduces the ATP level of the cells. This inhibition affects mitochondrial but not vesicular dynamics. However, inhibiting or knocking down glycolysis reduces the FAT of membranous vesicles without affecting the dynamics of mitochondria.

Although the glycolytic enzyme GAPDH is largely found in the cytosol, we demonstrate by different approaches that this enzyme is specifically associated with dynamic vesicles. Moreover, these dynamic vesicles are capable of generating ATP.

The mechanism underlying this association involves the scaffold protein huntingtin (HTT). Knocking down HTT in cells or in mouse brains leads to a specific loss of GAPDH on vesicles, without affecting cytosolic levels, suggesting that HTT tethers GAPDH to vesicles.

Taken together, these findings show that FAT is energetically sustained by the ATP generated by the glycolytic machinery located on the vesicles themselves

6.2 Article II

Zala D, <u>Hinckelmann MV</u>, Yu H, Lyra da Cunha MM, Liot G, Cordelières FP, Marco S, Saudou F. **Vesicular glycolysis provides on-board energy for fast axonal transport.** Cell, 2013.

Cell



Vesicular Glycolysis Provides On-Board Energy for Fast Axonal Transport

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SUMMARY

Fast axonal transport (FAT) requires consistent energy over long distances to fuel the molecular motors that transport vesicles. We demonstrate that glycolysis provides ATP for the FAT of vesicles. Although inhibiting ATP production from mitochondria did not affect vesicles motility, pharmacological or genetic inhibition of the glycolytic enzyme GAPDH reduced transport in cultured neurons and in Drosophila larvae. GAPDH localizes on vesicles via a huntingtin-dependent mechanism and is transported on fast-moving vesicles within axons. Purified motile vesicles showed GAPDH enzymatic activity and produced ATP. Finally, we show that vesicular GAPDH is necessary and sufficient to provide onboard energy for fast vesicular transport. Although detaching GAPDH from vesicles reduced transport, targeting GAPDH to vesicles was sufficient to promote FAT in GAPDH deficient neurons. This specifically localized glycolytic machinery may supply constant energy, independent of mitochondria, for the processive movement of vesicles over long distances in axons.

INTRODUCTION

Fast axonal transport (FAT) is mediated by the molecular motors dynein and kinesin that transport cargos over long distances in neurons (Hirokawa et al., 2010). This extremely rapid and efficient transport is essential to deliver molecules such as trophic factors to nerve terminals that can be distant up to 1 m from cell bodies in the case of human motor neurons (Salinas et al., 2008). FAT consumes energy because the dynein and kinesin motors require ATP to transport cargos along microtubules. Because kinesin hydrolyses one ATP per 8 nm step (Gennerich and Vale, 2009), nerve cells must have energy sources distributed throughout their axons for these molecular motors to function.

Glucose is the major source of brain energy and is required for normal brain function. Glucose breakdown occurs in the cytosol through the glycolytic pathway that converts glucose into pyruvate and generates free energy (Ames, 2000). The net energetic balance of glycolysis is relatively low, with two ATP being produced from one molecule of glucose. In contrast to glycolysis, mitochondria produce 34 ATP molecules (Ames, 2000) and provide most of the energy used by the brain. However, mitochondria are not evenly distributed within neurons, and are most abundant at sites with a high-energy demand such as synapses and nodes of Ranvier (MacAskill and Kittler, 2010).

One fundamental requirement for efficient FAT is a constant supply of ATP. How is ATP distributed within neurons to fuel the molecular motors? It also remains to be understood how ATP can be continuously provided to fast-moving vesicles in axonal segments devoid of mitochondria. These questions are of particular importance in the context of several neurodegenerative disorders, such as amyotrophic lateral sclerosis, Alzheimer's, Parkinson's, and Huntington's diseases, which are characterized by mitochondrial dysfunction and axonal transport defects (Hirokawa et al., 2010; Salinas et al., 2008). Here we investigated the relative contributions of glycolysis and the mitochondrial respiratory chain in supplying ATP for FAT.

RESULTS

Mitochondria, GAPDH, and Axonal ATP

To reliably analyze vesicular FAT, we designed microfluidicbased devices derived from a previously described microfluidic culture platform (Taylor et al., 2005). Embryonic rat cortical neurons can extend long processes into the channels, but only axons can reach the distal chamber located 450 μ m away from the proximal chamber containing the cell bodies and most of the dendrites, as shown by Tau staining (Figure S1A available online). We electroporated cortical neurons with





Figure 1. Microfluidic Device to Analyze **Transport and ATP Content in Axons**

(A) Transport of BDNE-mCherry in rat cortical axons. The top picture is a time projection of BDNF that reveals the presence of several axons in the microchannel. Top middle corresponds to the generated kymograph. Analyzed trajectories are shown with a color code, green for anterograde, red for retrograde, and blue for static vesicles. Bottom illustrates the distribution of velocity from ten analyzed kymographs (anterograde n = 174, retrograde n = 154).

(B) Rat cortical neurons expressing Mito-DR and GAPDH-eGFP. Top is an overlaid picture taken in the cell body chamber compartment and middle shows the distal part of the channel with two axons (differential interference contrast [DIC] in gray, Mito-DR in red, and GAPDH-eGFP in green). Bottom is a two-color kymograph.

(C) Rat cortical neurons expressing Perceval, pH reporter SNARF 5 was added to the medium and chambers were analyzed with confocal microscopy. Upper (1) DIC, reveals axons in the channel. SNARF5 labeling reveals two axons that were analyzed for their intracellular pH (2) and normalized to the isobastic point (iso, 3). The upper axon in the chamber shows high expression of Perceval that was used to analyze ADP (4) and ATP (5). The ADP, ATP overlay is shown in (o, 6).

(D) Perceval signal reveals small fluctuations of the ATP:ADP ratio within axons whereas the pH ratio remained constant.

See also Figure S1.

5

SNARF5 ratic

120

and Sheetz, 2004; Tristan et al., 2011), mitochondria were abundant in cell bodies and discontinuously distributed in neurites, whereas GAPDH was diffused throughout the cytoplasm (Figure 1B). The same pattern of distribution was

fluorescent-tagged cargos and plated the neurons in microchambers. We performed FAT assays 3-5 days after the cells were plated, when axons had reached the distal chamber. We used brain-derived neurotrophic factor tagged with mCherry (BDNF-mCherry), which displays the characteristic features of a FAT-dependent cargo and retains BDNF function (Kwinter et al., 2009). To selectively analyze axonal transport, we recorded vesicular dynamics in the distal part of the microchannels and generated kymographs from the video recordings. Analyzes of BDNF vesicle dynamics revealed fast and highly processive anterograde and retrograde movements (Figure 1A; Movie S1) at velocities similar to those previously reported (Kwinter et al., 2009).

To analyze the distribution of mitochondria and of the key glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the microfluidic devices, we used a mitochondrial signal peptide fused to dsRed (Mito-DR) and GAPDH tagged with GFP (GADPH-GFP) and verified that the respective behaviors of these constructs in axons grown in microchannels were similar to that in cultured neurons. As previously reported (Miller

observed in axons extending within the microchannels (Figure 1B). Mitochondria exhibited characteristic dynamic behavior within the microchambers, and were either stationary, very dynamic, or changing directions (Movie S2) as illustrated by the two-color kymograph (Figure 1B). We also investigated the trafficking of BDNF-mCherry-containing vesicles and of mitochondria in the same axons and found no overlap between stationary or dynamic populations (Figure S1B). In contrast to mitochondria, GAPDH was diffuse throughout the cytosol of the chambered axons and its distribution did not appear to change over time (Figure 1B).

Although there are many reliable methods to measure ATP content within cells, the subcellular location of ATP is more difficult to resolve. To selectively determine changes in ATP content within axons, we took advantage of a genetically encoded fluorescent sensor that monitors ATP:ADP ratio in living cells (Berg et al., 2009). The ATP:ADP ratio was constant over long distances within the axons (Figures 1C and 1D). As the signal emitted by Perceval is pH-dependent, we measured possible variations in intracellular pH using the SNARF 5 probe and

Cell

observed no major fluctuations throughout the axons. This suggests that there is a constant availability in ATP within axons that is provided by both mitochondria and glycolysis.

GAPDH but Not Mitochondria Is Critical for FAT

We next determined whether mitochondrial H⁺-ATP-synthase inhibition by oligomycin, which blocks ATP production from the mitochondria (Chang and Reynolds, 2006), or whether GAPDH inhibition by iodoacetate affected FAT in cultured cortical neurons. We first measured the efficacy of oligomycin and iodoacetate in modulating ATP content within neurons. We added 1 mM pyruvate to the iodoacetate-treated neurons to bypass glycolysis and to allow mitochondria to continue to produce ATP. A 5 min treatment with oligomycin (20 μ M) induced a 25% reduction in ATP that reached to 80% after 30 min (Figure 2A). In contrast, a 30 min iodoacetate treatment that leads to a strong inhibition of GAPDH (Figure 2B) reduced ATP by only 40%. We next measured ATP:ADP ratio using Perceval as in Figure 1A. We observed a dynamic response in the Perceval signal that correlated with the ATP content as measured within neuronal extracts. Oligomycin treatment produced a progressive and substantial reduction in the Perceval signal, whereas iodoacetate treatment was not as effective (Figure 2C). These results indicate that most of the ATP in neurons originated from the mitochondria and that mitochondria inhibition rather than GAPDH inhibition significantly reduced the ATP level in neurons.

We next assessed BDNF vesicle dynamics in these conditions. After 30 min oligomycin treatment, when ATP is maximally reduced (Figure 2A), mean anterograde and retrograde vesicle velocity was unaffected and the percentage of dynamic vesicles remained constant (Figures 2D and S2A). We next treated neurons with iodoacetate and pyruvate. In contrast to oligomycin-mediated inhibition of mitochondrial ATP, GAPDH inhibition reduced FAT of BDNF vesicles after as little as 5 min of iodoacetate treatment as shown in the kymograph and by the reduction in the velocities and dynamics of BDNF (Figures 2D and S2A; Movie S1). In support for a specific effect of iodoacetate, we found a 50% inhibition of FAT when using its IC₅₀ concentration (Schmidt and Dringen, 2009) (Figure S2B).

Although oligomycin had no effect on vesicle transport, it rapidly blocked mitochondria trafficking (Figures 2E and S2C; Movie S2). On the other hand, iodoacetate had no effect on mitochondria dynamics even after 30 min of treatment. This indicates the lack of off-target effects of iodoacetate on molecular motors because the same motors are used for mitochondria and vesicles (Hirokawa et al., 2010). These results are in agreement with previous studies indicating that loss of mitochondrial ATP production, but not the disruption of mitochondrial membrane potential or mitochondria morphology can induce the loss of mitochondria dynamics (Kaasik et al., 2007; Rintoul et al., 2003). Together, these experiments suggest that GAPDH rather than mitochondria is critical for FAT.

We next took advantage of the identification of two mitochondria adaptor proteins, milton and Miro (Glater et al., 2006; Wang and Schwarz, 2009), to specifically deplete mitochondria from axons and redistribute them into the cell bodies. Indeed, milton knockout flies show no mitochondria in their axons but have a normal axonal distribution of vesicles. This suggested that transport of vesicles may not strictly depend on mitochondria. Previous work showed that milton-C expression in neurons redistributes mitochondria into the cell bodies but does not alter mitochondria function (Glater et al., 2006). We therefore expressed milton-C in cortical neurons and observed that mitochondria clustered within cell bodies so that axons were depleted of mitochondria (Figure 3A). By transfecting Perceval into neurons, we observed a progressive but marked reduction of ATP:ADP ratio within axons of neurons coexpressing milton-C whereas the ratio in the cell body remained high (Figure 3B).

We next coexpressed milton-C, GFP, and mito-DR in neurons and plated them in microchambers. Having verified that mitochondria were also absent from axons in microchannels as a result of milton-C expression (Figure 3C), we assessed BDNF vesicle dynamics in neurons expressing milton-C and BDNFmCherry. To make sure these axons are depleted from mitochondria, we added mitotracker to the medium and recorded vesicular transport in the distal part of the mitochondriadepleted axons. We found that the velocities of vesicles were largely unaffected (Figure 3D) compared to control neurons (Figure 1A). Our results indicate that BDNF vesicles can move efficiently in axons both devoid of mitochondria and with reduced ATP:ADP ratio.

Silencing GAPDH Impairs FAT of BDNF, APP, and TrkB but Not Mitochondria Trafficking

We plated on coverslips cortical neurons silenced for GAPDH with a siRNA that specifically targets rat GAPDH mRNA. GAPDH knockdown substantially reduced GAPDH protein levels as shown by immunoblot analyzes (Figure 4A). We observed a significant reduction in both anterograde and retrograde transport of BDNF vesicles in GAPDH-silenced neurons (Figures 4B and 4C). We also silenced GAPDH with another siRNA sequence in cortical neurons that were plated in microchambers (Figure S3A). As with the first siRNA (Figures 4A-4C), we observed a significant decrease in transport (Figures S3B and S3C). To verify the efficacy of the two siRNAs, we determined GAPDH activity and protein levels and observed a similar reduction (Figure S3D). Finally, to control for any possible off-target effects, we restored GADPH protein levels in siRNA-treated neurons by coexpressing a mouse GAPDH-GFP construct that was insensitive to the rat GAPDH siRNA (Figure 4A). We observed a complete recovery of normal BDNF vesicle dynamics in axons (Figures 4B and 4C).

Besides its role in glycolysis, GAPDH functions in other cellular processes such as DNA repair, membrane fusion, cytoskeletal dynamics, and cell death (Tristan et al., 2011). To demonstrate that the inhibitory effect of silencing GAPDH on FAT is linked to its glycolytic activity, we coexpressed a siRNA-insensitive and catalytically inactive form of GADPH (GAPDH-GFP-C149G) (Figure 4A). In contrast to GAPDH-GFP, the catalytically inactive construct was unable to restore the defective axonal transport induced by the siRNA (Figures 4B and 4C). These results indicate that catalytically active GAPDH is required for FAT and that the mechanism involves sustained glycolytic activity.

We next analyzed the consequences of GAPDH silencing on the transport of other cargos. As for iodoacetate treatment we found no effect on mitochondria dynamics (Figure 4D) further



Figure 2. Inhibition of GAPDH Reduces BDNF Transport but Inhibition of Mitochondrial ATP Synthesis Does Not

(A and B) Cortical rat neurons were treated with 20 µM oligomycin or 1 mM iodoacetate and 1 mM pyruvate for five and 30 min. Total ATP level (A) and GAPDH activity (B) were recorded and expressed as percentages of the values found in untreated cultures (control) (n = 8).

(C) Time course of Perceval ratio measured in neurons treated or not with oligomycin or iodoacetate.

(D) Neurons expressing BDNF-mCherry plated in microchambers were treated with oligomycin or iodoacetate and pyruvate as in (A). Representative kymograph of BDNF, mean + SEM for anterograde and retrograde velocities and for the percentage of dynamics vesicles in control, 30 min oligomycin- and, 5 min (legend continued on next page)



Figure 3. Milton-C Depletes Mitochondria from Axons and Induces an ATP:ADP Ratio Drop in Axons but Does Not Affect FAT

(A) Rat cortical neurons expressing Mito-DR, GFP, and empty control vector (pcDNA) or milton-C were analyzed by confocal microscopy. Mitochondria are present in the cell body, dendrites and axon of centrol neurons. In most milton-C expressing neurons, mitochondria are clustered in the cell body and absent from dendrites and axons. (B) Neurons expressing Perceval and, empty control vector (pcDNA) or milton-C. Pseudocolor reveals the ATP:ADP distribution along the axons. Quantification of the ATP:ADP signal along the axons was performed using a line-scan from the cell body through the axon. The graph corresponds to the mean drop of ATP:ADP ratio in axons when neurons express milton-C (n = 3 axons).

(C) DIC picture of milton-C, GFP, and Mito-DR expressing neurons plated in microchambers with the axon entering the channel. No mitochondria were found in the axon.

(D) Neurons expressing BDNF-mCherry and milton-C with mitotracker green to stain mitochondria. In contrast to the top axon, the bottom one (*) has no mitochondria and was recorded for BDNF trafficking. The kymograph (bottom) and the quantification of anterograde and retrograde velocities (mean + SEM, anterograde vesicles n = 28, retrograde vesicles n = 17) revealed typical FAT values.

indicating that mitochondria transport does not depend on GAPDH. We then investigated two other types of cargos: the amyloid-precursor protein APP fused to GFP (APP-GFP), a characteristic marker of FAT in neurons whose transport is kinesin-1-dependent (Kaether et al., 2000) and TrkB, the BDNF receptor fused to RFP (TrkB-RFP) that is trafficked bidirectionally within axons (Salinas et al., 2008). Whereas, APP and TrkB-containing vesicles motilities were not modified upon oligomycin treatment (Figure S3E), we observed a marked reduction in the transport of both cargos after GAPDH silencing (Figures 4E and 4F) suggesting that GAPDH could participate to the transport of many different types of vesicles.

In Vivo Silencing of GAPDH Reduces FAT in Drosophila Larvae

Because glycolysis may have a preferentially predominant role in providing ATP in cultured neurons, we determined the consequences of GAPDH depletion on in vivo axonal transport in *Drosophila melanogaster*. We exploited the transparency of the larvae to record axonal transport (Pilling et al., 2006). Larvae at L3 stage were placed between two coverslips and examined by videomicroscopy (Figures 4G and 4H). We used the UAS-GAL4 system to express synaptotagmin-GFP in the segmental nerves of *Drosophila* using the *Ok6-Gal4* driver. We crossed these flies with *UAS-shGAPDH* flies to selectively deplete GAPDH in the motoneurons of the offspring. As control, we crossed the *synaptotagmin-GFP* flies to *UAS-shZPG* flies (see Extended Experimental Procedures). We observed a strong reduction in the velocity of synaptotagmin-GFP vesicles transport in flies expressing shGAPDH compared to that in flies expressing the shZPG (Figure 4I; Movie S3). The use of another *UAS-shGAPDH Drosophila* (shGAPDH(2)) line gave similar results (Figure 4I). We also found that the dynamics of mitochondria were not altered in flies that are silenced for GAPDH. (Figure 4J). This further demonstrates in vivo our findings that transport of vesicles depends on GAPDH whereas transport of mitochondria does not.

Together, our results in cortical neurons and in *Drosophila* larvae demonstrate that (1) GAPDH is required for FAT in vitro and in vivo, (2) this mechanism might be general, and (3) this critical function has been evolutionarily conserved.

GAPDH Localizes on Vesicles

Although GAPDH is mainly cytosolic, it has also been reported to be in specific subcellular compartments including the cytoskeleton, membranes, and fusion vesicles (Tristan et al., 2011).

iodoacetate-treated neurons (anterograde velocity, control: n = 275; oligomycin: n = 46; iodoacetate: n = 5; retrograde velocity, control: n = 235; oligomycin: n = 40; iodoacetate: n = 10; percentages of dynamic vesicles were calculated from respectively 13 (control), 7 (oligomycin) and 16 (iodoacetate) kymographs). (E) Neurons expressing Mito-DR plated in microchambers were treated with oligomycin or iodoacetate and pyruvate as in (A). Representative kymographs of Mito-DR, mean + SEM for anterograde and retrograde velocities and for the percentage of dynamic mitochondria in control, 5 min oligomycin- and 30 min iodoacetate-treated neurons (anterograde velocity, control: n = 44; oligomycin: n = 7; iodoacetate: n = 46; retrograde velocity, control: n = 51; oligomycin: n = 51; oligomycin: n = 44; oligomycin: n = 7; iodoacetate: n = 46; retrograde velocity, control: n = 51; oligomycin: n = 4; iodoacetate: n = 50; percentages of dynamic vesicles were calculated from respectively 7 (control), 10 (oligomycin) and 14 (iodoacetate) kymographs). *p < 0.5, ***p < 0.001. Error bars represent SEM. See also Figure S2, and Movies S1 and S2.



Figure 4. GAPDH Silencing In Rat Neurons and In Vivo Reduces FAT of Vesicles but Not of Mitochondria

A) Immunoblot of rat cortical neurons extracts showing GAPDH gene-replacement with mouse GAPDH-GFP.

(B) Rat neurons were electroporated as in (A) to replace endogenous GAPDH expression, together with the BDNF-mCherry vector and plated on coverslips. Kymographs were generated.

(C) Quantification of mean velocities from kymographs. These experiments were not performed in microchambers. The values represent neuritic velocities (axons and dendrites) that are generally lower than pure axonal velocities (anterograde: scramble siRNA [sc] + pcDNA: n = 75; siGAPDH + pcDNA: n = 290; siGAPDH + GAPDH-GFP: n =184; siGAPDH + GAPDH-GFP-C149G: n = 136; retrograde: sc + pcDNA: n = 50; siGAPDH + pcDNA: n = 296; siGAPDH + GAPDH-GFP: n =136; siGAPDH + GAPDH-GFP-C149G: n = 123). (D) Mean velocity of Mito-DR-labeled mitochondria in axons with sc or siGAPDH (anterograde: sc: n = 67; siGAPDH: n = 129; retrograde: sc: n = 82; siGAPDH: n = 144).

(E) Mean velocity of APP-GFP vesicles in axons with sc or siGAPDH (anterograde: sc: n = 440; siGAPDH II: n = 338; retrograde: sc: n = 415; siGAPDH II: n = 329).

(F) Mean velocity of TrkB-RFP in axons with sc or siGAPDH (anterograde: sc: n = 328; siGAPDH II: n = 220; retrograde: sc: n = 212; siGAPDH II: n = 191).

(G) Drosophila L3 larvae expressing synaptotagmin-GFP and shZPG (control) or shGAPDH in all motor neurons were collected and processed for videomicroscopy as illustrated.

(H) Low and high magnifications of confocal microscopy images of a synaptotagmin-GFP L3 larva. The green channel corresponds to GFP (488 nm) and the red channel to larva autofluorescence at 561 nm.

(I) Characteristic kymographs of synaptotagmin-GFP are shown as well as the quantification of mean vesicles velocity (left: n = 30 for *shZPG* and n = 27 for *shGAPDH* [3] and right: n = 64 for *wt* and n = 68 for *shGAPDH* [2] flies).

(J) Quantification of Mito-GFP mean velocity in control (shZPG) and shGAPDH (3) larvae (n = 35 for *shZPG* and n = 35 for *shGAPDH* [3] flies).

 $^{***}p < 0.001.$ Error bars represent SEM. See also Figure S3 and Movie S3.

Therefore we analyzed whether GAPDH is located on brain vesicles. We performed subcellular fractionation of mouse brain and found GAPDH in the "small vesicles" fraction (P3) that also contained synaptophysin, BDNF and the p50 and p150 subunits of dynactin. We also detected dynein intermediate chain (DIC) and kinesin heavy chain (KHC) that are subunits of the corresponding molecular motors, dynein and kinesin-1 (Figures 5A and S4). We next performed immunofluorescence experiments for endogenous GAPDH on neurons that were electroporated with BDNF-mCherry. We first permeabilized cells with saponin before fixation to remove the abundant cytosolic pool of GAPDH and selectively detect vesicle-associated GAPDH. We found a strong colocalization of endogenous GAPDH on BDNF-containing vesicles along axons as shown by confocal microscopy and reconstruction along the z axis. Quantification of the colocalization revealed a strong overlap between BDNF vesicles and GAPDH (Figure 5B) with 77.2% \pm 7.6% of BDNF vesicles being positive for endogenous GAPDH (n = 102).

To further demonstrate that GAPDH is present on "motile vesicles" and has a functional role, we adapted a previously reported protocol (Perlson et al., 2009) to purify dynamic vesicles from the brains of Thy1-p50-GFP transcenic mice (M30 line) that express
Cell



Figure 5. GAPDH Localizes on Motile Vesicles

(A) Adult mouse brains were processed to obtain different subcellular fractions as illustrated in Figure S4 (cytcsolic [S3], vesicular [P3], and cytosol + vesicles fraction [S2]) that were subsequently analyzed by immunoblotting.

(B) Confocal images of cortical axons with BDNFmCherry vesicles and immunostained for endogenous GAPDH.

(C) Immunoblotting analyzes of the motile vesicles preparation (IP), as illustrated in Figure S4. VF, vesicles fraction; IP, immunopurified vesicles; FT, flow through.

(D) Purification and staining process of motile vesicles. Native motile vesicles were stained with DIO (green) and immunostained for endogenous GAPDH (top row). Control condition is without first antibody (bottom row).

(E) (a) Immunocryoelectron microscopy reveals the presence of GAPDH antigens on the vesicle (black dots). (b) Negative control without anti-GAPDH antibody. (c) Representation of a tomogram projection (10 slices) computed from a 3D reconstruction of a GAPDH immunogold-labeled vesicle. (d) Color representation of the 3D volume of (c). Blue pixels depict the vesicle, reddish pixels show iron bead used for vesicles purification and yellow pixels correspond to the IgG-

conjugated gold particle. Analysis of micrographies revealed an average number of gold particles associated to vesicles of 0.2 ± 0.2 for negative control and 1.1 ± 0.5 for immunolabeled sample (p < 0.05). See also Movie S4 and Figure S4.

the p50 dynamitin subunit of dynactin fused to GFP under the Thy1 neuronal promoter. These mice express p50-GFP at low concentration so that it does not disrupt the dynactin complex and thereby does not alter axonal transport (Ross et al., 2006). We first performed a subcellular fractionation of the brain tissue and obtained a cytoplasmic fraction containing small organelles. which we then subjected to sucrose gradient purification to enrich for vesicles (VF) (Figures 5C and S4). This fraction contained GAPDH, endogenous p50, the transgenic p50-GFP, and the p150 dynactin subunit. This VF fraction also contained soluble proteins as shown by the presence of significant levels of tubulin. We next used an anti-GFP antibody to selectively immunopurify vesicles containing p50-GFP, which therefore represented neuronal motile vesicles (IP lane, Figures 5C and S4). This immunomagnetic purification of neuronal vesicles containing the motor complexes was effective as the IP fraction was greatly enriched in p50-GFP protein and eliminated soluble proteins as shown by the near to complete absence of tubulin in the IP fraction. In addition, no proteins were detected by BCA assay, Coomassie-stained gels or immunoblots from immunomagnetic purifications of nontransgenic mouse brains (data not shown). This indicates the lack of contamination by soluble proteins. The IP fraction contained BDNF, SNAP25, a vesicular protein, and components of the molecular motors complex such as DIC, endogenous p50, the p150 dynactin subunit, and low levels of kinesin. This is expected as a significant fraction of kinesin is in the cytosol and kinesin is recruited to vesicles upon activation (Figure 5A) (Colin et al., 2008). This method therefore allowed us to highly purify vesicles that contain p50-GFP and the mclecular motors complex, as well as proteins associated to these motile vesicles. Importantly, GAPDH was present in the IP fraction (Figure 5C). As phosphoglycerate kinase (PGK) is downstream of GAPDH and generates ATP during glycolysis, we tested whether PGK was also present with GAPDH on the vesicles. Like GAPDH, we also found PGK in the small vesicles fraction (Figure 5A) and in the immunopurified motile vesicles fraction (Figure 5C). Dynabeads coupled to anti-GFP antibodies were used to purify native intact vesicles from the vesicle fraction (VF) that were then labeled with a lipophilic marker (Figures 5D and S4). These motile vesicles were immunopositive for GAPDH (Figure 5D) suggesting localization of GAPDH on the cytosolic face of the vesicles. To unequivocally demonstrate GAPDH localization, we performed immunocryoelectron tomography and found a significant number of gold particles on vesicles in presence of GAPDH antibodies compared to the situation without primary antibody (Figure 5E, a and b; Movie S4). 3D reconstruction of tilt series and 3D volume rendering demonstrated the localization of GAPDH on the cytosolic face of the vesicles (Figure 5E, c and d; Movie S4).

Motile Vesicles Show GAPDH Activity and Produce ATP

We next investigated the role of vesicular GAPDH. First, purified motile vesicles were capable of substantial GAPDH activity (Figure 6A). Next, we incubated the motile vesicles fraction (IP) with glyceraldehyde-3-phosphate, ADP, P_i , and NAD+ to measure ATP production (Figure 6B). We observed a rapid and significant production of ATP under these conditions showing that both



Figure 6. Motile Vesicles Produce ATP and Vesicular GAPDH Is Sufficient for FAT

(A) GAPDH activity of motile vesicles is inhibited by iodoacetate (n = 6).

(B) Motile vesicles fraction incubated with glycolytic substrates shows ATP production (n = 3).

(C) Cortical neurons expressing GAPDH-GFP were plated in the microchambers and fluorescence was analyzed by confocal microscopy. The axon was photobleached prior to video recording. Stars indicate fast-moving GAPDH-GFP-positive vesicles. The corresponding kymograph is shown. (D) Cortical neurons expressing GAPDH-GFP and BDNF-mCherry were analyzed as in (C). The kymograph shows cotransport of the two proteins in axons.

(E) Cartoon showing the chimeric Synaptotagmin1-TM-GAPDH-GFP protein.

(F) Axons of neurons expressing BDNF-mCherry together with TM-GAPDH-GFP were analyzed by confocal microscopy.

(G) Immunoblotting of HEK cells expressing various constructs of active, inactive and vesicular GAPDH and fractionated as in Figure 5A.

(H) Velocity of BDNF vesicles in neurons electroporated with sc or siGAPDH and with active or inactive TM-GAPDH-GFP and plated in microchambers. (anterograde: scramble siRNA (sc) + GFP: n = 10; siGAPDH II + GFP: n = 43; siGAPDH II + TM-GAPDH-GFP: n = 108; siGAPDH II + TM-GAPDH-C149G-GFP: n = 31; retrograde: scramble siRNA (sc) + GFP: n = 11; siGAPDH II + GFP: n = 51; siGAPDH II + TM-GAPDH-C149G-GFP: n = 70; siGAPDH II + TM-GAPDH-C149G-GFP: n = 30). *p < 0.5, **p < 0.01,***p < 0.001. Error bars represent SEM. See also Movies S5 and S6 and Figure S4.

(Movie S5; Figure 6C). To further demonstrate that GAPDH is present on fastmoving vesicles, we determined whether GAPDH-GFP and BDNF-mCherry were being cotransported on the same vesicles. As shown by the movie and kymograph analyzes (Movie S6; Figure 6D), most of the dynamic vesicles containing BDNF-mCherry were also positive for

GAPDH and the downstream PGK are enzymatically active. ATP production required full GAPDH catalytic function, as it was completely blocked by iodoacetate. These results show that motile vesicles contain functional glycolytic machinery and produce ATP in an autonomous manner.

Vesicular GAPDH Is Transported within Axons

We next expressed GAPDH-GFP in cortical neurons and used videomicroscopy to analyze axons for the presence of GAPDH-GFP-labeled dynamic vesicles. As GAPDH is mainly cytosolic, we first bleached the axon within the microchannel to extinguish existing fluorescence. We observed fast-moving GAPDH-GFP-positive vesicles with high processive movements

GAPDH-GFP. We could also detect dynamic GAPDH-GFP-positive vesicles that were negative for BDNF-mCherry supporting a wider role of GAPDH in the transport of other vesicles such as synaptotagmin, APP, and TrkB-containing vesicles (Figure 4). The findings that GAPDH is present on motile vesicles that are actively transported in axons suggest that vesicular GAPDH could provide energy support for axonal transport.

Vesicular GAPDH Is Sufficient to Promote FAT of Vesicles

To unequivocally address whether vesicular GAPDH is sufficient for FAT we silenced endogenous GAPDH and addressed GAPDH to vesicles through its fusion to the transmembrane domain of synaptotagmin (Figure 6E). The fusion protein called TM-GAPDH-GFP colocalized with BDNF-mCherry vesicles in axons (Figure 6F). Quantification of the colocalization revealed a strong overlap between BDNF vesicles and GAPDH with 72.2% \pm 2.4% of BDNF vesicles found positive for TM-GAPDH (n = 326). TM-GAPDH-GFP was also detected in the vesicular fraction after subcellular fractionation (Figure 6G). However, in contrast to wild-type GAPDH and GAPDH-GFP, TM-GAPDH was not detected in the cytosol (S3 fraction, Figure 6G). Reexpressing TM-GAPDH in neurons silenced for endogenous GAPDH was found sufficient to restore axonal transport. In contrast, a catalytically inactive vesicular GAPDH (C149G) was unable to restore axonal transport in these conditions (Figure 6H). These results indicate that a strictly vesicular GAPDH is sufficient to propel vesicles in axons.

Huntingtin Scaffolds GAPDH on Vesicles

To investigate the requirement of GAPDH on vesicles for FAT, we searched for the mechanism(s) that maintain GAPDH on vesicles with the goal of blocking GAPDH attachment to vesicles and analyzing axonal transport. Identifying such a mechanism would add further evidence for the specificity and the physiological relevance of GAPDH localization on vesicles.

GAPDH interacts with huntingtin (HTT), the protein that when mutated causes Huntington's disease (Bae et al., 2006; Burke et al., 1996; Culver et al., 2012; Goehler et al., 2004; Kaltenbach et al., 2007; Ratovitski et al., 2012). HTT localizes on vesicles and is an essential scaffold protein regulating axonal transport of vesicles including BDNF (Caviston and Holzbaur, 2009; Colin et al., 2008; Gauthier et al., 2004). We detected HTT in the motile vesicle fraction with GAPDH (Figure 5C). We immunostained neurons electroporated with BDNF-eGFP for endogenous HTT and GAPDH and selectively assessed the pool of vesicular associated proteins by saponin treatment. Both proteins colocalized within axons on BDNF vesicles (Figure 7A). To investigate whether HTT could scaffold GAPDH on vesicles, we silenced HTT using previously characterized siRNA that show no offtarget effects (Colin et al., 2008). We observed a loss of GAPDH localization on BDNF vesicles (Figure 7B). We confirmed these findings in vivo by crossing Htt^{flox/flox} mice (Dragatsis et al., 2000) with the inducible CamKII-CreER^{T2} line that express the CRE recombinase in the cortex and hippocampus upon tamoxifen treatment (see Extended Experimental Procedures) and performed subcellular fractionation from their cortices (Figure 7C). This successfully reduced HTT in all fractions. However, the GAPDH levels were only decreased in the vesicular fraction (P3) demonstrating that depletion of HTT reduces GAPDH attachment to vesicles. These results agree with the observation that HTT is a phenocopy of GAPDH because the silencing of either protein decreases the transport of BDNF, APP, and TrkB (Colin et al., 2008; Gauthier et al., 2004; G.L., unpublished data) (Figure 4F). Our findings also provide further evidence for the specific localization of GAPDH on vesicles.

Vesicular GAPDH Is Necessary to Promote FAT of Vesicles

Our finding that HTT depletion leads to the detachment of GAPDH from vesicles but maintains GAPDH pool in the cytosol

allowed us to address whether vesicular GAPDH is necessary to propel vesicles within axons. Therefore, we selectively decreased vesicular GAPDH by depleting HTT in cortical neurons and analyzed FAT (Figures 7D and 7E). We used two siRNA targeting different sequences of HTT and found that both RNAi decreased FAT when compared to scramble siRNA. The observed decrease in transport was similar to that of GAPDH-silenced neurons (Figures 4C and 6H). We re-expressed TM-GAPDH in HTT-silenced neurons and found that for both siHTT used, TM-GAPDH rescued axonal transport in both directions. Interestingly, forcing the vesicular localization of GAPDH on vesicles further increased the anterograde velocity of the vesicles compared to the control condition (GFP) or the C149G inactive point mutant (Figure 7F). This suggests that increasing the stoichiometry of GAPDH and consequently the local energy supply on vesicles further enhances the efficacy of transport. Together, we conclude that vesicular but not cytosolic GAPDH is required to propel vesicles at high velocities in axons.

DISCUSSION

We show that glycolytic enzymes located on vesicles rather than mitochondria are critical to maintain the high vesicular velocities that are characteristic of FAT. Perturbing glycolysis decreases FAT but does not totally block transport. Conversely, we found that FAT does not depend on the ATP generated by the mitochondria. However, our findings do not exclude the possibility that mitochondria could insure a basic transport within cells.

GAPDH is a very abundant protein present both in the cytoplasm and the nucleus and is also reported to interact with microtubules and membranes (Tristan et al., 2011). Could GAPDH present on MTs contribute to generate ATP for FAT of vesicles? The exact function of GAPDH on MTs is not fully understood, but GAPDH was shown to induce MT bundling (Huitorel and Pantaloni, 1985). However, binding of the enzymatically active tetrameric form of GAPDH to MTs leads to its dissociation into a monomeric and inactive enzyme (Durrieu et al., 1987) suggesting a low glycolytic activity of GAPDH when bound to MTs. Another function for GAPDH on MTs could be linked to its vesicle fusion activity. Indeed, evidence suggests a role for GAPDH in the fusion of vesicles to membranes (Glaser and Gross, 1995; Tisdale, 2002) but this GAPDH-dependent membrane fusion is independent from its glycolytic activity (Tisdale et al., 2004). Although we cannot exclude the possibility that MT-associated GAPDH could contribute to FAT, our findings that glycolytic enzymes are present on motile vesicles and produce ATP and, that a strictly vesicular GAPDH is necessary and sufficient to propel vesicles at high velocities in GAPDH-silenced neurons suggest that molecular motors could use preferentially this local pool of ATP generated by the vesicle itself.

Because GAPDH localizes on fast moving vesicles within axons, what are the possible mechanisms linking GAPDH to vesicular membranes? We have found that HTT, the protein that when mutated causes Huntington's disease, scaffolds GAPDH on vesicles. Our findings are in agreement with the



Figure 7. Vesicular GAPDH Is Required for FAT

(A) Immunostaining of endogenous HTT and endogenous GAPDH of cortical neurons electroporated with BDNF-mChery and scramble siRNA were analyzed by confocal microscopy. The arrows show the axon that was resliced along the z axis (on the right).

(B) Neurons were silenced for HTT (siHTT) and processed as in (A).

(C) Immunoblotting of adult cortices from *Htt^{flox/flox}* and CamKCreER^{T2};*Htt^{flox/flox}* mice previously fractionated as in Figure 5A. The quantification corresponds to five animals per genotype.

(D) Cortical neurons electroporated with sc or two different siHTT and GFP or TM-GAPDH-GFP were analyzed by immunoblotting for endogenous HTT, GAPDH, and tubulin.

(E) Velocity of BDNF vesicles in neurons plated in microchambers electroporated as in (D). (anterograde: scramble siRNA(sc) + GFP: n = 35; siHTT I + GFP: n = 23; siHTT I + TM-GAPDH-GFP: n = 46; siHTT II + GFP: n = 61; siHTT II + TM-GAPDH-GFP: n = 91; retrograde: scramble siRNA (sc) + GFP: n = 39; siHTT I + GFP: n = 16; siHTT I + TM-GAPDH-GFP: n = 91; neuronal scramble siRNA (sc) + GFP: n = 39; siHTT I + GFP: n = 61; siHTT II + TM-GAPDH-GFP: n = 91; neuronal scramble siRNA (sc) + GFP: n = 39; siHTT I + GFP: n = 61; siHTT II + TM-GAPDH-GFP: n = 91; neuronal scramble siRNA (sc) + GFP: n = 39; siHTT I + GFP: n = 61; siHTT II + TM-GAPDH-GFP: n = 91; neuronal scramble siRNA (sc) + GFP: n = 39; siHTT I + GFP: n = 61; siHTT II + TM-GAPDH-GFP: n = 91; neuronal scramble siRNA (sc) + GFP: n = 39; siHTT I + GFP: n = 61; siHTT II + TM-GAPDH-GFP: n = 91).

(F) Velocity of BDNF vesicles in neurons plated in microchambers electroporated with GFP, active or inactive TM-GAPDH-GFP. (anterograde: GFP: n = 169; TM-GAPDH-GFP: n = 280; TM-GAPDH-C149G-GFP: n = 260; retrograde: GFP: n = 117; TM-GAPDH-GFP: n = 187; TM-GAPDH-C149G-GFP: n = 287). *p < 0.5, ***p < 0.001, Error bars represent SEM.

many studies that reported an interaction between HTT and GAPDH (Bae et al., 2006; Burke et al., 1996; Culver et al., 2012; Goehler et al., 2004; Kaltenbach et al., 2007; Ratovitski et al., 2012; Shirasaki et al., 2012). We also found PGK on the motile fraction. Whether HTT also scaffolds other key enzymes of the glycolytic pathway such as PGK remains to be determined although this may be a possibility given the identification of such enzymes by proteomic or yeast two-hybrid approaches (Culver et al., 2012; Kaltenbach et al., 2007; Shirasaki et al., 2012) Also, it will be of interest to determine whether such scaffolding function is altered in Huntington's disease. Such studies may shed light on the mechanisms by which HTT regulates axonal transport in Huntington's disease and reconcile the defects in axonal transport and the observed defects in energetic homeostasis.

Although we show a crucial role for HTT in GAPDH attachment to vesicles, it is still possible that other mechanisms coexist. Indeed, the Rab2 GTPase that is required for protein transport within the early secretory pathway binds to and recruits GAPDH to vesicular membranes (Tisdale et al., 2004). Similarly to the transport from the endoplasmic reticulum to Golgi, specific Rabs that remain to be identified could recruit GAPDH onto fast moving vesicles in axons. Another mechanism for the vesicular recruitment of GAPDH could involve posttranslational modifications of GAPDH, such as palmitoylation at cysteine 244 that was shown to increase recruitment of GAPDH to vesicles (Yang et al., 2005).

The local vesicular ATP production by the glycolytic enzymes and its consumption by the molecular motors present on the same vesicles may provide rapid feedback between ATP production and changes in consumption, as has been shown in erythrocytes (Ames, 2000). This concept of vesicular energetic coupling is also supported by a proteomic study that identified GAPDH and PGK on synaptic vesicles (Burré and Volknandt, 2007) and by the observation that glycolytic machinery activates the vesicular proton pump required for vesicle glutamate reuptake at the synapse (Ikemoto et al., 2003). In addition, our results show that contrary to vesicular transport, mitochondrial traffic does not rely on glycolysis, but depends on mitochondrial ATP. Although similar to vesicular motors, the motors that transport mitochondria preferentially use ATP produced by the mitochondria rather than ATP generated by nonmitochondrial sources, such as the cytoplasmic glycolytic machinery. How the same motors-kinesin-1 is involved in the transport of both mitochondria and vesicles (Hirokawa et al., 2010)-discriminate between these different sources of ATP is likely to depend on the proximity of the energetic source and ensures the full efficacy of the cellular system.

In neurons, mitochondria are scattered in axons but enriched at subcellular locations that require high energy levels, such as synapses and nodes of Ranvier. Thus, along an axon, mitochondria can thereby be spared and may even be absent from large regions. Our findings that glycolytic machinery is present on fast-moving vesicles in axons indicate that neurons have constructed an autonomous transport system that is independent of the fluctuating energy levels that could occur in some regions of the axon.

EXPERIMENTAL PROCEDURES

Mice

All experimental procedures were performed in strict accordance with the recommendations of the European Community (86/609/EEC) and the French National Committee (87/848) for care and use of laboratory animals.

Flies

Recombinant *ok6-Gal4:UAS-Syt-GFP* (X) flies and *CCAP-G4/CyOA-G;UAS-mitoGFP/TM3SAG* flies are gifts from M.-L. Parmentier and T. Schwarz, respectively. *UAS-shGAPDH* and *UAS-shZPG* strains were provided by Vienna *Drosophila* RNAi Center (VDRC). Generation of crosses and in vivo transport assay in fly larvae are detailed in Extended Experimental Procedures.

Microchambers and Neurons Plating

Microchambers were made according to Taylor et al. (2005). For videomicroscopy or immunostaining, we first electroporated cortical neurons with fluorescent-tagged cargos and plated the neurons in microchambers. Microchambers fabrication and electroporation are detailed in Extended Experimental Procedures.

Videomicroscopy and Video Analyses

Live videomicroscopy of neuronal cultures or *Drosophila* larvae were carried out using an imaging system previously detailed (Gauthier et al., 2004). Videomicroscopy and video analyses are detailed in Extended Experimental Procedures.

Immunoisolation of Vesicles and Immunocryoelectron Tomography

We selectively purified vesicles that contain p50-GFP from Thy:p50-GFP mouse brains (Ross et al., 2006) through successive subcellular fractionation, sucrose gradient fractionation, and immunoisolation. Vesicles were either immunostained in native conditions and analyzed by confocal microscopy or processed for immunocryoelectron tomography (Extended Experimental Procedures).

Perceval Assay, GAPDH Activity, and ATP Production

Neurons expressing Perceval were aralyzed by Leica SP5 laser scanning confocal microscope. Perceval signal was quantified as the ratio of the ATP/ADP channels and controlled for pH variations (Extended Experimental Procedures). GAPDH activity was measured using the KDalert GAPDH Assay (Invitrogen). ATP production was measured using the CellTiter-Glo Luminescent Viability Assay (Promega) as detailed in Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, four figures, and six movies and can be found with this article online at http://dx. doi.org/10.1016/j.cell.2012.12.029.

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Supplemental Information

EXTENDED EXPERIMENTAL PROCEDURES

Statistical Analyses

Statview 4.5 software (SAS Institute Inc.) or InfoStat software (InfoStat Group, Cordoba National University) were used for statistical analysis. Groups were compared by ANOVA followed by Fisher's PLSD post hocs analyses. The criterion for statistical significance was set at p < 0.05.

Mice

Wild-type mice are from the C57Bl6/J strain (Charles River Laboratories, France). Thy1:p50-GFP (Ross et al., 2006), $Htt^{flox/flox}$ (Dragatsis et al., 2000) and CamKII-CreER^{T2} (Erdmann et al., 2007) were previously described and are on the C57Bl6/J background. Animals were maintained with access to food and water *ad libitum* in a colony room kept at a constant temperature (19°C–22°C) and humidity (40%–50%) on a 12:12 hr light/dark cycle. All experimental procedures were performed in strict accordance with the recommendations of the European Community (86/609/EEC) and the French National Committee (87/848) for care and use of laboratory animals. Mice depleted for HTT in the forebrain were obtained by crossing $Htt^{flox/flox}$ mice with the inducible CamKII-CreER^{T2} line that expresses the CRE recombinase in the cortex and hippocampus upon tamoxifen treatment. Tamoxifen preparation and injection were performed as described in (Erdmann et al., 2007). Briefly, 3- to 6-month-old mice were injected intraperitoneally with 1 mg tamoxifen twice a day for 5 consecutive days. Mice were sacrificed 10 days after the end of tamoxifen treatment.

Reagents and Antibodies

ATP, ADP, NADH, GAP, iodoacetate and oligomycin are from Sigma-Aldrich. Mitotracker Green is from Invitrogen. Neurons were electroporated with Amaxa rat neuron nucleofactor (Lonza). The following antibodies and dilutions were used for immunoblotting: mouse anti-GAPDH (MAB374 Chemicon, 1:1,000); rabbit anti-GAPDH (G9545 Sigma, 1:1,000); mouse anti-HTT (4C8, Euromedex, 1:1,000); mouse anti-PGK-1 (Abcam ab90787, 1:1,000); mouse anti-p150^{Glued} (BD Transduction Laboratories 610474, 1:1,000); rabbit anti-p50 dynamitin (Millipore AB5869P, 1:250); mouse anti-DIC (Millipore ab1618, 1:500); mouse anti-KHC (Chemicon MAB1614, 1:500), rabbit anti-BDNF (Chemicon AB1779SP, 1:1,000); mouse anti-Synaptophysin (Sigma S5768, 1:1,000); mouse anti-SNAP25 (Abcam ab24737, 1:1,000); mouse anti-GFP (Zymed laboratories Inc, 1:500) and mouse anti-α-tubulin (Sigma T6199, 1:1,000).

Constructs and siRNAs

The following expressing plasmids were previously published: BDNF-mCherry displays the characteristic features of a FAT-dependent cargo and retains BDNF function (Kwinter et al., 2009), GAPDH-GFP (Dastoor and Dreyer, 2001), Perceval (Berg et al., 2009), milton-C (Glater et al., 2006), APP-GFP (Kaether et al., 2000), pDsRed2-Mito is from Clontech, TrkB-RFP is a gift from M Chao and H. Yano. GAPDH-GFP-C149G was generated by PCR using the following oligonucleotides TGT-CCG-TCG-TGG-ATC-TGA-CGG-GCC-GCC-TGG-AGA-AAC-CTG-CC and GGC-AGG-TTT-CTC-CAG-GCG-CGT-CAG-ATC-CAC-GAC-GGA-CA. To obtain TM-GAPDH we generated a synthetic construct encoding the first 100 nucleotides of mouse synaptotagmin 1 (P46096) fused to a TEV site linker and the first 45 nucleotides of the mouse GAPDH (until the BstEll site) (GenScript). Chimeric GAPDH was obtained by insertion of the synthetic fragment at the Spel and BstEll sites of the GAPDH-GFP expression construct.

Rat GAPDH was silenced with annealed siRNA I GCG-AGA-UCC-CGC-UAA-CAU-CAA-TT and UUG-AUG-UUA-GCG-GGA-UCU-CGC-TT (Eurogentec) or siRNA II GGU-CGG-UGU-GAA-CGG-AUU-UTT and AAA-UCC-GUU-CAC-ACC-GAC-CTT (QIAGEN). Rat huntingtin was silenced with annealed siRNA I CAG-GGU-UUC-UAA-AAC-UAC-ATT and UGU-AGU-UUU-AGA-AAC-CCU-GTT (Eurogentec) or siRNA II UUA-CUG-UCU-ACU-GGA-UUC-ATT and UGA-AUC-CAG-UAG-ACA-GUA-ATT (Eurogentec).

Flies

CCAP-GAL4 and UAS-mito-GFP strains were previously described (Park et al., 2003; Pilling et al., 2006). Flies were maintained at 25°C for the experiments. For synaptotagmin-GFP transport assay, virgin *ok6-Gal4:UAS-Syt-GFP* were crossed with *UAS-shZPG* (3), *UAS-shGAPDH* (2) or *UAS-shGAPDH* (3) males. The *Ok6-Gal4:UAS-Syt-GFP* flies and the *Ok6-Gal4:UAS-Syt-GFP* flies; *UAS-shZPG* flies are used as controls. *Zero Population Growth* gene (ZPG) is a protein expressed only in the germline (Tazuke et al., 2002). For Mito-GFP transport assay, virgin *UAS-shZPG* (3) or *UAS-shGAPDH* (3) were crossed with *CCAP-GAL4/CyOA-G;UAS-mitoGFP/TM3SAG* males. Offspring L3 larvae were collected for videomicroscopy. 3-5 larvae from the same genotype were laid on an 18 mm round coverslip. A second coverslip was added to form a sandwich and the system was mounted in a modified home-made Ludin chamber with a gentle compression of the larvae. Larvae were then observed by fast-videomicroscopy.

Microchambers and Neurons Plating

Master was made with SU8 resin on silicon wafer using photolithography. Fabrication was done in two steps: a first layer for the microchannels (3 µm high) being crossed by neuronal processes, a second layer for the chamber (50 µm high) where cell bodies are seeded. Replicates of the master were made with epoxy resin. A mixture of silicone elastomer (PDMS, Sylgard 184, Dow corning) and its curing agent was poured on the masters. Air bubbles were removed in a dessicator under vacuum during 1 hr and polymerization was performed by incubating 3 hr at 60°C. PDMS microchambers and Iwaki boxes with a glass bottom were placed into a plasma cleaner for surface activation during 30 s. After a rapid passage at 60°C, PDMS pieces and Iwaki boxes were brought

together to form an irreversible tight seal. The microfluidic devices were coated with a mix of poly-D-lysin (0.5 mg/ml) + laminin (10 mg/ml) and incubated overnight at 4°C then washed three times with Neurobasal medium. One million neurons were plated in the upper chambers in neuronal media. Embryonic rat cortical neurons extend long processes into the channels but only axons can reach the distal chamber located 450 μ m away from the proximal one that contains the cell bodies and the dendrites (Taylor et al., 2005).

For videomicroscopy or immunostaining, we first electroporated cortical neurons with fluorescent-tagged cargos and plated the neurons in microchambers. Neurons were analyzed after 3 to 6 DIV. Electroporation had no effect on neurite growth.

Videomicroscopy and Videoanalyses

Live videomicroscopy of neuronal cultures or *Drosophila* larvae were carried out using an imaging system previously detailed (Gauthier et al., 2004). The microscope and the chamber were kept at 37°C. Images were recorded every 200 ms with a 100 X PlanApo N.A. 1.4 oil immersion objective. Images were collected in stream mode using a Micromax camera (Roper Scientific) set at 2 X 2 binning with an exposure time of 100 to 150 ms. Projections, animations and analyses were generated using ImageJ software (http://rsb.info. nih.gov/ij/, NIH, USA). Maximal projection was performed to identify the vesicles paths, which in our system corresponds to vesicle movements in axons. Kymographs and analyses were generated with the KymoToolBox, a home-made plug-in. This plug-in allows the generation of multiple kymographs that are calibrated in space (*x* axis in µm) and time (*y* axis in s). Kymographs were then analyzed manually by drawing the vesicles trajectories. The plug-in extracts the dynamic parameters directly from the trajectories and also reports the tracked particles on the original video with a color directionality code (red for anterograde, green for retrograde and blue for stationary dynamics). The *n* values reported in the figures represent the number of segments measured within multiple kymographs. KymoToolBox is available upon request. To visualize GAPDH-GFP-dynamics vesicles within axons, we expressed GAPDH-GFP in cortical neurons and bleached the axon within the microchannel to extinguish existing fluorescence due to cytosolic GAPDH.

Mouse Brain Subcellular Fractionation and Vesicles Purification

A cartoon showing the various steps of purification of the vesicles is shown in Figure S4. Thy1:p50-GFP adult mice (Ross et al., 2006) (3–6 months) or $Htt^{flox/flox}$; CamKII-CreER^{T2} tamoxifen-treated mice (3–6 months) were decapitated, and the cortices were rapidly removed and homogenized in preparation buffer (320 mM Sucrose, 4 mM HEPES, pH 7.4). The homogenate was centrifuged for 10 min at 1,000 g. The supernatant was collected, and the pellet was resuspended and homogenized in preparation buffer and recentrifuged for 10 min at 1,000 g. Both supernatants were pooled (S1). The S1 was recentrifuged for 40 min at 12,000 g and the supernatant was collected (S2). Then, the S2 was recentrifuged for 90 min at 57,000 rpm (Beckman TL-100). The supernatant was collected (S3); each pellet was resuspended and well homogenized by sonication in preparation buffer containing 1% NP40, corresponding to P1, P2, and P3 respectively.

In the case of extracts from Thy1:p50-GFP mouse brains, discontinuous sucrose gradients were prepared by layering 1 ml supernatant (S2) onto 6 layers of 11 ml HEPES / sucrose solution (ranging from 1.10 to 0.32 M sucrose, pH 7.4). After centrifugation for 90 min at 170,000 g (Beckman Coulter, Optima L-80XP ultracentrifugation), 24 fractions were collected from bottom to top of the gradient. The vesicles are contained in the 19–20 fractions (VF) corresponding to sucrose concentration of 0.4–0.5 M. The VF fraction therefore contains a pool of vesicles. A fraction of these vesicles possess the molecular motors complex with some of the dynactin subunit p50 being the p50-GFP transgene.

Immunoisolation of Vesicles

We selectively purified from Thy:p50-GFP mouse brains vesicles that contain p50-GFP and therefore have the molecular motor complex. The vesicles fraction (VF) was incubated with μ MACS anti-GFP magnetic iron microbeads (MACS molecular) for 30 min at 4°C. A microcolumn was placed in the magnetic field of the μ MACS separator, prepared by applying the lysis buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris HCl, pH 8.0) and washed three times with preparation buffer. The incubated solution was applied onto the column. The flow-through was collected (FT) and the column washed three times. The vesicular proteins were eluted (IP) by removing the column out of the magnetic field and by addition of 150 μ l preparation buffer. The same volume of each fraction was loaded on acrylamide gels for western blot analyses. As shown on Figure 5C, the IP fraction is enriched in p50-GFP. Vesicles containing the molecular motor complexes without p50-GFP are not retained in the column. This step ensures that the IP fraction is not contaminated by soluble proteins such as tubulin (Figure 5C).

Immunofluorescence of Vesicles in Native Conditions

The vesicles fraction from sucrose gradient was incubated with Dynabeads M-280 sheep anti-Mouse IgG (Invitrogen) coupled to anti GFP antibody. We purified and immunolabeled the vesicles with anti rabbit GAPDH antibody and secondary Alexa A555 anti-rabbit antibody. Vesicles were labeled with the lipophilic tracer DIO (Invitrogen) and then analyzed by confocal microscopy.

Immunofluorescence of Cortical Neurons

Cortical neurons were washed for 5 min in PBS with 0.01% of saponin and fixed with paraformaldehyde 4% in PBS at 37°C for 20 min, treated with 1% NGS and 0.1% Triton X-100 for 1 hr. Cultures were stained with rabbit anti-GAPDH (G9545 Sigma, 1:200) and mouse anti-HTT (4C8, Euromedex, 1:200) for 1 hr followed by secondary antibody (Invitrogen Alexa 488 and Alexa 647, 1:500).

Electron Microscopy, Immunolabeling, and Electron Tomography

The vesicles fraction (VF) was incubated with μ MACS anti-GFP magnetic iron microbeads (MACS molecular) for 30 min at 4°C. Vesicles bound to microbeads were precipitated by centrifugation (TLS55) at 40,000 rpm for 10 min. After centrifugation the sample was resuspended in 10 μ I HEPES 4 mM buffer and put on glow discharged lacey carbon coated grids for plunge freezing in liquid ethane on a Leica EM CPC (Leica).

For immunolabeling, samples were incubated at 4°C with rabbit IgG anti-GAPDH (1:200), washed in HEPES 4 mM buffer and incubated with goat IgG anti-rabbit conjugated to 5 nm gold (1:100). After each step, the sample was recovered on a μMACS column.

Frozen aqueous suspensions (Dubochet et al., 1982) of vitrified immunolabeled vesicles were observed in a JEOL JEM-2200FS (JEOL Europe) at 200kV with an omega filter operated with a Z-loss centered 10eV window. Images were recorded on a $2k \times 2k$ Gatan CCD Camera (Gatan) with Digital Micrograph software (Gatan). Tomographic tilt series were acquired each 2 degrees from -60 to +60 degrees with TEMography software (JEOL), reconstructed with IMOD (Mastronarde, 1997) using WBP algorithm and processed in ImageJ (Abramoff et al., 2004). 3D rendering and movie were made with Amira (Visage Imaging). The number of gold particles observed on micrographies from the negative control (secondary antibody only; n = 18) and the immunolabelled sample (first and secondary antibodies; n = 25) were counted, as a total number and as particles associated to vesicles (gold beads observed within a distance of no more than 10 nm of the vesicle outer border). Considering alpha = 0.05, the values obtained for the number of gold particles dispersed in an area of 6.5×105 nm² were 4.5 ± 1.0 for negative control and 3.0 ± 0.8 for immunolabelled sample indicating that in both cases background is equivalent. However, when the average number of gold particles associated to vesicle associated to v

HEK Subcellular Fractionation

HEK cells were grown in 10 cm Petri dishes and transfected by calcium phosphate method. 36 hr later cells were submitted to subcellular fractionation as described above. Fractions were analyzed by western blot.

Western Blot

Extract proteins were electrophoresed under reducing conditions on 6%, 8%, 10%, or 15% polyacrylamide gels. Proteins were transferred to a nitrocellulose membrane (Bio-Rad). The immunodetection buffer used was Tris-buffered saline (10 mM Tris HCl, NaCl 200 mM [pH 7.4], and 0.1% Tween 20). Nonspecific binding was saturated by incubation with 5% nonfat dried milk for 1h. Primary antibodies were diluted with 5% milk. Membranes were washed and incubated for 1 hr with HRP conjugated secondary antibodies (Jackson: 1/10,000). Immunoreactive proteins were detected according to the enhanced chemiluminescence protocol (Pierce Biotechnology).

Perceval Assay

Neurons expressing Perceval were analyzed by Leica SP5 laser scanning confocal microscope equipped with a x63 oil-immersion objective. Thirty minutes before images acquisition 1:1,000 of pH reporter SNARF 5 was added to the medium. Images were acquired in sequence mode. For SNARF 5, excitation was set at 546 nm and detection of PMT was set at 575–595 nm (pH sensitive) and 610–630 nm (pH insensitive). For Perceval signal, excitation was set at 405 nm (ADP sensitive) and 496 nm (ATP sensitive) and detection of PMT was set at 520–560 nm. Analyses were done with ImageJ; pH and Perceval was quantified by the ratio of the two channels.

GAPDH Activity and ATP Production

To measure activity in vesicles, 1 μ g of purified "motile vesicles" (IP) were incubated with 100 μ l of KDalert GAPDH Assay. GAPDH activity was measured following the instructions of the supplier. For ATP production, 1 μ g of purified "motile vesicles" (IP) were incubated with 2 mM GAP, 2 mM Pi, 2 mM NAD, and 0.2 mM ADP in a volume of 100 μ l at room temperature for one min. Reaction was stopped by adding 100 μ l of CellTiter-Glo Luminescent Viability Assay (Promega) and luminescence was monitored. The same assays were used to measure GAPDH activity and ATP content within cortical neurons plated in 96 wells plates and treated with oligomycin or with iodoacetate and pyruvate for five and 30 min.

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Figure S1. Microchannels Contain Axons with Distinct Fast-Moving BDNF Vesicles and Dynamic Mitochondria, Related to Figure 1 (A) Confocal images of axons at the distal part of the 450 µm channels (top, DIC) immunostained with the axonal marker Tau-1 (middle) and overlay (bottom). (B) Two-color kymograph of BDNF-mCherry vesicles (red) and mitochondria (green).



Figure S2. Time Course of GAPDH and Mitochondrial ATP Inhibition on BDNF Vesicles and Mitochondria Trafficking, Related to Figure 2 (A) Representative kymographs of BDNF-mCherry axonal trafficking in control, 5 and 30 min oligomycin 20 μ m and iodoacetate 1 mM treated neurons. (B) Time course of anterograde and retrograde BDNF-mCherry velocity of neurons treated with 100 μ M iodoacete (for anterograde velocity: 0 min n = 68; 5 min n = 39; 10 min n = 42; 20 min n = 67; 30 min n = 36; for retrograde velocity: 0 min n = 51; 5 min n = 28; 10 min n = 31; 20 min n = 64; 30 min n = 12). (C) Representative kymographs of Mito-DR axonal trafficking in control, 5 and 30 min oligomycin 20 μ m and iodoacetate 1 mM treated neurons. **p < 0.01; ***p < 0.001, error bars, SEM.



Figure S3. GAPDH Silencing Reduces GAPDH Levels and Enzymatic Activity and Decreases FAT, Related to Figure 4

(A) Immunoblotting analysis of rat cortical neurons extracts showing GAPDH silencing with siGAPDH II.

(B) Quantification of mean velocities from kymographs of control neurons and GAFDH silenced neurons with siGAPDH II. (for anterograde velocity: scramble siRNA (sc) n = 474 ; siGAPDH II: n = 563; for retrograde velocity: sc: n = 508; siGAPDH: n = 563).

(C) Characteristic kymographs of (B).

(D) GAPDH activity of neurons silenced with siGAPDH I and siGAPDH II and expressed as percentage of control.

(E) Quantification of mean velocities from kymographs of neurons expressing APP-GFP or TrkB-RFP in control and 20 µM oligomycin treated neurons for 10 to 30 min (for APP-GFP: control n = 81; oligomycin n = 20; for TrkB: control: n = 114; oligomycin n = 109).

***p < 0.001, error bars, SEM.

Cell



Figure S4. Protocol for Motile Vesicle Purification, Related to Figures 5 and 6

Cartoon showing the protocol to obtain the different fractions used for western blot, immunofluorescence, immunocryo electron microscopy (Cryo EM), GAPDH activity and in vitro glycolysis.

7 Vesicles are energetically autonomous for transport

7.1 Study presentation

Axonal transport is essential for determining a neuron's fate. Although many studies focused on understanding the mechanisms regulating the fine-tuning of this process, the identity of this moving organelle is yet to be defined. This can lead to identifying new regulatory partners and mechanisms that can govern the transport of dynamic vesicles, and to a more precise understanding of the mechanisms of axonal transport.

To characterize this novel type of vesicles, the dynamic vesicles, we performed mass spectrometry analysis from purified dynamic vesicles from mouse brains. The protein profile detected is consistent with vesicles: there is an enrichment of transmembrane proteins, molecular motors and adaptor proteins; and many vesicular markers are well represented in the sample. Strikingly, we found by biochemical and super-resolution microscopy that the whole glycolytic machinery was associated to vesicles. These findings extend our previous work on the role of GAPDH in sustaining fast axonal transport. Importantly, we show that Pyruvate Kinase, the last ATP producing step of glycolysis is important for generating energy to sustain FAT. Knocking down PK reduces the velocity of BDNF vesicles. Additionally, the transport deficit due to silencing of Gapdh is restored by the addition of PK's substrate. This suggests that the presence of glycolytic enzymes on vesicles is pivotal for regulating the efficiency of transport.

However, whether the ATP produced by glycolysis on vesicles is sufficient to support transport is still not known. Native purified vesicles are able to produce ATP when incubated with phosphoenolpyruvate (PEP). Additionally, these vesicles retain kinase activity, reflecting the presence of functional motors.

We then incubated dynamic vesicles purified from mouse brains with *in vitro* polymerized microtubules. Using TIRF microscopy, we could see that in the presence of ATP, they are capable of moving along MTs, further proving that vesicles contain a functional set of molecular motors. In this environment completely devoid of

cytoplasmic influence, when we incubated motile vesicles in an ATP deprived environment, but with the addition of ADP and PEP, vesicles were dynamic. This unequivocally demonstrates that dynamic vesicles are energetically autonomous for trafficking.

7.2 Article III

<u>Hinckelmann MV</u>, Nieaghe C, Poujol C, Choquet D, Hoflack B, Zala D, Saudou F. **Molecular Profiling of Brain Motile Vesicles Reveals Their Energetic Autonomy for Microtubule-Based Transport**. Manuscript in preparation

Molecular Profiling of Brain Motile Vesicles Reveals Their Energetic Autonomy for Microtubule-Based Transport

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Summary

Intracellular transport of vesicles requires energy. This is especially important in neurons in which transport occurs over long. While many proteins regulating transport in neurons have been identified, a comprehensive molecular composition of transported vesicles in neurons has yet to be established. Here, we report a procedure to selectively enrich motile vesicles that were subjected to semiquantitative proteomic analysis. In addition to the expected molecular motors, synaptic vesicles proteins and components of the endo-exocytic machineries, we found the ten glycolytic enzymes that break down glucose into pyruvate generating ATP. We show through biochemical approaches and super-resolution microscopy that, most glycolytic enzymes are selectively associated to vesicles and that the glycolytic cascade ensures proper axonal transport of vesicles in neurons. Finally, we provide evidence that vesicles can move with only ADP and the appropriate glycolytic substrates in a reconstituted in vitro transport assay of native vesicles. Together, we conclude that transport of vesicles along microtubules can be autonomous.

Results

Purification of Motile Vesicles

Many studies have investigated the proteome of synaptic or secretory vesicles leading to an exhaustive list of proteins specifically present in these various organelles (Ahmed et al., 2013; Au et al., 2007; Blondeau et al., 2004; Boyken et al., 2013; Burré et al., 2006; Coughenour et al., 2004; Grønborg et al., 2010; Takamori et al., 2006). However, the molecular identity of the vesicles that are transported through long distances in neurons is not defined. Especially, constitution of the dynamics or motile vesicles, in terms of their luminal constituents, transmembrane proteins, and associated complexes is still unknown. The identification of these components could lead to a better understanding of the mechanisms regulating transported vesicles in neurons. To obtain a highly enriched fraction of motile vesicles, we modified a previously described protocol (Zala et al., 2013) that take advantage from the Thy1:p50-GFP transgenic mice. These mice express the dynactin subunit, dynamitin, fused to GFP under the neuronal promoter Thy1, in levels below the threshold that disrupts the dynactin complex (Ross et al., 2006). Therefore, the vesicles associated with p50-GFP can be considered as motile vesicles as they are likely to associate with the molecular motors.

Mouse forebrains were lysed and subjected to subcellular fractionation, in order to obtain the 100,000g fraction, or P3 fraction, known to be enriched in small vesicles. To selectively enrich for motile vesicles, we performed a magnetic immunopurification against GFP thereby selectively purifying small vesicles associated with the molecular motors (Figure 1A). This motile vesicle fraction (Figure **1B**, IP-GFP lane) is enriched in p50-GFP as well as in the endogenous p50. This is expected since GFP-dynamitin incorporates into dynactin in a ratio of 2.2 labeled subunits out of four total dynamitin subunit per complex (Hendricks et al., 2010). Importantly, it also contains the dynactin subunit p150^{Glued}, all components of the dynactin complex. Both retrograde and anterograde molecular motors are associated to this fraction, as shown by the presence of dynein intermediate chain (DIC) and kinesin heavy chain (KHC). We also controlled our purification protocol by analyzing the presence of proteins that are known to be transported along microtubules (MTs) in axons and dendrites. As expected, vesicular markers as SNAP25, synaptophysin known to be present as an integral membrane protein of synaptic vesicle, as well as the luminal vesicular protein pro-BDNF that is present in large dense core vesicles are present in the fraction (Altar and DiStefano, 1998; Fawcett et al., 1997; Haubensak et al., 1998; Michael et al., 1997). Huntingtin (HTT), a regulator of axonal transport known to be associated to motile vesicles, is also found in the fraction (Colin et al., 2008; DiFiglia et al., 1995; Gauthier et al., 2004; Gutekunst et al., 1995; Trottier et al., 1995). Importantly, none of these components were significantly detected in the control IP (Figure 1B), showing the specificity of the immunoisolation. Cytoplasmic protein tubulin is specifically associated, although in low quantity, to the motile vesicle fraction. This association is expected, as tubulin is known to interact with molecular motors as well as with the dynactin complex. We conclude we have developed and validated a protocol to selectively enrich for brain motile vesicles.

Protein Composition of Motile Vesicles

The protein composition of the purified motile vesicles was further analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) after separation by SDS-PAGE. A total of 1291 proteins were identified as being constituents of motile vesicles. Consistent with the motile nature of purified vesicles, among the most abundant components detected are members of the dynein complex including dynein heavy chain (DHC), DIC and dynein light chains (DLC), as well as subunits of the dynein-associated dynactin complex: p150^{Glued}, p50, p62, p24, p27, Arp11, Capza and Capz β (Figure 1C and Table 2). Although less represented in the sample, both the conventional anterograde motor Kif5C and the neuronal motor responsible for synaptic vesicle precursor anterograde trafficking Kif1A were identified (Figure 1C and Table 2). We then ranked the identified proteins by intensity and grouped them into bins of 100 proteins. The number of proteins classified to the Gene Ontology term "transmembrane proteins" in a bin was then compered to the number present in the cytoplasmic fraction. As shown in Figure 1D, the motile vesicle proteins are present in high levels in the high intensity bins, and are much more represented when compared to the number of proteins identified in the cytosolic fraction that are represented in high levels only in the low intensity bins. The variety of different vesicular compartments found to be associated to the molecular motor complex can be shown by the diversity of the different small GTPases and associated proteins (Table 3). These include Rab3A and C known to be associated to synaptic vesicles, several small GTPases associated to ER-Golgi vesicular trafficking (Rab1B, RAB2A, RAB6A and B, Rab8A and B, rab14), endolysosomal trafficking (RAB7A) and

122

endosomes (Rab4B, Rab18, Rab21, Rab5B, C and A, Rab1A, Rab7), and cilia transport (Rab23 and Rab 28). In line with these findings, a great variety of proteins known to be resident of the endocytic compartment were identified, as Clathrins, Dynamin, components of AP complex, Synaptojanine. The purified fraction contained as well synaptic vesicles residents as synapsin and synaptophysin, among many other markers of different vesicular compartments (Table 1). Finally, a large number of proteins not previously reported to be part of vesicles were also detected. These include categories such as signaling proteins like phosphatases and kinases, components of the cytoskeleton, chaperones, ribosomal proteins, proteasome components and some metabolic proteins. These findings suggest that a large set of proteins are present on vesicles and that could play a role in regulating vesicular trafficking.

The Whole Glycolytic Machinery is Associated to Motile Vesicles

We previously reported that GAPDH plays a role in regulating FAT but whether the whole glycolytic machinery is present on vesicles remains to be established (Zala et al., 2013). Glycolysis consists in ten sequential enzymatic reactions that break glucose into pyruvate (Fig S1). We found the ten enzymes of the glycolytic machinery to be present in the proteomic analysis of purified motile vesicles. Furthermore, they were among the most important in terms of abundance (Figure 2A), and upon ranking, they were enriched in the motile vesicle fraction (Figure 2B). Normally considered as mere contaminants, it has been recently suggested that some of the glycolytic enzymes might associate to specific structures providing efficiently the ATP needed for certain functions (Ikemoto et al., 2003; Takeda and Ueda, 2012; Zala et al., 2013). We further validated the specificity of the association of glycolytic enzymes to motile vesicles by performing immunoprecipitation experiments followed by Western Blot analysis. As shown in Figure 2C, most of the glycolytic enzymes were found to be selectively associated to the motile fraction as compared to control immunoprecipitation where their levels are much lower.

To better assess their localization, we performed immunofluorescence studies in axons of primary cortical neurons. To eliminate the cytosolic free portion of proteins, cells were permeabilized before fixation ensuring that only the proteins associated to cytoskeletal structures or organelles are present. This approach is key for abundant cytosolic proteins such as glycolytic enzymes. We aimed to analyze the subcellular distribution of Hexokinase (HK), PhosphoGlucose Isomerase (GPI), PhosphoFructo Kinase (PFK), Aldolase (ALDO), Glyceraldehyde 3-phosphate Dehydrogenase (GAPDH), PhosphoGlycerate Kinase (PGK), PhosphoGlycerate Mutase (PGM), Enolase (ENO) and Pyruvate Kinase (PK). We first verified the best conditions of use and specificity for each of these antibodies, and analyzed their distribution in axons using Total Internal Reflection Fluorescence (TIRF) microscopy. (Figure 2D, Fig S1B). Using this approach, we observed co-localization of all the glycolytic enzymes tested with vesicles. To unequivocally demonstrate this association, we analyzed the distribution of the same proteins by high-resolution microscopy. Using Ground State Depletion (GSD) microscopy, with a spatial resolution of 20 nm, we observed a significant co-localization between endogenous glycolytic enzymes and SNAP25 positive vesicles (Figure 2D). For all the analyzed enzymes, the colocalization is above the 25% of the vesicles (Fig S1C). Unfortunately, we could not reliably assess the distribution of HK and GPI using the last technique, as the quality of the staining was not sufficient for this type of experiments. Together, our results using complementary biochemical approach and super-resolution microscopy indicate that most of the glycolytic enzymes are selectively localized on motile vesicles in neurons.

Glycolytic ATP is Required for FAT

It is commonly thought that ATP dependent mechanisms rely on mitochondria as a source of energy. The main role of glycolysis in energy production is to generate pyruvate, which will be taken up by mitochondria to generate the majority of cellular ATP. However, in recent years, evidence suggests that glycolytic enzymes may have an important role in locally generating ATP. For instance, GAPDH/PGK and PK present on synaptic vesicles produce enough ATP to sustain neurotransmitter uptake (Ikemoto et al, 2003; Takeda et al 2012). We have recently shown that ATP production through the activation of the glycolytic enzyme GAPDH, and not mitochondrial ATP, facilitates FAT (Zala et al., 2013). However, GAPDH does not produce directly ATP that is generated by the PGK and PK reactions. For this reason, our findings that all the glycolytic enzymes of the pay-off phase (Figure S1A) are associated to motile vesicles suggests that a functional glycolytic cascade is necessary to produce on board ATP and to promote axonal transport. We took advantage of microfluidic chambers that allows us to isolate neuronal cell bodies from axons (Taylor et al., 2005; Zala et al., 2013). Embryonic cortical neurons were

124

transfected with BDNF-mCherry, a well-described FAT cargo (Kwinter et al., 2009; Zala et al., 2013) that we showed to co-purify with motile vesicles (Figure 1B). Transfected neurons were plated in microchambers and BDNF containing axons were imaged. Glycolysis has two ATP producing steps, PGK and PK (Figure S1A). PK is the last glycolytic step and is downstream of GAPDH/PGK. We therefore examined the consequences of knocking down PK, by using 3 different siRNAs and studied FAT (Figure 3A). Silencing of the second ATP producing enzyme also reduced affects BDNF transport as shown in the kymographs (Figure 3B) by reducing their velocity (Figure 3C). This highlights the importance of all the ATP producing glycolytic enzymes in sustaining FAT. To determine if a functional pay-off glycolytic phase is required for FAT, we tested whether FAT reduction induced by the silencing of GAPDH can be rescued by addition of phospho-enol pyruvate (PEP) that is the substrate of PK reaction. We confirmed that reducing levels of GAPDH (Figure 3D) affects BDNF vesicles velocity. However, when we bypass this step by the addition of PK substrate, velocities go back to normal values (Figures 3E and 3F). Together, these findings provide further evidence of the importance of glycolysis in sustaining FAT in axons.

Native Motile Vesicles Produce Glycolytic ATP

Decreasing levels GAPDH or inhibiting its catalytic activity slows down FAT in axons, suggesting that this ATP source is essential for transport. Since we found all the glycolytic enzymes to specifically associate to motile vesicles and that the ATP producing glycolytic enzymes PK and Gapdh/PGK are required for transport, we evaluated whether purified motile vesicles are capable of generating ATP. Purified vesicles were incubated with the PK's substrate, PEP, ADP and cofactors necessary for the reaction to occur. ATP content was measured at different time points after incubation. These purified organelles generated rapidly substantial amounts of ATP (Figure 4A). This production is sustained in time, as the ATP content keeps increasing with time, showing that this process is continuously occurring. This demonstrates that motile vesicles contain functional PK capable of self-generating ATP.

Native Motile Vesicles Contains Functional Molecular Motors

Purified motile vesicles are associated to molecular motors (Figure 1B). To further analyze the relevance of glycolysis in transport, we tested the functionality of these associated motors. Therefore, we measured the ability of purified motile vesicles to hydrolyze ATP in a MT-dependent assay. As shown in Figure 4B, purified vesicles hydrolyze ATP in the presence of polymerized MTs. No ATP hydrolysis was observed in the absence of MTs, consistent with the fact that molecular motors ATPases require MTs. This suggests that motile vesicles contain molecular motors that retain their functionality.

Native Motile Vesicles are Energetically Independent for Transport

Having demonstrated that native purified motile vesicles have functional molecular motors and a full glycolytic cascade, we aim to demonstrate that this vesicular ATP production is able to self-propel vesicles in an environment devoid of any cytosolic contaminants. To make sure that vesicular movement is not a consequence of bulk ATP diffusion, we performed *in vitro* motility tests with purified brain motile vesicles. For these experiments we used a flow chamber that consists of a silanized coverslip adhered with double-sided tape to a glass slide, generating a flow chamber with a volume of approximately 10 µl. Rhodamine *in vitro* polymerized microtubules were specifically attached to the coverslip using anti-tubulin antibodies. Finally, purified motile vesicles from mouse brains were fluorescently labeled with a lipophylic die and flown into the chamber. In this cytosolic-free condition we used TIRF microscopy to study whether vesicular MT-based transport can be sustained by glycolytically-produced ATP.

In the absence of exogenously added ATP, native vesicles did not show processive movement along MTs. However, they stably bind to MTs, consistent with dynein association to MTs in the non-nucleotide state (Figure 4D). As previously reported (Hendricks et al., 2010), the addition of 1mM ATP, resulted in the movement of dynamic vesicles along MTs. Kymograph analysis showing the trajectories of these vesicles along MTs, show they maintain their association and move bidirectionally on MTs (Figure 4E). Finally, we tested whether the production of glycolytic ATP is sufficient to sustain vesicular transport. We focused on the last step of the glycolytic cascade and incubated native motile vesicles with ADP and PEP, the substrate of the PK reaction. We next analyzed movement of vesicles in the flow chamber by TIRF microscopy. We found that in condition where ATP is absent but replaced by ADP

126

and the substrates of an endogenous on-board ATP producing enzyme, PK, vesicles are able to self propel as processively as in presence of 1 mM ATP (Figure 4F). Our findings unequivocally demonstrate that vesicles can move independently of the bulk ATP present in the cytosol but have some energetic autonomy due to the presence of a fully functional endogenous glycolytic cascade.

Experimental Procedures

Mice

Thy1:p50-GFP transgenic mice expressing low levels of dynamitin fused to GFP were described previously (Ross et al., 2006). Animals were maintained with access to food and water *ad libitum* and kept at a constant temperature (19°C-22°C) and humidity (40%–50%) on a 12:12 hr light/dark cycle. All experimental procedures were performed in an authorized establishment (Institut Curie, Orsay facility license #C91471108, Feb 2011) in strict accordance with the recommendations of the European Community (86/609/EEC) and the French National Committee (2010/63) for care and use of laboratory animals under the supervision of authorized investigators (permission #91-448 to S. Humbert). This study was evaluated and approved by the Comité d'Ethique en matière d'Expérimentation Animale Paris Centre et Sud (National registration number: #59) presided by Pascal Bigey.

Mouse Brain Vesicle Purification

Motile Vesicles were purified as shown in Fig.1A. Thy1:p50-GFP mouse forebrains were homogenized in Lysis Buffer (Hepes 4 mM, Sucrose 320 mM, pH 7.4) containing protease inhibitor cocktail (Sigma). The homogenate was first spun for 10 min at 800 x g to remove unlysed cells and nuclei. The supernatant was then centrifuged for 40 min at 12.000 x g in order to pellet mitochondria and large membranes. The resulting supernatant was then further centrifuged for 90 min at 100.000 x g (Beckman, TLA 100.1). The cytosol-enriched supernatant was kept for mass spectrometry analysis, whereas the high-speed pellet enriched in small vesicles (Vesicle Fraction) was resuspended in Lysis Buffer.

In order to selectively purify small vesicles bound to the molecular motor complex, we immunupurified dynamitin-GFP-positive vesicles. For this purpose, the Vesicle Fraction was incubated with anti-GFP iron μ Beads or iron μ Beads not bound to antibodies as control (Miltenyi) for 30 min at 4°C. Separation μ Columns were placed in a magnetic field, and the incubated solution applied onto the column. The flow through (FT) containing unbound material was collected, and then the column was rinsed three times with Lysis Buffer. The Motile Vesicle fraction (IP) was eluted from

the column by applying 150 μ l of elution buffer (0.1M triethylamine, pH 11.8, 0.1% Triton X-100) in a tube containing 9 μ l of of 1 M MES pH 3 for neutralization.

For mass spectrometry, proteins were precipitated using 10% TCA, and resuspended in Laemmli buffer.

For native *in vitro* motility tests, and in order to keep the complex molecular motorvesicle-associated proteins intact but removing cytosolic contaminants, the Vesicle Faction was further fractionated by flotation through a sucrose step gradient as previously described (Perlson et al., 2009).

Mass Spectrometry and Enrichment Analysis

Samples were fractionated by cutting full lanes after SDS-PAGE into 10 slices. Proteins in each slice were reduced, carbamidomethylated at the cystein-residues and in-gel digested by trypsin in a 1:50 ratio. Tryptic peptides were separated on a 15 cm reversed-phase column (C18, 15 cm x 75 µm, 2 µm beads of 100-Å pore size; Dionex) within a 60 min gradient from 0.1% formic acid / 5% acetonitrile to 0.1% formic acid / 40% acetonitrile operated by an Ultimate 3000 HPLC system (Dionex). Mass spectrometry was performed with a LTQ Orbitrap XL mass spectrometer (Thermo Scientific), equipped with a nanoESI source (Proxeon). The top eight peaks in the mass spectra (Orbitrap; resolution, 60,000) were selected for fragmentation (CID; normalized collision energy, 35%; activation time, 30 ms, q-value, 0.25). Dynamic exclusion was enabled (repeat count, 2; repeat duration, 10 s; exclusion duration, 20 s). MS/MS spectra were acquired in the LTQ in centroid mode. Proteins were identified using the MaxQuant software package version 1.2.2.5 (MPI for UniProt Biochemistry, Germany) and database version 04/2013. Carbamidomethylation of cysteine was chosen as a fixed modification; acetylation of the N-terminus, deamidation of asparagine and oxidation of methionine as variable modifications. Proteins with at least one unique and two razor peptides were kept when present in at least two of three replicates. An in-house R-script (www.rproject.org) was used to average protein intensities within sample types, rank proteins decreasingly hereby and count number of proteins within bins of 100 proteins that are associated with a particular gene ontology or criteria.

Microfluidic chambers and neuronal plating

129

Microfluidic chambers were obtained as described previously (Zala et al., 2013). Briefly, PDMS replicates were done from a master mold, and laid on a previously activated bottom glass dish to form an irreversible tight seal. The microfluidic chamber was then coated with Ploy-D-lysine (0.5 mg/ml) and laminine (10 mg/ml). Embrionic rat cortical neurons (E17) were electroporated with BDNFm-Cherry using Amaxa Nucleofactor kit (Lonza). Neurons were then plated in the proximal chamber in Neurobasal supplemented with 2% B27, 1% Glutamax and 1% penicillin/streptomycin. Videomicroscopy acquisitions fixation for and immmunolabeling were done 3-5 DIV, when axons had already crossed the microchannels.

Live-cell imaging and Analysis

In vivo axonal transport of BDNF-mCherry was imaged in cortical neurons plated in microfluidic chambers. Neuronal processes can grow into the microchannels, but only axons can reach the distal part of the microchannel located 450 μ m away from the proximal chamber where cell bodies are located. Acquisitions were done in the distal part of the microchannels, using an inverted microscope (Elipse T*i*, Nikon) with a 60 x 1.42 NA oil immersion objective coupled to a spinning-disk confocal system (CSU-X1; Yookogawa) connected to an EM-CDD camera (eVolve, Photometrics) and maintained at 37°C and 5 %CO₂. Images were taken every 0.2 sec for one minute. Kymographs and velocity analysis were done using KymoToolBox (Zala et al., 2013), a home made plugin for ImageJ (NIH, USA).

Native in Vitro Motility test

Rhodamine labeled microtubules were generated by incubating for 20 min at 37°C unlabeled tubulin purified from porcine brain and rhodhamine labeled tubulin (Cytoskeleton) in BRB80 buffer (Pipes 80 mM, MgCl₂ 1mM, EGTA 1mM) containing 1mM GTP to allow polymerization. Polymerized microtubules were then stabilized by adding Taxol 50 μ M and incubated again for 20 min at 37°C. Free tubulin was then eliminated by centrifuging the polymerized microtubules for 10 min at 14.000 x *g*. The pellet was resuspended in BRB80 buffer containing 40 μ M Taxol.

Native *in vitro* assays were carried out in a flow chamber made of a slide attached to a silanized coverslip by two-sided tape. Flow chambers were first incubated for 5 min with 2% anti-tubulin antibody (Sigma Aldrich), and then blocked with 5% Pluronic

Results

F127 (Sigma Aldrich). Microtubules were allowed to bind in the flow chamber for 10 min, and the excess was removed by washing with BRB80 100mM DTT 20 μ MTaxol. Finally, purified Native Vesicles, previously incubated or not with MgATP or PEP and ADP in Motility Buffer (BRB80, 0.05% Pluronic F127, 20 μ M Taxol, 0.25 mg/ml BSA, 50 mM DTT, 0.5 mg/ml Glucose Oxidase, 470 U/ml Catalase, 15 mg/ml Glucose) were added to the flow chamber (Fig 4A)

Acquisitions were made using an inverted microscope (Elipse T*i*, Nikon) with a 60 x 1.42 NA APO TIRF oil immersion objective (Nikon) coupled to CDD camera (CoolSnap, Photometrics) and maintained at 37° C and 5 %CO₂. A first picture of rhodamine labeled microtubules was taken, and then vesicular movement was recorded by acquiring every 0.2 s.

ATP Production and Consumption Analysis

To test molecular motor activity after vesicular purification, 10µl of "motile vesicles" were incubated with 1mg of polymerized microtubules and 1 mM MgATP at 30°C. 10µl aliquots were removed at different time points, and reaction was stopped by storing them at 4°C. To measure ATP content, 2ul of Cell Titer-Glo Luminescent Viability Assay (Promega) was added, and luminescence was recorded.

To assess ATP production by PK that specifically associates to " motile vesicles", 10µl of this fraction was incubated at 37°C with PEP, ADP, NADH Pi in motility buffer. ATP content of the samples was measured at different time point as described above.

Western Blot

Proteins concentration was assed using BCA kit (Pierce). 10µg of proteins were denatured at 95°C for 10minutes in loading buffer. 6%, 8%, 10% 12% and 15% acrylamide gels were loaded. Proteins were transferred onto nitrocellulose membranes and the blocked in 5% non-fat milk in TBS buffer 0.1%Tween. Primary antibodies were incubated for one hour at room temperature as well as secondary antibodies.

Immunolabeling and STORM microscopy

Neurons were fixed 3-5 days after plating in the microchambers. A 2 minutes prelysis was done using 0.5% saponine in PBS, after which cells were fixed for 20 minutes

131

using 4%PFA 4%sucrose in PBS. Once fixed, microchambers were rinsed 3 times with PBS, and blocked for one hour at room temperature with 3%BSA + 0.3% Triton X-100. Primary antibodies were incubated overnight at 4°C in blocking buffer and secondary antibodies were incubated for one hour at room temperature.

Superresolution images were taken in a Leica SR GSD (Leica Microsystems, Mannheim, Germany) GSD microscope, equipped with a HCX PL APO 100X objective (Leica Microsystems, Mannheim, Germany) with a 1.47 NA for TIRF illumination. Images were acquired on a sensitive EMCCD iXon3 camera (ANDOR, Belfast, United Kingdom). The lasers used were 405 nm, 488 nm, 532 nm and 642 nm diodes with a 30 mW, 300 mW, 1W and 500 mW rough power respectively (Coherent, Santa Clara, CA, United States).

Constructs and si-RNA

BDNF-mCherry construct has been previously published (Kwinter et al., 2009). Rat GAPDH was silenced with a previously published siRNAII (Zala et al., 2013) Rat PK was silenced with annealed siRNA I CAU-CUC-CCU-GCA-GGU-GAA-GGA-GAT-TT and UCU-CCU-UCA-CCU-GCA-GGG-AGA-UGT-TT (Eurogentec), siRNA II GAU-UUU GGA-GGC-CAG-CGA-UGG-AAT-TT and UUC-CAU-CGC-UGG-CCU-CCA-AAA-UCT-TT (Eurogentec), or siRNAIII GAG-CCU-CCA-GUC-AAU-CCA-CAG-AC5-5 and GUC-UGU-GGA-UUG-ACU-GGA-GGC-UC5-5 (Eurogentec).

Reagents and Antibodies

ATP, ADP and PEP are from Sigma-Aldrich. The following antibodies and dilutions were used for western blotting and immunofluorescence; mouse anti-GFP HRP (Miltenyi, 1:5.000 WB), mouse anti-p150^{g/ued} (BD Transduction Laboratories, 1:1,000 WB), mouse anti-KHC (Chemicon, 1:500 WB), mouse anti-DIC (Millipore, 1:500 WB), mouse anti-synaptophysin (Sigma, 1:1,000 WB), mouse anti-α-tubulin (Sigma, 1:1,000 WB), rabbit anti-BDNF (Chemicon, 1:1,000 WB), rabbit anti-p50 dynamitin (Millipore, 1:250 WB), mouse anti-SNAP25 (Abcam, 1:2,000 WB and IF), rabbit anti-Hexokinase I (Cell Signaling, 1:1000 WB, 1:100 IF), mouse anti GPI (Santa Cruz biotechnology, 1:500 WB), goat anti-Aldolase (Santa Cruz Biotecnology 1:200 WB, 1:50 IF), rabbit anti-GAPDH (Sigma, 1:1,000 WB, 1:100 IF), mouse anti-PGK-1 (Abcam, 1:1,000 WB) goat anti-PGK (Santa Cruz, 1:100 IF), goat anti-PGAM (Santa Cruz biotechnology, 1:200 WB, 1:100 IF), rabbit anti-GAPDH (Sigma, 1:100 IF), rabbit anti-PGAM (Santa Cruz biotechnology, 1:200 WB, 1:100 IF), rabbit anti-PGAM (Santa Cruz biotechnology, 1:200 WB, 1:100 IF), rabbit anti-PGAM (Santa Cruz biotechnology, 1:200 WB, 1:100 IF), rabbit anti-PGAM (Santa Cruz biotechnology, 1:200 WB, 1:100 IF), rabbit anti-PGAM (Santa Cruz biotechnology, 1:200 WB, 1:100 IF), rabbit anti-PGAM (Santa Cruz biotechnology, 1:200 WB, 1:100 IF), rabbit anti-PGAM (Santa Cruz biotechnology, 1:200 WB, 1:100 IF), rabbit anti-PGAM (Santa Cruz biotechnology, 1:200 WB, 1:100 IF), rabbit anti-PGAM (Santa Cruz biotechnology, 1:200 WB, 1:100 IF), rabbit anti-PGAM (Santa Cruz biotechnology, 1:200 WB, 1:100 IF), rabbit anti-PGAM (Santa Cruz biotechnology, 1:200 WB, 1:100 IF), rabbit anti-PGAM (Santa Cruz biotechnology, 1:200 WB, 1:100 IF), rabbit anti-Enolase (Santa Cruz

biotechnology, 1:200 WB, 1:100 IF), rabbit anti-Pyruvate Kinase (Cell signaling, 1:1000 WB, 1:100 IF) and rabbit anti-LDH (Cell signaling, 1:1000 WB).

Statistical Analysis

Statistical analyses were done using GraphPad Prism (GraphPad software Inc). Different groups were compared using ANOVA followed by Tukey's post hoc anlaysis. All data are presented as mean ± SEM.

Figure Legends

Figure 1: Motile vesicles purification.

(A) Isolation of motile vesicles. Thy:1 p50 GFP brains were gently homogenized. The homogenate was subjected to sequential centrifugation steps in order to obtain the high-speed pellet containing small vesicles (P3). Motile Vesicles were isolated from non-motile ones through magnetic immunopurification against GFP. (B) The Motile Vesicle fraction (IP-GFP) is enriched in the transgenic and endogenous p50. It contains anterograde and retrograde molecular motors, as well as vesicular markers. (C) Motile Vesicles and cytoplasm composition was studied by LC-MS/MS. Comparison of these two fractions show that motile vesicles are enriched in transmembrane proteins. (D) Motile Vesicle fraction identified proteins were ranked by intensity, and plotted according to their relative abundance (gray spots). Molecular motors and associated proteins (green spots) are among the most abundant proteins. Numerous components previously identified as vesicular residents are represented in the motile fraction (red spots). Different small GTPases were also found to be associated to the Motile Vesicles (blue spots).

Figure 2: Glycolytic enzymes are associated to Motile Vesicles.

(A) The glycolytic enzymes (greed spots) are present in the motile vesicles, and are among the most abundant proteins. (B) Glycolysis is enriched in the Motile Vesicles.
(C) The presence of all the glycolytic machinery is specific, as shown by the absence of these enzymes in the control IP. (D) Glycolytic enzymes co-localize with vesicles in axons. SNAP25 staining (red channel) co-localizes with different glycolytic enzymes (green channel). Co-staining was analyzed by TIRF microscopy (upper panels) and by super resolution microscopy (lower panels).

Figure 3: FAT relies on ATP-producing glycolytic enzymes.

(A) Representative Western blot showing the silencing of PK in cortical neurons. (B) Representative kymographs showing the trajectories of BDNF-mCherry vesicles in axons (left panels) and analyzed trajectories with color code red for retrograde, green for anterograde and blue for static vesicles (right panels). Down-regulation of pyruvate kinase reduces de velocity of BDNF-mCherry vesicles. (C) Mean and SEM for anterograde and retrograde velocities of control and 3 different si-PK

(anterograde velocity, control: n= 237, si-PK 1: n= 245, si-PK 2: n= 100, si-PK 3: n=139; retrograde velocity, control: n=276, si-PK 1: n= 318, si-PK 2: n= 107, si-PK 3: n=204. *** = p<0.001). (D) Western blot showing the silencing of Gapdh in cortical neurons (E) Representative Kymographs (left panels) and analysed kymographs (right panels) of BDNF-mCherry vesicles in axons. Silencing Gapdh slows down vesicles velocities, and this defects is restored by incubation of 1mM PEP. (F) Anterograde and retrograde velocities represented as mean + SEM (anterograde velocity, control: n= 115, si-GAPDH: n= 93, si-GAPDH + PEP: n=85. *** = p<0.001).

Figure 4: Motile Vesicles are autonomous for transport.

(A) Motile Vesicles are capable of producing their ATP. The graph shows the basal level of luminescence of the substrate of glycolysis and Motile Vesicles without substrates. Then we observe the ATP production overtime of purified Motile Vesicles when incubated with PK's substrate PEP. (B). Molecular motors retain their kinase activity in purified Motile Vesicles. Motile Vesicles are incubated with polymerized microtubules and 1mM ATP. The ATP reduction overtime in presence of microtubules reflects the presence of active molecular motors. (C) Representation of native in vitro motility test. Flow chamber was assembled using a silanized coverslips and glass slide, spaced by double-sided tape. Anti-tubulin antibodies, blocking solution and rhodamine labeled MTs are sequentially flown into the chamber, to ensure specific attachment of MT. Purified motile vesicles purified from mouse brain are finally incubated into the flow chamber. Microtubules and Motile Vesicles are imaged using TIRF microscopy. (D) In the absence of ATP, Motile Vesicles are able to attach to microtubules, but are not dynamic. (E) The addition of ATP into the chambers, results in vesicular movement. (F) Vesicles incubated with PEP and not with ATP, are dynamic (MTs in red, Motile Vesicles in green, kymograph representing movement right panel).

Results








Bins of intensity-ranked proteins

С



ΗK GPI PFK ALDO GAPDH PGK PGM ENO ΡK































BDNF m-Cherry

10 un

D

Е

Control si-RNA



С





📕 si-Gapdh 📕 si-Gapdh + PEP

А



В

С



D

Е







Table 1

Cytoplasmic Vesicle Resident

	Endocytic Vesicle			
Rank	Protein Name	Gene Name	Acc. Number	Avrg LFQ
16	91 kDa synaptosomal-associated protein;Clathrin coat assembly protein AP180	Snap91	Q61548	31.71097331
43	Dynamin-1	Dnm;Dnm1;Kiaa4093	P39053	30.13128181
46	100 kDa coated vesicle protein A;Adapter-related protein complex 2 alpha-1 subunit	Adtaa;Ap2a1;Clapa1	P17426	29.70256275
53	Adapter-related protein complex 2 beta subunit; Adaptor protein complex AP-2 subunit beta	Ap2b1;Clapb1	Q9DBG3	29.56938286
78	Synaptic inositol-1,4,5-trisphosphate 5-phosphatase 1;Synaptojanin-1	Kiaa0910;Synj1	Q8CHC4	28.86713636
80	Clathrin Interactor 1;MCG22297, Isoform CKA_D;Putative uncharacterized protein	An2m1:Clanm1:mCG	2Q99KN9	28.83995118
90 110	Fordermal growth factor recentor nathway substrate 15-related sequence	Enc15l1.Enc15B.Enc15	.060902	28.30344777
114	AP1 subunit gamma-binding protein 1:Gamma-synergin:Synergin gamma	An1ghn1.Svng.Svnrg	055V85	28 2263884
115	EH and SH3 domains protein 1:Intersectin-1	Ese1:ltsn:ltsn1	09Z0R4	28.19463428
119	100 kDa coated vesicle protein C;Adapter-related protein complex 2 alpha-2 subunit	Adtab;Ap2a2	P17427	28.16077193
122	Adaptor-associated kinase 1;AP2-associated protein kinase 1	Aak1;Kiaa1048	Q3UHJ0	28.1356995
135	Clathrin assembly lymphoid myeloid leukemia; Phosphatidylinositol-binding clathrin assembly protein	Calm;Fit1;Picalm	Q7M6Y3	27.90624892
138	EH domain-containing protein 3	Ehd2;Ehd3	Q9QXY6	27.82999058
140	Dynamin family member proline-rich carboxyl-terminal domain less;Dynamin-1-like protein	Dnm1l;Drp1	Q8K1M6	27.78354659
145	SH3-domain GRB2-like (Endophilin) interacting protein 1;Putative uncharacterized protein Sgip1	RP23-66O4.1-007;Sgip	Q8VD37	27.73942577
157	Stromal membrane-associated protein 1-like;Stromal membrane-associated protein 2	Smap1l;Smap2	Q7TN29	27.54658017
192	Itsn2 protein;EH domain and SH3 domain regulator of endocytosis 2;Intersectin-2	ltsn2;Ese2;Sh3d1B	Q9Z0R6	27.08386693
236	EPS-15-Interacting protein 1;Epsin-1;Intersectin-EH-binding protein 1	Epn1	Q80VP1	26.57560268
268	SH3-domain GRB2-like 2;Endopnilin-1;Endopnilin-A1;SH3 domain protein ZA	KP23-158E10.3-002;Sr	008520	26.27454992
2/3	Amphiphysin II;Amphiphysin-like protein;Bridging integrator 1	Ampni;Bin1;Sn3p9	008539	26.24543114
255	Dynamin-2	Vps50 Dpm2·Kiaa0820	091700	25.61502927
408	Adaptin ear-hinding cost-associated protein 1:NECAP endocytosis-associated protein 1	Necan1	Q66296	25.3936426
400	Dynamin-1	Necapi	P39053	25 24976488
425	Ig mu chain C region membrane-bound form Ig mu chain C region secreted form	løh-6	P01873	25.24570400
443	Early endosome antigen 1	Eea1	08BL66	25.06167575
495	ESCRT-I complex subunit TSG101:Tumor susceptibility gene 101 protein	Tsg101	Q61187	24.81171531
525	Putative uncharacterized protein; Hepatocyte growth factor-regulated tyrosine kinase substrate	Hgs;Hrs;RP23-82I5.13	(Q99LI8	24.63441078
541	Vacuolar proton pump subunit E 1;V-ATPase 31 kDa subunit;V-type proton ATPase subunit E 1	Atp6e;Atp6e2;Atp6v1	eP50518	24.53887915
572	Sorting nexin-1;Putative uncharacterized protein;Sorting nexin 1	Snx1	Q9WV80	24.37017193
617	ADP-ribosylation factor-binding protein GGA3;Golgi-localized, gamma ear-containing	Gga3;Kiaa0154;RP23-3	3 Q8BMI3	24.17655708
624	SDP3 protein;Sorting nexin-3;Putative uncharacterized protein;Sorting nexin 3;Sorting nexin 3	Snx3;mCG_121767	070492	24.1480493
626	Signal transducing adapter molecule 1;Putative uncharacterized protein	Stam;Stam1;RP23-211	(P70297	24.1377429
646	EH domain-containing protein 1;PAST homolog 1	Ehd1;Past1	Q9WVK4	24.02199297
653	Annexin A2;Annexin II;Annexin-2;Calpactin I heavy chain;Calpactin-1 heavy chain	Anx2;Anxa2;Cal1h;CT0	P07356	23.99909256
659	Adapter-related protein complex 2 sigma subunit; Adaptor protein complex AP-2 subunit sigma	Ap17;Ap2s1;Claps2	P62743	23.9840975
696	H beta>58 protein;Vacuolar protein sorting-associated protein 26A;Vesicle protein sorting 26A	Vps26;Vps26a;mCG_1	1P40336	23.83932452
/1/	Neural RAP guanine nucleotide exchange protein	Kiaa0313;Pdzgef1;Rap	EQ8CHG7	23./2439616
//5	GRIP and colled-coll domain containing 2,185 kDa Golgi colled-coll protein	GCC2;mCG_19017;Klaa		23.40705559
828	ADP-ribosylation factor-like protein 10c;ADP-ribosylation factor-like protein 88	Ariiuc;Ari80;Glei		23.10/83814
043	Adapter protein containing PH domain, PTB domain and redcine 2000 motion 1	Appi1;Dip15d;Kidd142		23.019/7237
040 910	Recircleted protein Rel-A	vµssza Ral·Rala·Ral-a	D63321	23.01377723
921	Charged multivesicular body protein 1a:Chromatin-modifying protein 1a	Chmn1·Chmn1a·Pcoln	20921W0	22.71500118
931	Clavesin-2:Retinaldehyde-hinding protein 1-like 2	Clys2.Blbn1l2	088692	22.03003331
964	ADP-ribosvlation factor 6	Arf6	P62331	22.46625275
966	Charged multivesicular body protein 2a;Chromatin-modifying protein 2a	Chmp2a	Q9DB34	22.45822169
974	Coated vesicle-associated kinase of 104 kDa;SCY1-like protein 2	Cvak104;D10Ertd802e	;Q8CFE4	22.4168348
990	ESCRT-II complex subunit VPS22;Vacuolar-sorting protein SNF8;SNF8	D11Moh34;Snf8;RP23	- Q9CZ28	22.3237211
992	Ras-related protein Krev-1;Ras-related protein Rap-1A;GTP-binding protein smg p21B	Krev-1;Rap1a;Rap1b	P62835	22.31306699
1000	RING finger protein 22; RING finger protein HAC1; Tripartite motif-containing protein 3	Hac1;Rnf22;Trim3	Q9R1R2	22.27161804
1006	Sorting nexin-2	Snx2	Q9CWK8	22.22426199
1017	Charged multivesicular body protein 6; Chromatin-modifying protein 6	Chmp6;RP24-225K18.4	4P0C0A3	22.18272209
1038	Adapter protein GRB2; Growth factor receptor-bound protein 2; SH2/SH3 adapter GRB2	Grb2;RP23-28I4.1-004	Q60631	22.05980697
1085	Adapter-related protein complex 3 sigma-1 subunit; AP-3 complex subunit sigma-1	Ap3s1	Q9DCR2	21.74706202
1089	Charged multivesicular body protein 2b;Chromatin-modifying protein 2b	Chmp2b	Q8BJF9	21.71551407
1111	vacuolar protein sorting 25 (Yeast);ESCK1-II complex subunit VPS25	KP23-281C18.7-007;V		21.58162846
1121	ESCK1-I COMPLEX SUBURIT MVB12B;MUITIVESICULAR DODY SUBURIT 12B;Protein FAM125B	Fam1250;RP23-421L7		21.52402959
1132	Centaurin Beta 2;An-GAP with coneu-coll, ANK repeat and PH domain-containing protein 2 Charged multiverieular body protein 4b/Chromatin modifiling protein 4b	Chmp4b		21.4131430/
1141	WASH complex subunit strumpellin	Kiaa0106	080252	21.3/199992
1212	wash complex subulit su umperin Cyclin-dependent kinase inhibitor 18:Cyclin-dependent kinase inhibitor p37:p37Kin1	Cdkn1h	QOCZE/ P/6/1/	21.13418089
1215	Charged multivesicular hody protein 1h-2:Chromatin-modifying protain 1h-2	Chmn1h2	090004	20.00200089
1213	Charged multivesicular body protein 72 Chromatin-modifying protein 7	Chmp7	08R1T1	20.55062155
	Sunantic Vesicle			23.00002100
Rank	Protein Name	Gene Name	Acc. Number	Avrg LFO
18	Dihydropyrimidinase-related protein 2;Unc-33-like phosphoprotein 2	Crmp2;Dpvsl2:Ulip2	DPYL2	31.6972
56	Synapsin II;Synapsin-2;Putative uncharacterized protein	Syn2	SYN2	29.50070265
144	Synapsin I;Synapsin-1	Syn1;Syn-1	SYN1	27.741776

Results

	147	65 kDa glutamic acid decarboxylase:Glutamate decarboxylase 2	Gad2.Gad65	DCF2	27 72669045
	100		Amph:Amph1		27.72003043
	202	Ampinpinpinsin DM90 optigenet Major superticusciele protein n20:Supertenburin	Amph, Amphi		27.34904828
	303	Biologianigen, wajor synaphic vesicie protein pso, synaphophysin	syp	STPH	20.03587028
	435	Alpha-synuclein;Non-A beta component of AD amyloid;Non-A4 component of amyloid precursor	Snca;Syn	SYUA	25.14000259
	457	Beta-soluble NSF attachment protein;Brain protein I47	Napb;Snapb	SNAB	25.02794288
	504	p65;Synaptotagmin I;Synaptotagmin-1	Syt1	SYT1	24.76329383
	548	Calcium regulator SV2A;Synaptic vesicle glycoprotein 2A	Kiaa0736;Sv2;Sv2a	SV2A	24.49083603
	590	Clavesin-1;Retinaldehyde-binding protein 1-like 1	Clvs1;Rlbp1l1	CLVS1	24.28376211
	627	Synaptogyrin-3	Syngr3	SNG3	24.13607451
	640	HCNPpp:Hippocampal cholinergic neurostimulating peptide	Pbp:Pebp:Pebp1	PEBP1	24.06656159
	796	Mossy fiber terminal-associated vertebrate-specific presynaptic protein:Protein FAM79A	Fam79a:Mover:Tprg1	TPRGL	23.26143366
	803	Adanter-related protein complex 3 subunit delta-1:AP-3 complex subunit delta	An3d·An3d1	ΔP3D1	23 2143543
	005	Pab 2 interacting malacula 2:Pag allating curantic membrane avagutaria protain 2:PIM2 gamma	Kinn O 227, Bime 2	DIMES	22.2143343
	033	Radio-s-interfacting molecule s, Regulating synaptic memorialite exocytosis protein s, Nivis gamma	Fadly Fak Fak 1 King 420		23.07340270
	919	Focal addression kinase 1;pp125FAX;Pf0tein-tyrosine kinase 2		TRRE	22.05801044
	958	Syndindin; Fratticking protein particle complex subunit 4; EKS23 nomolog	Sbdn;Trappc4	TPPC4	22.4/5/3244
	996	Synaptogyrin-1	Syngr1	SNG1	22.29825447
	1114	Synaptic vesicle membrane protein VAT-1 homolog-like;Putative uncharacterized protein Vat1l	Kiaa1576;Vat1l	VAT1L	21.57234372
	1139	Vacuolar proton pump subunit G 2;V-ATPase 13 kDa subunit 2;V-type proton ATPase subunit G 2	Atp6g2;Atp6v1g2;Ng3	EVATG2	21.3839795
	1290	AMPA-selective glutamate receptor 2;GluR-B;GluR-K2;Glutamate receptor 2	Glur2;Gria2	GRIA2	
		Vesicle Coating			
	Rank	Protein Name	Gene Name	Acc Number	Avrg LEO
	208	Alpha-coat protein: Coatomer subunit alpha-Protein: Yanonsin-related pentide	Cona		26 84578625
	200	Aprile-Oat protein, Coatomer subunit aprila, Froxenin, Aenin, Aenin, Aeniopsin-Felated peptide			20.84378023
	214	Secto nomolog A (S. cerevisiae)	RP23-306D20.10-001;:		26.7619549
	215	Cyclin-G-associated kinase	Gak	GAK	26.75927752
	249	Protein transport protein Sec31A;SEC31-like protein 1;SEC31-related protein A	Sec31a;Sec31l1	SC31A	26.43082982
	285	Putative uncharacterized protein	Ap1b1	Q8CC13	26.15291079
	306	Epsin 2	Epn2;RP23-155J3.7-00	EPN2	26.0255653
	411	Protein SEC13 homolog;SEC13-like protein 1;SEC13-related protein	Sec13;Sec13l1	SEC13	25.2715848
	463	Adaptor protein complex AP-1, gamma 1 subunit;Putative uncharacterized protein	Ap1g1;Adtg;Clapg1	AP1G1	24.97421291
	498	Beta-coat protein:Coatomer subunit beta:p102	Copb2	COPB2	24.80076551
	499	Adaptor protein complex AP-1 mu-1 subunit: Adaptor-related protein complex 1 mu-1 subunit	An1m1·Cltnm	AP1M1	24 79420179
	F07	Clatherin light nolymonida (Leo). Clatherin light nolymonida (Leo) isofarm CRA c. Clatherin light chain A			24.75420175
	507	Clathin light polypeptide (LCa), Clathini, light polypeptide (LCa), isoform CKA_C, Clathini light Chain A	Cita, NP25-2091010.4-00	CLCD	24.75541462
	012			CLUB	24.20893347
	647	Coatomer subunit gamma;Gamma-coat protein	Copg;Copg1	COPG	24.01883338
	684	Adapter-related protein complex 3 subunit beta-2;Adaptor protein complex AP-3 subunit beta-2	Ap3b2	AP3B2	23.88825549
	719	Beta-coat protein;Coatomer subunit beta	Copb;Copb1	COPB	23.71564324
					22 72644644
	905	Archain;Coatomer subunit delta;Delta-coat protein	Arch1;Copd	COPD	22.73644611
	905 970	Archain;Coatomer subunit delta;Delta-coat protein SEC23-interacting protein	Arch1;Copd Sec23ip	COPD S23IP	22.73644611 22.44803764
	905 970 1082	Archain;Coatomer subunit delta;Delta-coat protein SEC23-interacting protein Sec24 related gene family, member B (S. cerevisiae)	Arch1;Copd Sec23ip Sec24b	COPD S23IP Q80ZX0	22.73644611 22.44803764 21.75531793
	905 970 1082	Archain;Coatomer subunit delta;Delta-coat protein SEC23-interacting protein Sec24 related gene family, member B (S. cerevisiae)	Arch1;Copd Sec23ip Sec24b	COPD S23IP Q80ZX0	22.73644611 22.44803764 21.75531793
	905 970 1082 Bank	Archain;Coatomer subunit delta;Delta-coat protein SEC23-interacting protein Sec24 related gene family, member B (S. cerevisiae) Lysosome Protein Name	Arcn1;Copd Sec23ip Sec24b	COPD S23IP Q80ZX0	22.73644611 22.44803764 21.75531793
	905 970 1082 Rank	Archain;Coatomer subunit delta;Delta-coat protein SEC23-interacting protein Sec24 related gene family, member B (S. cerevisiae) Lysosome Protein Name Nethylmaleimide sensitive fusion protein:Suppressor of K(A) transport growth defect 2	Arch1;Copd Sec23ip Sec24b Gene Name	COPD S23IP Q80ZX0 Acc. Number	22.73644611 22.44803764 21.75531793 Avrg LFQ 21.08857858
	905 970 1082 Rank 24	Archain; Coatomer subunit delta; Delta-coat protein SEC23-interacting protein Sec24 related gene family, member B (S. cerevisiae)	Arcn1;Copd Sec23ip Sec24b Gene Name Nsf;Skd2	COPD S23IP Q80ZX0 Acc. Number NSF	22.73644611 22.44803764 21.75531793 Avrg LFQ 31.08857858
	905 970 1082 Rank 24 181 102	Archain; Coatomer subunit delta; Delta-coat protein SEC23-interacting protein Sec24 related gene family, member B (S. cerevisiae)	Arcn1;Copd Sec23ip Sec24b Gene Name Nsf;Skd2 Atp6c;Atp6c1;Atp6v1c	COPD S23IP Q80ZX0 Acc. Number NSF :VATC1	22.43644611 22.44803764 21.75531793 Avrg LFQ 31.08857858 27.25772837
	905 970 1082 Rank 24 181 193	Archain;Coatomer subunit delta;Delta-coat protein SEC23-interacting protein Sec24 related gene family, member B (S. cerevisiae)	Arch1;Copd Sec23ip Sec24b Gene Name Nsf;Skd2 Atp6c;Atp6c1;Atp6v1c Mem3;Vps35	COPD S23IP Q80ZX0 Acc. Number NSF :VATC1 VPS35	22.43844611 22.44803764 21.75531793 Avrg LFQ 31.08857858 27.25772837 27.08244637
	905 970 1082 Rank 24 181 193 283	Archain;Coatomer subunit delta;Delta-coat protein SEC23-interacting protein Sec24 related gene family, member B (S. cerevisiae)	Arch1;Copd Sec23ip Sec24b Gene Name Nsf;Skd2 Atp6c;Atp6c1;Atp6v1c Mem3;Vps35 Napg;Snapg	COPD S23IP Q80ZX0 Acc. Number NSF VATC1 VPS35 SNAG	22.43644611 22.44803764 21.75531793 Avrg LFQ 31.08857858 27.25772837 27.08244637 26.18923927
	905 970 1082 Rank 24 181 193 283 438	Archain;Coatomer subunit delta;Delta-coat protein SEC23-interacting protein Sec24 related gene family, member B (S. cerevisiae)	Arch;Copd Sec23ip Sec24b Gene Name Nsf;Skd2 Atp6c;Atp6c1;Atp6v1c Mem3;Vps35 Napg;Snapg Cp	COPD S23IP Q80ZX0 Acc. Number NSF :VATC1 VPS35 SNAG CERU	22.43644611 22.44803764 21.75531793 Avrg LFQ 31.08857858 27.25772837 27.08244637 26.18923927 25.13069069
	905 970 1082 Rank 24 181 193 283 438 446	Archain;Coatomer subunit delta;Delta-coat protein SEC23-interacting protein Sec24 related gene family, member B (S. cerevisiae)	Arcn1;Copd Sec23ip Sec24b Gene Name Nsf;Skd2 Atp6c;Atp6c1;Atp6v1c Mem3;Vps35 Napg;Snapg Cp Atp6v1h	COPD S23IP Q80ZX0 Acc. Number NSF :VATC1 VPS35 SNAG CERU VATH	22.73644611 22.44803764 21.75531793 Avrg LFQ 31.08857858 27.25772837 27.08244637 26.18923927 25.13069069 25.04805809
_	905 970 1082 Rank 24 181 193 283 438 446 550	Archain;Coatomer subunit delta;Delta-coat protein SEC23-interacting protein Sec24 related gene family, member B (S. cerevisiae)	Arcn1;Copd Sec23ip Sec24b Gene Name Nsf;Skd2 Atp6c;Atp6c1;Atp6v1c Mem3;Vps35 Napg;Snapg Cp Atp6v1h Atp6m;Atp6v1d;Vatd	COPD S23IP Q80ZX0 Acc. Number NSF :VATC1 VPS35 SNAG CERU VATH VATD	22.43644611 22.44803764 21.75531793 31.08857858 27.25772837 27.08244637 26.18923927 25.13069069 25.04805809 24.46355921
_	905 970 1082 Rank 24 181 193 283 438 446 550 585	Archain;Coatomer subunit delta;Delta-coat protein SEC23-interacting protein Sec24 related gene family, member B (S. cerevisiae)	Arcn1;Copd Sec23ip Sec24b Gene Name Nsf;Skd2 Atp6c;Atp6c1;Atp6v1c Mem3;Vps35 Napg;Snapg Cp Atp6v1h Atp6m;Atp6v1d;Vatd Arl0b;Arl8a;Gie2	COPD S23IP Q80ZX0 Acc. Number NSF VATC1 VPS35 SNAG CERU VATH VATD ARL8A	22.43644611 22.44803764 21.75531793 31.08857858 27.25772837 27.08244637 26.18923927 25.04805809 25.04805809 24.46355921 24.29841064
	905 970 1082 Rank 24 181 193 283 438 446 550 585 655	Archain;Coatomer subunit delta;Delta-coat protein SEC23-interacting protein Sec24 related gene family, member B (S. cerevisiae) Protein Name N-ethylmaleimide-sensitive fusion protein;Suppressor of K(+) transport growth defect 2 Vacuolar proton pump subunit C 1;V-type proton ATPase subunit C 1 Maternal-embryonic 3;Vacuolar protein sorting-associated protein 35;Vesicle protein sorting 35 Gamma-soluble NSF attachment protein;N-ethylmaleimide-sensitive factor attachment protein gamma Ceruloplasmin;Ferroxidase Vacuolar proton pump subunit H;V-type proton ATPase subunit H Vacuolar proton pump subunit D;V-ATPase 28 kDa accessory protein;V-type proton ATPase subunit D ADP-ribosylation factor-like protein 10B;ADP-ribosylation factor-like protein 8A G protein subunit beta-2;Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2	Arcn1;Copd Sec23ip Sec24b Gene Name Nsf;Skd2 Atp6c;Atp6c1;Atp6v1c Mem3;Vps35 Napg;Snapg Cp Atp6v1h Atp6m;Atp6v1d;Vatd Arl10b;Arl8a;Gie2 Gnb2;Gnb4	COPD S23IP Q80ZX0 Acc. Number NSF VATC1 VPS35 SNAG CERU VATH VATD ARL8A GBB2	22.43644611 22.44803764 21.75531793 31.08857858 27.25772837 27.08244637 26.18923927 25.13069069 25.04805809 24.46355921 24.29841064 23.99565238
	905 970 1082 24 181 193 283 438 446 550 585 655 797	Archain;Coatomer subunit delta;Delta-coat protein SEC23-interacting protein Sec24 related gene family, member B (S. cerevisiae) Protein Name N-ethylmaleimide-sensitive fusion protein;Suppressor of K(+) transport growth defect 2 Vacuolar proton pump subunit C 1;V-type proton ATPase subunit C 1 Maternal-embryonic 3;Vacuolar protein sorting-associated protein 35;Vesicle protein sorting 35 Gamma-soluble NSF attachment protein;N-ethylmaleimide-sensitive factor attachment protein gamma Ceruloplasmin;Ferroxidase Vacuolar proton pump subunit H;V-type proton ATPase subunit H Vacuolar proton pump subunit D;V-ATPase 28 kDa accessory protein;V-type proton ATPase subunit D ADP-ribosylation factor-like protein 10B;ADP-ribosylation factor-like protein 8A G protein subunit beta-2;Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2 Palmitoyl-protein hydrolase 1;Palmitoyl-protein thioesterase 1	Arch;Copd Sec23ip Sec24b Gene Name Nsf;Skd2 Atp6c;Atp6c1;Atp6v1c Mem3;Vps35 Napg;Snapg Cp Atp6v1h Atp6m;Atp6v1d;Vatd Arl10b;Arl8a;Gie2 Gnb2;Gnb4 Ppt;Ppt1;RP23-232N10	COPD S23IP Q802X0 Acc. Number NSF VATC1 VPS35 SNAG CERU VATH VATH VATD ARL8A GBB2 SPPT1	22.43644611 22.44803764 21.75531793 Avrg LFQ 31.08857858 27.25772837 27.08244637 26.18923927 25.13069069 25.04805809 24.46355921 24.29841064 23.99565238 23.24753131
	905 970 1082 24 181 193 283 438 446 550 585 655 797 1166	Archain;Coatomer subunit delta;Delta-coat protein SEC23-interacting protein Sec24 related gene family, member B (S. cerevisiae) Protein Name N-ethylmaleimide-sensitive fusion protein;Suppressor of K(+) transport growth defect 2 Vacuolar proton pump subunit C 1;V-type proton ATPase subunit C 1 Maternal-embryonic 3;Vacuolar protein sorting-associated protein 35;Vesicle protein sorting 35 Gamma-soluble NSF attachment protein;N-ethylmaleimide-sensitive factor attachment protein gamma Ceruloplasmin;Ferroxidase Vacuolar proton pump subunit H;V-type proton ATPase subunit H Vacuolar proton pump subunit D;V-ATPase 28 kDa accessory protein;V-type proton ATPase subunit D ADP-ribosylation factor-like protein 10B;ADP-ribosylation factor-like protein 8A G protein subunit beta-2;Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2 Palmitoyl-protein hydrolase 1;Palmitoyl-protein thioesterase 1 Acid ceramidase:Acid ceramidase subunit albna:Acid ceramidase subunit beta	Arcn1;Copd Sec23ip Sec24b Gene Name Nsf;Skd2 Atp6c;Atp6c1;Atp6v1c Mem3;Vps35 Napg;Snapg Cp Atp6v1h Atp6m;Atp6v1d;Vatd Arl10b;Arl8a;Gie2 Gnb2;Gnb4 Ppt;Ppt1;RP23-232N16 Asah:Asah1	COPD S23IP Q80ZX0 Acc. Number NSF VATC1 VPS35 SNAG CERU VATC1 VATD ARL8A GBB2 SPPT1 ASAH1	22.43644611 22.44803764 21.75531793 31.08857858 27.25772837 27.08244637 26.18923927 25.13069069 25.04805809 24.46355921 24.29841064 23.99565238 23.24753131 21.12993699
	905 970 1082 Rank 24 181 193 283 438 446 550 585 655 797 1166	Archain;Coatomer subunit delta;Delta-coat protein SEC23-interacting protein Sec24 related gene family, member B (S. cerevisiae) Protein Name N-ethylmaleimide-sensitive fusion protein;Suppressor of K(+) transport growth defect 2 Vacuolar proton pump subunit C 1;V-type proton ATPase subunit C 1 Maternal-embryonic 3;Vacuolar protein sorting-associated protein 35;Vesicle protein sorting 35 Gamma-soluble NSF attachment protein;N-ethylmaleimide-sensitive factor attachment protein gamma Ceruloplasmin;Ferroxidase Vacuolar proton pump subunit H;V-type proton ATPase subunit H Vacuolar proton pump subunit D;V-ATPase 28 kDa accessory protein;V-type proton ATPase subunit D ADP-ribosylation factor-like protein 10B;ADP-ribosylation factor-like protein 8A G protein subunit beta-2;Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2 Palmitoyl-protein hydrolase 1;Palmitoyl-protein thioesterase 1 Acid ceramidase;Acid ceramidase subunit apha;Acid ceramidase subunit beta	Arch;Copd Sec23ip Sec24b Gene Name Nsf;Skd2 Atp6c;Atp6c1;Atp6v1c Mem3;Vps35 Napg;Snapg Cp Atp6v1h Atp6m;Atp6v1d;Vatd Arl10b;Arl8a;Gie2 Gnb2;Gnb4 Ppt;Ppt1;RP23-232N16 Asah;Asah1	COPD S23IP Q80ZX0 Acc. Number NSF :VATC1 VPS35 SNAG CERU VATH VATD ARL8A GBB2 SPPT1 ASAH1	22.43644611 22.44803764 21.75531793 31.08857858 27.25772837 27.08244637 26.18923927 25.13069069 25.04805809 24.46355921 24.29841064 23.99565238 23.24753131 21.1299369
	905 970 1082 Rank 24 181 193 283 438 446 550 585 655 797 1166	Archain;Coatomer subunit delta;Delta-coat protein SEC23-interacting protein Sec24 related gene family, member B (S. cerevisiae) Protein Name N-ethylmaleimide-sensitive fusion protein;Suppressor of K(+) transport growth defect 2 Vacuolar proton pump subunit C 1;V-type proton ATPase subunit C 1 Maternal-embryonic 3;Vacuolar protein sorting-associated protein 35;Vesicle protein sorting 35 Gamma-soluble NSF attachment protein;N-ethylmaleimide-sensitive factor attachment protein gamma Ceruloplasmin;Ferroxidase Vacuolar proton pump subunit H;V-type proton ATPase subunit H Vacuolar proton pump subunit D;V-ATPase 28 kDa accessory protein;V-type proton ATPase subunit D ADP-ribosylation factor-like protein 10B;ADP-ribosylation factor-like protein 8A G protein subunit beta-2;Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2 Palmitoyl-protein hydrolase 1;Palmitoyl-protein thioesterase 1 Acid ceramidase;Acid ceramidase subunit alpha;Acid ceramidase subunit beta	Arch;Copd Sec23ip Sec24b Gene Name Nsf;Skd2 Atp6c;Atp6c1;Atp6v1c Mem3;Vps35 Napg;Snapg Cp Atp6v1h Atp6m;Atp6v1d;Vatd Arl10b;Arl8a;Gie2 Gnb2;Gnb4 Ppt;Ppt1;RP23-232N10 Asah;Asah1	COPD S23IP Q80ZX0 Acc. Number NSF VATC1 VPS35 SNAG CERU VATH VATD ARL8A GBB2 5PPT1 ASAH1 ASAH1	22.43644611 22.44803764 21.75531793 31.08857858 27.25772837 27.08244637 26.18923927 25.04805809 25.04805809 25.04805809 24.46355921 24.29841064 23.99565238 23.24753131 21.12993699
	905 970 1082 70 8 8 8 4 4 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5	Archain;Coatomer subunit delta;Delta-coat protein SEC23-interacting protein Sec24 related gene family, member B (S. cerevisiae) Protein Name N-ethylmaleimide-sensitive fusion protein;Suppressor of K(+) transport growth defect 2 Vacuolar proton pump subunit C 1;V-type proton ATPase subunit C 1 Maternal-embryonic 3;Vacuolar protein sorting-associated protein 35;Vesicle protein sorting 35 Gamma-soluble NSF attachment protein;N-ethylmaleimide-sensitive factor attachment protein gamma Ceruloplasmin;Ferroxidase Vacuolar proton pump subunit H;V-type proton ATPase subunit H Vacuolar proton pump subunit D;V-ATPase 28 kDa accessory protein;V-type proton ATPase subunit D ADP-ribosylation factor-like protein 10B;ADP-ribosylation factor-like protein 8A G protein subunit beta-2;Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2 Palmitoyl-protein hydrolase 1;Palmitoyl-protein thioesterase 1 Acid ceramidase;Acid ceramidase subunit alpha;Acid ceramidase subunit beta Protein Name Cithkrin harmenshire 1	Archi;Copd Sec23ip Sec24b Gene Name Nsf;Skd2 Atp6c;Atp6c1;Atp6v1c Mem3;Vps35 Napg;Snapg Cp Atp6v1h Atp6m;Atp6v1d;Vatd Arl10b;Arl8a;Gie2 Gnb2;Gnb4 Ppt;Ppt1;RP23-232N10 Asah;Asah1	COPD S23IP Q802X0 Acc. Number NSF VATC1 VPS35 SNAG CERU VATH VATD ARL8A GBB2 5PPT1 ASAH1 Acc. Number CEU	22.43644611 22.44803764 21.75531793 31.08857858 27.25772837 27.08244637 26.18923927 25.04805809 25.04805809 24.46355921 24.29841064 23.99565238 23.24753131 21.12993699 Avrg LFQ
	905 970 1082 Rank 24 181 193 283 438 446 550 585 655 797 1166 Rank 22	Archain;Coatomer subunit delta;Delta-coat protein SEC23-interacting protein Sec24 related gene family, member B (S. cerevisiae) Protein Name N-ethylmaleimide-sensitive fusion protein;Suppressor of K(+) transport growth defect 2 Vacuolar proton pump subunit C 1;V-type proton ATPase subunit C 1 Maternal-embryonic 3;Vacuolar protein sorting-associated protein 35;Vesicle protein sorting 35 Gamma-soluble NSF attachment protein;N-ethylmaleimide-sensitive factor attachment protein gamma Ceruloplasmin;Ferroxidase Vacuolar proton pump subunit H;V-type proton ATPase subunit H Vacuolar proton pump subunit D;V-ATPase 28 kDa accessory protein;V-type proton ATPase subunit D ADP-ribosylation factor-like protein 10B;ADP-ribosylation factor-like protein 8A G protein subunit beta-2;Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2 Palmitoyl-protein hydrolase 1;Palmitoyl-protein thioesterase 1 Acid ceramidase;Acid ceramidase subunit alpha;Acid ceramidase subunit beta Clathrin, heavy polypeptide (Hc);Clathrin heavy chain 1	Arch1;Copd Sec23ip Sec24b Gene Name Nsf;Skd2 Atp6c;Atp6c1;Atp6v1c Mem3;Vps35 Napg;Snapg Cp Atp6v1h Atp6m;Atp6v1d;Vatd Arl10b;Arl8a;Gie2 Gnb2;Gnb4 Ppt;Ppt1;RP23-232N16 Asah;Asah1 Gene Name Cltc;RP23-366M19.3-0	COPD S23IP Q802X0 Acc. Number NSF VATC1 VPS35 SNAG CERU VATD VATD ARL8A GBB2 SPPT1 ASAH1 Acc. Number (CLH OCCH	22. 43644611 22. 44803764 21. 75531793 31.08857858 27.25772837 27.08244637 26.18923927 25.13069069 25.04805809 24.46355921 24.29841064 23.99565238 23.24753131 21.12993699 Avrg LFQ 31.16046741
	905 970 1082 24 181 193 283 438 446 550 585 655 505 585 655 797 1166 22 Rank 22 89	Archain;Coatomer subunit delta;Delta-coat protein SEC23-interacting protein Sec24 related gene family, member B (S. cerevisiae) Protein Name N-ethylmaleimide-sensitive fusion protein;Suppressor of K(+) transport growth defect 2 Vacuolar proton pump subunit C 1;V-type proton ATPase subunit C 1 Maternal-embryonic 3;Vacuolar protein sorting-associated protein 35;Vesicle protein sorting 35 Gamma-soluble NSF attachment protein;N-ethylmaleimide-sensitive factor attachment protein gamma Ceruloplasmin;Ferroxidase Vacuolar proton pump subunit H;V-type proton ATPase subunit H Vacuolar proton pump subunit D;V-ATPase 28 kDa accessory protein;V-type proton ATPase subunit D ADP-ribosylation factor-like protein 10B;ADP-ribosylation factor-like protein 8A G protein subunit beta-2;Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2 Palmitoyl-protein hydrolase 1;Palmitoyl-protein thioesterase 1 Acid ceramidase;Acid ceramidase subunit alpha;Acid ceramidase subunit beta Clathrin, heavy polypeptide (Hc);Clathrin heavy chain 1 Crmp1 protein	Arcn1;Copd Sec23ip Sec24b Gene Name Nsf;Skd2 Atp6c;Atp6c1;Atp6v1c Mem3;Vps35 Napg;Snapg Cp Atp6v1h Atp6m;Atp6v1d;Vatd Arl10b;Arl8a;Gie2 Gnb2;Gnb4 Ppt;Ppt1;RP23-232N16 Asah;Asah1 Gene Name Cltc;RP23-366M19.3-0 Crmp1	COPD \$23IP Q80ZXO Acc. Number NSF VATC1 VPS35 SNAG CERU VATH VATD ARL8A GB82 SPPT1 ASAH1 ASAH1 ACC. Number ICLH Q6P1J1	22.43644611 22.44803764 21.75531793 31.08857858 27.25772837 27.08244637 26.18923927 25.13069069 25.04805809 24.46355921 24.29841064 23.99565238 23.24753131 21.12993699 Avrg LFQ 31.16046741 28.66768859
	905 970 1082 Rank 24 181 193 283 438 446 550 585 655 797 1166 555 797 1166 22 89 96	Archain;Coatomer subunit delta;Delta-coat protein SEC23-interacting protein Sec24 related gene family, member B (S. cerevisiae) Protein Name N-ethylmaleimide-sensitive fusion protein;Suppressor of K(+) transport growth defect 2 Vacuolar proton pump subunit C 1;V-type proton ATPase subunit C 1 Maternal-embryonic 3;Vacuolar protein sorting-associated protein 35;Vesicle protein sorting 35 Gamma-soluble NSF attachment protein;N-ethylmaleimide-sensitive factor attachment protein gamma Ceruloplasmin;Ferroxidase Vacuolar proton pump subunit H;V-type proton ATPase subunit H Vacuolar proton pump subunit D;V-ATPase 28 kDa accessory protein;V-type proton ATPase subunit D ADP-ribosylation factor-like protein 10B;ADP-ribosylation factor-like protein 8A G protein subunit beta-2;Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2 Palmitoyl-protein hydrolase 1;Palmitoyl-protein thioesterase 1 Acid ceramidase;Acid ceramidase subunit alpha;Acid ceramidase subunit beta Clathrin, heavy polypeptide (Hc);Clathrin heavy chain 1 Crmp1 protein Calcium-dependent activator protein for secretion 1;Calcium-dependent secretion activator 1	Arcn1;Copd Sec23ip Sec24b Gene Name Nsf;Skd2 Atp6c;Atp6c1;Atp6v1c Mem3;Vps35 Napg;Snapg Cp Atp6v1h Atp6m;Atp6v1d;Vatd Arl10b;Arl8a;Gie2 Gnb2;Gnb4 Ppt;Ppt1;RP23-232N16 Asah;Asah1 Gene Name Cltc;RP23-366M19.3-0 Crmp1 Cadps;Caps;Caps1;Kiaz	COPD \$231P Q80ZX0 Acc. Number NSF VATC1 VPS35 SNAG CERU VATH VATD ARL8A GBB2 SPPT1 ASAH1 ACC. Number (CLH Q6P1J1 aCAPS1	22. 43644611 22. 44803764 21. 75531793 31.08857858 27.25772837 27.08244637 26.18923927 25.13069069 25.04805809 24.46355921 24.29841064 23.99565238 23.24753131 21.12993699 Avrg LFQ 31.16046741 28.66768859 28.59765052
	905 970 1082 Rank 24 181 193 283 438 446 550 585 655 797 1166 Rank 22 89 89 66 100	Archain;Coatomer subunit delta;Delta-coat protein SEC23-interacting protein Sec24 related gene family, member B (S. cerevisiae) Protein Name N-ethylmaleimide-sensitive fusion protein;Suppressor of K(+) transport growth defect 2 Vacuolar proton pump subunit C 1;V-type proton ATPase subunit C 1 Maternal-embryonic 3;Vacuolar protein sorting-associated protein 35;Vesicle protein sorting 35 Gamma-soluble NSF attachment protein;N-ethylmaleimide-sensitive factor attachment protein gamma Ceruloplasmin;Ferroxidase Vacuolar proton pump subunit H;V-type proton ATPase subunit H Vacuolar proton pump subunit D;V-ATPase 28 kDa accessory protein;V-type proton ATPase subunit D ADP-ribosylation factor-like protein 10B;ADP-ribosylation factor-like protein 8A G protein subunit beta-2;Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2 Palmitoyl-protein hydrolase 1;Palmitoyl-protein thioesterase 1 Acid ceramidase;Acid ceramidase subunit alpha;Acid ceramidase subunit beta Clathrin, heavy polypeptide (Hc);Clathrin heavy chain 1 Crmp1 protein Clathrin, heavy polypeptide (Hc);Clathrin for secretion 1;Calcium-dependent secretion activator 1 ADP-ribosylation factor 3;ADP-ribosylation factor 1	Arch1;Copd Sec23ip Sec24b Gene Name Nsf;Skd2 Atp6c;Atp6c1;Atp6v1c Mem3;Vps35 Napg;Snapg Cp Atp6v1h Atp6m;Atp6v1d;Vatd Ar10b;Arl8a;Gie2 Gnb2;Gnb4 Ppt;Ppt1;RP23-232N16 Asah;Asah1 Gene Name Cltc;RP23-366M19.3-0 Crmp1 Cadps;Caps;Caps1;Kiaa Arf3;Arf1	COPD \$231P Q802X0 Acc. Number NSF VATC1 VPS35 SNAG CERU VATH VATD ARL8A GBB2 5PPT1 ASAH1 ACc. Number (CLH Q6P1J11 3CAPS1 ARF3	22. 43644611 22. 44803764 21. 75531793 31.08857858 27.25772837 27.08244637 26.18923927 25.04805809 25.04805809 25.04805809 24.46355921 24.29841064 23.99565238 23.24753131 21.12993699 31.16046741 28.66768859 28.59765052 28.54470728
	905 970 1082 Rank 24 181 193 283 438 438 446 550 585 655 797 1166 Kank 22 89 96 100 100 160	Archain;Coatomer subunit delta;Delta-coat protein SEC23-interacting protein Sec24 related gene family, member B (S. cerevisiae) Protein Name N-ethylmaleimide-sensitive fusion protein;Suppressor of K(+) transport growth defect 2 Vacuolar proton pump subunit C 1;V-type proton ATPase subunit C 1 Maternal-embryonic 3;Vacuolar protein sorting-associated protein 35;Vesicle protein sorting 35 Gamma-soluble NSF attachment protein;N-ethylmaleimide-sensitive factor attachment protein gamma Ceruloplasmin;Ferroxidase Vacuolar proton pump subunit H;V-type proton ATPase subunit H Vacuolar proton pump subunit D;V-ATPase 28 kDa accessory protein;V-type proton ATPase subunit D ADP-ribosylation factor-like protein 10B;ADP-ribosylation factor-like protein 8A G protein subunit beta-2;Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2 Palmitoyl-protein hydrolase 1;Palmitoyl-protein thioesterase 1 Acid ceramidase;Acid ceramidase subunit alpha;Acid ceramidase subunit beta Clathrin, heavy polypeptide (Hc);Clathrin heavy chain 1 Crmp1 protein Clathrin, heavy polypeptide (HC);Clathrin for secretion 1;Calcium-dependent secretion activator 1 ADP-ribosylation factor 3;ADP-ribosylation factor 1 14-3-3 protein psilon	Arcn1;Copd Sec23ip Sec24b Gene Name Nsf;Skd2 Atp6c;Atp6c1;Atp6v1c Mem3;Vps35 Napg;Snapg Cp Atp6v1h Atp6m;Atp6v1d;Vatd Arl10b;Arl8a;Gie2 Gnb2;Gnb4 Ppt;Ppt1;RP23-232N16 Asah;Asah1 Gene Name Cltc;RP23-366M19.3-0 Crmp1 Cadps;Caps;Caps1;Kiaz Arf3;Arf1 Ywhae	COPD \$231P Q802X0 Acc. Number NSF VATC1 VPS35 SNAG CERU VATD VATD ARL8A GBB2 5PPT1 ASAH1 ACC. Number (CLH Q6PIJ1 aCAPS1 ARF3 1433E	22. 43644611 22. 44803764 21. 75531793 31.08857858 27.25772837 27.08244637 26.18923927 25.04805809 24.46355921 24.29841064 23.99565238 23.24753131 21.12993699 Avrg LFQ 31.16046741 28.56768595 28.59765052 28.54470728 27.49644716
	905 970 1082 Rank 24 181 193 283 438 438 438 438 446 550 585 655 797 1166 585 655 797 1160 Rank 22 89 96 100 1600	Archain;Coatomer subunit delta;Delta-coat protein SEC23-interacting protein Sec24 related gene family, member B (S. cerevisiae)	Arcn1;Copd Sec23ip Sec24b Gene Name Nsf;Skd2 Atp6c;Atp6c1;Atp6v1c Mem3;Vps35 Napg;Snapg Cp Atp6v1h Atp6m;Atp6v1d;Vatd Arl10b;Arl8a;Gie2 Gnb2;Gnb4 Ppt;Ppt1;RP23-232N16 Asah;Asah1 Gene Name Cltc;RP23-366M19.3-0 Crmp1 Cadps;Caps;Caps1;Kiaz Arf3;Arf1 Ywhae Ywhag	COPD \$23IP Q80ZXO Acc. Number NSF VATC1 VPS35 SNAG CERU VATD ARL8A GBB2 SPPT1 ASAH1 ACC. Number ICLH Q6P1J1 GAPS1 ARF3 1433E 1433E	22. 43644611 22. 44803764 21. 75531793 21. 75531793 31.08857858 27. 25772837 27. 08244637 26. 18923927 25. 13069069 25. 04805809 24. 46355921 24. 29841064 23. 99565238 23. 24753131 21. 12993699 24. 66768859 28. 59765052 28. 59765052 28. 594470728 27. 49644716 27. 30222692
	905 970 1082 Rank 24 181 193 283 438 446 550 585 655 797 1166 555 797 1166 889 96 100 100 100 100 177 262	Archain;Coatomer subunit delta;Delta-coat protein SEC23-interacting protein Sec24 related gene family, member B (S. cerevisiae) Protein Name N-ethylmaleimide-sensitive fusion protein;Suppressor of K(+) transport growth defect 2 Vacuolar proton pump subunit C 1;V-type proton ATPase subunit C 1 Maternal-embryonic 3;Vacuolar protein sorting-associated protein 35;Vesicle protein sorting 35 Gamma-soluble NSF attachment protein;N-ethylmaleimide-sensitive factor attachment protein gamma Ceruloplasmin;Ferroxidase Vacuolar proton pump subunit H;V-type proton ATPase subunit H Vacuolar proton pump subunit D;V-ATPase 28 kDa accessory protein;V-type proton ATPase subunit D ADP-ribosylation factor-like protein 10B;ADP-ribosylation factor-like protein 8A G protein subunit beta-2;Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2 Palmitoyl-protein hydrolase 1;Palmitoyl-protein thioesterase 1 Acid ceramidase;Acid ceramidase subunit alpha;Acid ceramidase subunit beta Clathrin, heavy polypeptide (HC);Clathrin heavy chain 1 Crmp1 protein Clathrin, heavy polypeptide (HC);Clathrin heavy chain 1 Crmp1 protein Clathrin, heavy solypeptide (HC);Clathrin heavy chain 1 ADP-ribosylation factor 3;ADP-ribosylation factor 1 AA-3-3 protein gamma;14-3-3 protein gamma, N-terminally processed Annexin A7;Annexin VII;Annexin-7;Synexin	Archi,Copd Sec23ip Sec24b Gene Name Nsf;Skd2 Atp6c;Atp6c1;Atp6v1c Mem3;Vps35 Napg;Snapg Cp Atp6v1h Atp6m;Atp6v1d;Vatd Arl10b;Arl8a;Gie2 Gnb2;Gnb4 Ppt;Ppt1;RP23-232N10 Asah;Asah1 Gene Name Cltc;RP23-366M19.3-0 Crmp1 Cadps;Caps;Caps1;Kiaz Arf3;Arf1 Ywhae Ywhag Anx7;Anxa7	COPD \$23IP Q80ZX0 Acc. Number NSF VATC1 VPS35 SNAG CERU VATH VATD ARL8A GBB2 SPPT1 ASAH1 ACC. Number (CLH Q6P1J1 Q6P1 Q6P1J1 Q6P1 Q	22. 43644611 22. 44803764 21. 75531793 21. 75531793 21. 75531793 21. 08857858 27. 25772837 27. 08244637 26. 18923927 25. 13069069 25. 04805809 24. 46355921 24. 29841064 23. 99565238 23. 24753131 21. 12993699 Avrg LFQ 31. 160467411 28. 66768859 28. 59765052 28. 54470728 27. 49644716 27. 30222692 26. 3141609
	905 970 1082 Rank 24 181 193 283 438 446 550 585 655 797 1166 Kank 22 89 96 100 160 160 177 262 274	Archain;Coatomer subunit delta;Delta-coat protein SEC23-interacting protein Sec24 related gene family, member B (S. cerevisiae) Protein Name N-ethylmaleimide-sensitive fusion protein;Suppressor of K(+) transport growth defect 2 Vacuolar proton pump subunit C 1;V-type proton ATPase subunit C 1 Maternal-embryonic 3;Vacuolar protein sorting-associated protein 35;Vesicle protein sorting 35 Gamma-soluble NSF attachment protein;N-ethylmaleimide-sensitive factor attachment protein gamma Ceruloplasmin;Ferroxidase Vacuolar proton pump subunit H;V-type proton ATPase subunit H Vacuolar proton pump subunit D;V-ATPase 28 kDa accessory protein;V-type proton ATPase subunit D ADP-ribosylation factor-like protein 10B;ADP-ribosylation factor-like protein 8A G protein subunit beta-2;Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2 Palmitoyl-protein hydrolase 1;Palmitoyl-protein thioesterase 1 Acid ceramidase;Acid ceramidase subunit alpha;Acid ceramidase subunit beta Clathrin, heavy polypeptide (Hc);Clathrin heavy chain 1 Crmp1 protein Clathrin, heavy polypeptide (Hc);Clathrin for secretion 1;Calcium-dependent secretion activator 1 ADP-ribosylation factor 3;ADP-ribosylation factor 1 14-3-3 protein gamma;14-3-3 protein gamma, N-terminally processed Annexin A7;Annexin VII;Annexin-7;Synexin 14-3-3 protein partici heat/alpha. N-terminally processed	Arch;Copd Sec23ip Sec24b Gene Name Nsf;Skd2 Atp6c;Atp6c1;Atp6v1c Mem3;Vps35 Napg;Snapg Cp Atp6v1h Atp6m;Atp6v1d;Vatd Arl10b;Arl8a;Gie2 Gnb2;Gnb4 Ppt;Ppt1;RP23-232N10 Asah;Asah1 Cene Name Cltc;RP23-366M19.3-0 Crmp1 Cadps;Caps;Caps1;Kiaa Arf3;Arf1 Ywhae Ywhag Anx7;Anxa7 Ywhab;RP33-321M14	COPD \$23IP Q802X0 Acc. Number NSF VATC1 VPS35 SNAG CERU VATD ARL8A GBB2 5PPT1 ASAH1 Acc. Number (CLH Q6P1J1 Q6P1 Q6P1J1 Q6P1 Q	22. 43644611 22. 44803764 21. 75531793 31.08857858 27. 25772837 27.08244637 26. 18923927 25. 13069063 25. 04805809 25. 04805809 24.46355921 24.29841064 23.99565238 23.24753131 21.12993699 Avrg LFQ 31.16046741 28.66768859 28.59765052 28.59765052 28.54470728 27.49644716 27.49644716 27.49644716 27.49644716 27.49644716
	905 970 1082 Rank 24 181 193 283 438 446 550 585 655 797 1166 Rank 22 89 6 Rank 22 89 96 100 160 177 262 274	Archain;Coatomer subunit delta;Delta-coat protein SEC23-interacting protein Sec24 related gene family, member B (S. cerevisiae) Protein Name N-ethylmaleimide-sensitive fusion protein;Suppressor of K(+) transport growth defect 2 Vacuolar proton pump subunit C 1;V-type proton ATPase subunit C 1 Maternal-embryonic 3;Vacuolar protein sorting-associated protein 35;Vesicle protein sorting 35 Gamma-soluble NSF attachment protein;N-ethylmaleimide-sensitive factor attachment protein gamma Ceruloplasmin;Ferroxidase Vacuolar proton pump subunit H;V-type proton ATPase subunit H Vacuolar proton pump subunit D;V-ATPase 28 kDa accessory protein;V-type proton ATPase subunit D ADP-ribosylation factor-like protein 10B;ADP-ribosylation factor-like protein 8A G protein subunit beta-2;Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2 Palmitoyl-protein hydrolase 1;Palmitoyl-protein thioesterase 1 Acid ceramidase;Acid ceramidase subunit alpha;Acid ceramidase subunit beta Clathrin, heavy polypeptide (Hc);Clathrin heavy chain 1 Crmp1 protein Clathrin, heavy polypeptide (Hc);Clathrin for secretion 1;Calcium-dependent secretion activator 1 ADP-ribosylation factor 3;ADP-ribosylation factor 1 14-3-3 protein gamma;14-3-3 protein gamma, N-terminally processed Annexin A7;Annexin VII;Annexin-7;Synexin 14-3-3 protein beta/alpha;14-3-3 protein beta/alpha, N-terminally processed Neuroendorcine-specific proteins Peticulop-1	Arch1;Copd Sec23ip Sec24b Gene Name Nsf;Skd2 Atp6c;Atp6c1;Atp6v1c Mem3;Vps35 Napg;Snapg Cp Atp6v1h Atp6m;Atp6v1d;Vatd Ar10b;Arl8a;Gie2 Gnb2;Gnb4 Ppt;Ppt1;RP23-232N16 Asah;Asah1 Gene Name Cltc;RP23-36M19.3-0 Crmp1 Cadps;Caps;Caps1;Kiaz Arf3;Arf1 Ywhae Ywhag Anx7;Anxa7 Ywhab;RP23-321M14. Nsn:Rtn1	COPD S23IP Q802X0 Acc. Number NSF VATC1 VPS35 SNAG CERU VATD VATD ARL8A GBB2 5PPT1 ASAH1 ACC. Number (CLH Q6P1J11 3CAPS1 4X35 14336 ANXA7 11433B RTN1	22.43644611 22.44803764 21.75531793 31.08857858 27.25772837 27.08244637 26.18923927 25.04805809 25.04805809 24.46355921 24.29841064 23.99565238 23.24753131 21.12993699 28.59765052 28.59470728 27.49644716 27.30222692 26.2413089 26.2015421
	905 970 1082 Rank 24 181 193 283 438 438 438 450 585 655 797 1166 Rank 22 89 96 100 100 160 177 262 274 277 216	Archain;Coatomer subunit delta;Delta-coat protein SEC23-interacting protein Sec24 related gene family, member B (S. cerevisiae) Protein Name N-ethylmaleimide-sensitive fusion protein;Suppressor of K(+) transport growth defect 2 Vacuolar proton pump subunit C 1;V-type proton ATPase subunit C 1 Maternal-embryonic 3;Vacuolar protein sorting-associated protein 35;Vesicle protein sorting 35 Gamma-soluble NSF attachment protein;N-ethylmaleimide-sensitive factor attachment protein gamma Ceruloplasmin;Ferroxidase Vacuolar proton pump subunit H;V-type proton ATPase subunit H Vacuolar proton pump subunit D;V-ATPase 28 kDa accessory protein;V-type proton ATPase subunit D ADP-ribosylation factor-like protein 10B;ADP-ribosylation factor-like protein 8A G protein subunit beta-2;Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2 Palmitoyl-protein hydrolase 1;Palmitoyl-protein thioesterase 1 Acid ceramidase;Acid ceramidase subunit alpha;Acid ceramidase subunit beta Cathrin, heavy polypeptide (Hc);Clathrin heavy chain 1 Crmp1 protein Clathrin, heavy polypeptide (HC);Clathrin heavy chain 1 Crmp1 protein Clathrin, heavy polypeptide (HC);Clathrin for secretion 1;Calcium-dependent secretion activator 1 ADP-ribosylation factor 3;ADP-ribosylation factor 1 14-3-3 protein gamma;14-3-3 protein gamma, N-terminally processed Annexin A7;Annexin VII;Annexin-7;Synexin 14-3-3 protein beta/alpha;14-3-3 protein beta/alpha, N-terminally processed Neuroendocrine-specific protein;Reticulon-1	Arcn1;Copd Sec23ip Sec24b Gene Name Nsf;Skd2 Atp6c;Atp6c1;Atp6v1c Mem3;Vps35 Napg;Snapg Cp Atp6v1h Atp6m;Atp6v1d;Vatd Ar110b;Arl8a;Gie2 Gnb2;Gnb4 Ppt;Ppt1;RP23-232N10 Asah;Asah1 Gene Name Cltc;RP23-366M19.3-0 Crmp1 Cadps;Caps;Caps1;Kiaz Arf3;Arf1 Ywhae Ywhag Anx7;Anxa7 Ywhab;RP23-321M14. Nsp;Rtn1	COPD S23IP Q802X0 Acc. Number NSF VATC1 VPS35 SNAG CERU VATD VATD ARL8A GBB2 5PPT1 ASAH1 ACC. Number ICLH Q6PIJ1 aCAPS1 ARF3 1433E 1433E 1433B RTN1 ACCE 1433B	22.43644611 22.44803764 21.75531793 31.08857858 27.25772837 27.08244637 26.18923927 25.13069069 25.04805809 24.46355921 24.29841064 23.99565238 23.24753131 21.12993699 000000000000000000000000000000000
	905 970 1082 Rank 24 181 193 283 438 446 550 585 655 797 1166 555 797 1166 797 1160 797 1160 777 262 274 277 316	Archain;Coatomer subunit delta;Delta-coat protein SEC23-interacting protein Sec24 related gene family, member B (S. cerevisiae)	Arcn1;Copd Sec23ip Sec24b Gene Name Nsf;Skd2 Atp6c;Atp6c1;Atp6v1c Mem3;Vps35 Napg;Snapg Cp Atp6v1h Atp6m;Atp6v1d;Vatd Arl10b;Arl8a;Gie2 Gnb2;Gnb4 Ppt;Ppt1;RP23-232N16 Asah;Asah1 Gene Name Cltc;RP23-366M19.3-0 Crmp1 Cadps;Caps;Caps1;Kiaz Arf3;Arf1 Ywhae Ywhag Anx7;Anxa7 Ywhab;RP23-321M14. Nsp;Rtn1 Agfg1;Hrb;Rip A-fr	COPD S23IP Q80ZX0 Acc. Number NSF VATC1 VPS35 SNAG CERU VATH VATD ARL8A GBB2 SPPT1 ASAH1 ACC. Number ICLH Q6P1J1 GAPS1 ARF3 14336 14336 ANXA7 14338 RTN1 AGFG1 ADDC	22.43644611 22.44803764 21.75531793 31.08857858 27.25772837 27.08244637 26.18923927 25.13069069 25.04805809 24.46355921 24.29841064 23.99565238 23.24753131 21.12993699 28.66768859 28.66768859 28.59765052 28.54470728 27.49644716 27.30222692 26.3141609 26.24413089 26.21015431 25.9235514
	905 970 1082 Rank 24 181 193 283 438 446 550 585 655 797 1166 555 797 1166 22 89 96 100 160 100 160 177 262 274 277 316 320	Archan;Coatomer subunit delta;Delta-coat protein SEC23-interacting protein Sec24 related gene family, member B (S. cerevisiae) Verthylmaleimide-sensitive fusion protein;Suppressor of K(+) transport growth defect 2 Vacuolar proton pump subunit C 1;V-type proton ATPase subunit C 1 Maternal-embryonic 3;Vacuolar protein sorting-associated protein 35;Vesicle protein sorting 35 Gamma-soluble NSF attachment protein;N-ethylmaleimide-sensitive factor attachment protein gamma Ceruloplasmin;Ferroxidase Vacuolar proton pump subunit H;V-type proton ATPase subunit H Vacuolar proton pump subunit D;V-ATPase 28 kDa accessory protein;V-type proton ATPase subunit D ADP-ribosylation factor-like protein 10B;ADP-ribosylation factor-like protein 8A G protein subunit beta-2;Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2 Palmitoyl-protein hydrolase 1;Palmitoyl-protein thioesterase 1 Acid ceramidase;Acid ceramidase subunit alpha;Acid ceramidase subunit beta Clathrin, heavy polypeptide (Hc);Clathrin heavy chain 1 Crmp1 protein Calcium-dependent activator protein for secretion 1;Calcium-dependent secretion activator 1 ADP-ribosylation factor 3;ADP-ribosylation factor 1 14-3-3 protein gamma;14-3-3 protein gamma, N-terminally processed Annexin A7;Annexin VII;Annexin-7;Synexin 14-3-3 protein beta/alpha;14-3-3 protein beta/alpha, N-terminally processed Neuroendocrine-specific protein;Reticulon-1 Arf-GAP domain and FG repeats-containing protein 1;HIV-1 Rev-binding protein homolog ADP-ribosylation factor 5	Arch;Copd Sec23ip Sec24b Gene Name Nsf;Skd2 Atp6c;Atp6c1;Atp6v1c Mem3;Vps35 Napg;Snapg Cp Atp6v1h Atp6m;Atp6v1d;Vatd Arl10b;Arl8a;Gie2 Gnb2;Gnb4 Ppt;Ppt1;RP23-232N16 Asah;Asah1 Gene Name Cltc;RP23-366M19.3-0 Crmp1 Cadps;Caps;Caps1;Kiaz Arf3;Arf1 Ywhae Ywhag Anx7;Anxa7 Ywhab;RP23-321M14. Nsp;Rtn1 Agfg1;Hrb;Rip Arf5	COPD \$23IP Q80ZX0 Acc. Number NSF VATC1 VPS35 SNAG CERU VATH ARL8A GBB2 5PPT1 ARL8A GBB2 5PPT1 ARL8A GBB2 5PPT1 ACC. Number (CH CGP1J1 CCH CGP1J1 CCH CGP1J1 CCH CAPS1 ARF3 14336 ANXA7 11433B 14336 ANXA7 11433B 14336 ANXA7 11433B 14336 ANXA7 11433B CERU CAPS1 CERU CCH CCH CCH CCH CCH CCH CCH CC	22. 43644611 22. 44803764 21. 75531793 31.08857858 27.25772837 27.08244637 26.18923927 25.13069069 25.04805809 24.46355921 24.29841064 23.99565238 23.24753131 21.12993699 Avrg LFQ 31.16046741 28.66768859 28.59765052 28.54470728 27.49644716 27.30222692 26.3141609 26.24413089 26.2413089 26.2413089 26.2413089
	905 970 1082 Rank 24 181 193 283 438 446 550 585 655 797 1166 89 655 797 1166 40 22 89 96 100 160 177 262 274 277 316 320 325	Archan;Coatomer subunit delta;Delta-coat protein SEC23-interacting protein Sec24 related gene family, member B (S. cerevisiae)	Arch1;Copd Sec23ip Sec24b Gene Name Nsf;Skd2 Atp6c;Atp6c1;Atp6v1c Mem3;Vps35 Napg;Snapg Cp Atp6v1h Atp6m;Atp6v1d;Vatd Arl10b;Arl8a;Gie2 Gnb2;Gnb4 Ppt;Ppt1;RP23-232N10 Asah;Asah1 Gene Name Cltc;RP23-366M19.3-0 Crmp1 Cadps;Caps;Caps1;Kiaa Arf3;Arf1 Ywhae Ywhag Anx7;Anxa7 Ywhab;RP23-321M14. Nsp;Rtn1 Agfg1;Hrb;Rip Arf5 Epb4.9	COPD S23IP Q802X0 Acc. Number NSF VATC1 VPS35 SNAG CERU VATD ARL8A GBB2 5PPT1 ASAH1 ACC. Number (CLH Q6P111 3CAPS1 433E 1433E 1433B 1433B 1433B RTN1 AGFG1 ARF5 Q9WVM2	22.43644611 22.44803764 21.75531793 31.08857858 27.25772837 27.08244637 26.18923927 25.04805809 25.04805809 25.04805809 24.46355921 24.29841064 23.99565238 23.24753131 21.12993699 31.16046741 28.66768859 28.59765052 28.59470728 27.49644716 27.30222692 26.2413089 26.21015431 25.9235514 25.90907747 25.82761432
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	905 970 1082 Rank 24 181 193 283 438 446 550 585 655 797 1166 555 797 1166 797 1160 797 1160 777 262 274 277 316 320 325 342 360	Archan;Coatomer subunit delta;Delta-coat protein SEC23-interacting protein Sec24 related gene family, member B (S. cerevisiae) Protein Name N-ethylmaleinide-sensitive fusion protein;Suppressor of K(+) transport growth defect 2 Vacuolar proton pump subunit C 1;V-type proton ATPase subunit C 1 Maternal-embryonic 3;Vacuolar protein sorting-associated protein 35;Vesicle protein sorting 35 Gamma-soluble NSF attachment protein;N-ethylmaleimide-sensitive factor attachment protein gamma Ceruloplasmin;Ferroxidase Vacuolar proton pump subunit H;V-type proton ATPase subunit H Vacuolar proton pump subunit D;V-ATPase 28 kDa accessory protein;V-type proton ATPase subunit D ADP-ribosylation factor-like protein 10B;ADP-ribosylation factor-like protein 8A G protein subunit beta-2;Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2 Palmitoyl-protein hydrolase 1;Palmitoyl-protein thioesterase 1 Acid ceramidase;Acid ceramidase subunit alpha;Acid ceramidase subunit beta Others Clathrin, heavy polypeptide (Hc);Clathrin heavy chain 1 Crmp1 protein Clathrin, heavy polypeptide (Hc);Clathrin heavy chain 1 Crmp1 protein Clathrin, heavy polypeptide (Hc);Clathrin heavy chain 1 Crmp1 protein 14-3-3 protein gamma;14-3-3 protein gamma, N-terminally processed Annexin A7;Annexin VII;Annexin-7;Synexin 14-3-3 protein beta/alpha;14-3-3 protein beta/alpha, N-terminally processed Neuroendocrine-specific protein;Reticulon-1 Arf-GAP domain and FG repeats-containing protein 1;HIV-1 Rev-binding protein homolog ADP-ribosylation factor 5 Dematin 48 kDa subuni;Erythrocyte protein band 4.9;Putative uncharacterized protein GTP-binding protein SAR1b Lysosomal-trafficking regulator 2;Neurobeachin	Arcn1;Copd Sec23ip Sec24b Gene Name Nsf;Skd2 Atp6c;Atp6c1;Atp6v1c Mem3;Vps35 Napg;Snapg Cp Atp6v1h Atp6m;Atp6v1d;Vatd Arl10b;Arl8a;Gie2 Gnb2;Gnb4 Ppt;Ppt1;RP23-232N16 Asah;Asah1 Gene Name Cltc;RP23-366M19.3-0 Crmp1 Cadps;Caps;Caps1;Kiaz Arf3;Arf1 Ywhae Ywhag Anx7;Anxa7 Ywhab;RP23-321M14. Nsp;Rtn1 Agfg1;Hrb;Rip Arf5 Epb4.9 Sar1b;Sara1b;Sara2 Lyst2;Nbea	COPD S23IP Q80ZXO Acc. Number NSF VATC1 VPS35 SNAG CERU VATH VATD ARL8A GBB2 SPPT1 ASAH1 CERU CCRU VATH VATD ARL8A GBB2 SPPT1 ASAH1 CERU CUH Q6P1J1 GAPS1 ARF3 14336 14336 ANXA7 14338 RTN1 AGFG1 AGFG1 ARF5 Q9WVM2 SAR18 NBEA	22. 43644611 22. 44803764 21. 75531793 31.08857858 27. 25772837 27. 08244637 26. 18923927 25. 13069069 25. 04805809 24. 46355921 24. 29841064 23. 29565238 23. 24753131 21. 12993699 24. 66768859 28. 59765052 28. 54470728 27. 49644716 27. 30222692 26. 3141609 26. 24413089 26. 21015431 25. 9235514 25. 59235514 25. 59235514 25. 57787526
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	905 970 1082 Rank 24 181 193 283 438 446 550 585 655 797 1166 555 797 1166 797 1160 797 1160 777 262 274 277 3160 325 342 342 342 342 342 342 342 342 342 342	Archan;Coatomer subunit delta;Delta-coat protein SEC23-interacting protein Sec24 related gene family, member B (S. cerevisiae) Protein Name N-ethylmaleimide-sensitive fusion protein;Suppressor of K(+) transport growth defect 2 Vacuolar proton pump subunit C 1;V-type proton ATPase subunit C 1 Maternal-embryonic 3;Vacuolar protein sorting-associated protein 35;Vesicle protein sorting 35 Gamma-soluble NSF attachment protein;N-ethylmaleimide-sensitive factor attachment protein gamma Ceruloplasmin;Ferroxidase Vacuolar proton pump subunit H;V-type proton ATPase subunit H Vacuolar proton pump subunit D;V-ATPase 28 kDa accessory protein;V-type proton ATPase subunit D ADP-ribosylation factor-like protein 108;ADP-ribosylation factor-like protein 8A G protein subunit beta-2;Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2 Palmitoyl-protein hydrolase 1;Palmitoyl-protein thioesterase 1 Acid ceramidase;Acid ceramidase subunit alpha;Acid ceramidase subunit beta Clathrin, heavy polypeptide (HC);Clathrin heavy chain 1 Crmp1 protein Calcium-dependent activator protein for secretion 1;Calcium-dependent secretion activator 1 ADP-ribosylation factor 3;ADP-ribosylation factor 1 14-33 protein gamma;14-3-3 protein gamma, N-terminally processed Annexin A7;Annexin VII;Annexin-7;Synexin 14-33 protein deta/alpha;4-3-3 protein beta/alpha, N-terminally processed Neuroendocrine-specific protein;Reticulon-1 Arf-GAP domain and FG repeats-containing protein 1;HIV-1 Rev-binding protein homolog ADP-ribosylation factor 5 Dematin 48 kDa subunit;Erythrocyte protein band 4.9;Putative uncharacterized protein GTP-binding protein SA1b Lysosomal-trafficking regulator 2;Neurobeachin 14-3-3 protein eta Coronin-1A;Coronin-like protein A;Coronin-like protein p57 Dmx-like 2:DmX-like protein 2;Rabconnertin-3	Arch;Copd Sec23ip Sec24b Gene Name Nsf;Skd2 Atp6c;Atp6c1;Atp6v1c Mem3;Vps35 Napg;Snapg Cp Atp6v1h Atp6m;Atp6v1d;Vatd Arl10b;Arl8a;Gie2 Gnb2;Gnb4 Ppt;Ppt1;RP23-232N10 Asah;Asah1 Gene Name Cltc;RP23-366M19.3-0 Crmp1 Cadps;Caps;Caps1;Kiaz Arf3;Arf1 Ywhag Anx7;Anxa7 Ywhab;RP23-321M14. Nsp;Rtn1 Agfg1;Hrb;Rip Arf5 Epb4.9 Sar1b;Sara1b;Sara2 Lyst2;Nbea Ywhah Coro1;Coro1a	COPD S23IP Q80ZXO Acc. Number NSF VATC1 VPS35 SNAG CERU VATH VATD ARL8A GBB2 SPPT1 ASAH1 ACC. Number ICLH Q6P1J1 GAPS1 ARF3 1433E 1433E 1433B RTN1 AGFG11 AGFG1 ARF5 Q9WVM2 SAR1B NBEA 1433F COR1A CDC42 IDMX12	22.43644611 22.44803764 21.75531793 21.75531793 21.75531793 21.08857858 27.25772837 27.08244637 26.18923927 25.13069069 25.04805809 24.46355921 24.29841064 23.99565238 23.24753131 21.12993699 28.59765052 28.54470728 27.49644716 27.3022692 26.3141609 26.24413089 26.24015431 25.9235514 25.9235514 25.9235514 25.9235514 25.5928552 25.54857312 25.54857312 25.39490766
	905 970 1082 Rank 24 181 193 283 438 446 550 585 655 797 1166 555 797 1166 100 100 100 100 177 262 274 277 316 320 325 342 360 361 363 385 385 385	Archan;Coatomer subunit delta;Delta-coat protein SEC23-interacting protein Sec24 related gene family, member B (S. cerevisiae) Protein Name N-ethylmaleimide-sensitive fusion protein;Suppressor of K(+) transport growth defect 2 Vacuolar proton pump subunit C 1,V-type proton ATPase subunit C 1 Maternal-embryonic 3;Vacuolar protein sorting-associated protein 35;Vesicle protein sorting 35 Gamma-soluble NSF attachment protein;N-ethylmaleimide-sensitive factor attachment protein gamma Ceruloplasmin;Ferroxidase Vacuolar proton pump subunit D;V-ATPase 28 kDa accessory protein;V-type proton ATPase subunit D ADP-ribosylation factor-like protein 108;ADP-ribosylation factor-like protein ATPase subunit D ADP-ribosylation factor-like protein 108;ADP-ribosylation factor-like protein RA G protein subunit beta-2;Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2 Palmitoyl-protein hydrolase 1;Palmitoyl-protein thioesterase 1 Acid ceramidase;Acid ceramidase subunit alpha;Acid ceramidase subunit beta Dthers Protein Name Clathrin, heavy polypeptide (Hc);Clathrin heavy chain 1 Crmp1 protein Clathrin, heavy polypeptide (Hc);Clathrin heavy chain 1 Crmp1 protein Clathrin, heavy polypeptide (Hc);Clathrin heavy chain 1 Calcium-dependent activator protein for secretion 1;Calcium-dependent secretion activator 1 ADP-ribosylation factor 3;ADP-ribosylation factor 1 14-3-3 protein egsilon 14-3-3 protein beta/alpha;14-3-3 protein gamma, N-terminally processed Annexin A7;Annexin VII;Annexin-7;Synexin 14-3-3 protein beta/alpha;14-3-3 protein beta/alpha, N-terminally processed Neuroendocrine-specific protein;Reticulon-1 Arf-GAP domain and FG repeats-containing protein 1;HIV-1 Rev-binding protein homolog ADP-ribosylation factor 5 Dematin 48 kDa subunit;Erythrocyte protein band 4.9;Putative uncharacterized protein GTP-binding protein SAR1b Lysosomal-trafficking regulator 2;Neurobeachin 14-3-3 protein eta Coronin-1A;Coronin-like protein A;Coronin-like protein p57 Dmx-like 2;DmX-like protein 2;Rabc	Arcn1;Copd Sec23ip Sec24b Gene Name Nsf;Skd2 Atp6c;Atp6c1;Atp6v1c Mem3;Vps35 Napg;Snapg Cp Atp6v1h Atp6m;Atp6v1d;Vatd Arl10b;Arl8a;Gie2 Gnb2;Gnb4 Ppt;Ppt1;RP23-232N10 Asah;Asah1 Gene Name Cltc;RP23-366M19.3-0 Crmp1 Cadps;Caps;Caps1;Kiaz Arf3;Arf1 Ywhae Ywhag Anx7;Anxa7 Ywhab;RP23-321M14. Nsp;Rtn1 Agfg1;Hrb;Rip Ant75 Epb4.9 Sar1b;Sara1b;Sara2 Lyst2;Nbea Ywhah Coro1;Coro1a Dmxl2;RP24-383M20.;	COPD S23IP Q80ZX0 Acc. Number NSF VATC1 VPS35 SNAG CERU VATT VATD ARL8A GBB2 SPPT1 ASAH1 AGB2 SPPT1 ACC. Number (CLH Q6P1J1 AAR53 14336 14336 14338 RTN1 AGFG1 AAXA7 1433B RTN1 AGFG1 AAXA7 1433B RTN1 AGFG1 AAXA7 1433B RTN1 AGFG1 AAXA7 1433B RTN1 AGFG1 AAXA7 1433B RTN1 AGFG1 AAXA7 1433B RTN1 AGFG1 AAXA7 1433B RTN1 AGFG1 AAXA7 1433B RTN1 AGFG1 AAXA7 1433B COR1A CDCA2 IDMXL2 VP26B	22.43644611 22.44803764 21.75531793 31.08857858 27.25772837 27.08244637 26.18923927 25.13069069 25.04805809 24.46355921 24.29841064 23.99565238 23.24753131 21.12993699 28.66768859 28.59765052 28.54470728 27.49644716 27.30222692 26.3141609 26.2413089 26.21015431 25.9235514 25.9235514 25.9235514 25.57787526 25.57787526 25.57787526 25.5628552 25.54857312 25.39490766 25.39197259
	905 970 1082 Rank 24 181 193 283 438 446 550 585 655 797 1166 555 797 1166 22 89 96 100 160 100 160 177 262 274 277 316 320 325 342 360 361 363 385 387 394	Archam;Loatomer subunit delta;Delta-coat protein SEC23-interacting protein Sec24 related gene family, member B (S. cerevisiae) Protein Name N-ethylmaleimide-sensitive fusion protein;Suppressor of K(+) transport growth defect 2 Vacuolar proton pump subunit C 1;V-type proton ATPase subunit C 1 Maternal-embryonic 3;Vacuolar protein sorting-associated protein 35;Vesicle protein sorting 35 Gamma-soluble NSF attachment protein;N-ethylmaleimide-sensitive factor attachment protein gamma Ceruloplasmin;Ferroxidase Vacuolar proton pump subunit H;V-type proton ATPase subunit H Vacuolar proton pump subunit D;V-ATPase 28 kDa accessory protein;V-type proton ATPase subunit D ADP-ribosylation factor-like protein 10B;ADP-ribosylation factor-like protein 8A G protein subunit beta-2;Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2 Palmitoyl-protein hydrolase 1;Palmitoyl-protein thioesterase 1 Acid ceramidase;Acid ceramidase subunit alpha;Acid ceramidase subunit beta Clathrin, heavy polypeptide (Hc);Clathrin heavy chain 1 Crmp1 protein Clathrin, heavy polypeptide (Hc);Clathrin heavy chain 1 Crmp1 protein Clathrin, heavy polypeptide (Hc);Clathrin heavy chain 1 Crmp1 protein 14-3-3 protein gamma;14-3-3 protein for secretion 1;Calcium-dependent secretion activator 1 ADP-ribosylation factor 3;ADP-ribosylation factor 1 14-3-3 protein gamma;14-3-3 protein gamma, N-terminally processed Neuroendocrine-specific protein;Reticulon-1 Arf-GAP domain and FG repeats-containing protein 1;HIV-1 Rev-binding protein homolog ADP-ribosylation factor 5 Dematin 48 kDa subunit;Erythrocyte protein band 4.9;Putative uncharacterized protein GTP-binding protein SAR1b Lysosomal-trafficking regulator 2;Neurobeachin 14-3-3 protein eta Coronin-1A;Coronin-like protein A;Coronin-like protein p57 Dmx-like 2;DmX-like protein 2;Rabconnectin-3 Vacuolar protein sorting-associated protein 268;Vesicle protein sorting 268 Protein trapsort moreing 268	Archi,Copd Sec23ip Sec24b Gene Name Nsf;Skd2 Atp6c;Atp6c1;Atp6v1c Mem3;Vps35 Napg;Snapg Cp Atp6v1h Atp6m;Atp6v1d;Vatd Arl10b;Arl8a;Gie2 Gnb2;Gnb4 Ppt;Ppt1;RP23-232N16 Asah;Asah1 Gene Name Cltc;RP23-366M19.3-0 Crmp1 Cadps;Caps;Caps1;Kiaz Arf3;Arf1 Ywhae Ywhag Anx7;Anxa7 Ywhab;RP23-321M14. Nsp;Rtn1 Agfg1;Hrb;Rip Arf5 Epb4.9 Sar1b;Sara1b;Sara2 Lyst2;Nbea Ywhah Coro1;Coro1a Dmxl2;RP24-383M20.: Vps26b Sar23;Scr22a;Scr22a;Scr22	COPD S23IP Q80ZX0 Acc. Number NSF VATC1 VPS35 SNAG CERU VATD ARL8A GBB2 5PPT1 CCH CCH CCH CCH CCH CCH CCH CC	22.43644611 22.44803764 21.75531793 31.08857858 27.25772837 27.08244637 26.18923927 25.13069069 24.46355921 24.29841064 23.99565238 23.24753131 21.12993699 24.46355921 24.29841064 23.99565238 23.24753131 21.12993699 28.59765052 28.54470728 27.3022692 26.3141609 26.24413089 26.24413089 26.24413089 26.3141609 26.34413089 26.3141609 25.525514 25.5925514 25.56787526 25.57787526 25.57787526 25.57787526 25.57787526 25.57787526 25.5285522 25.54857312 25.39490766 25.39197259 25.3243269
	905 970 1082 Rank 24 181 193 283 438 446 550 585 655 797 1166 885 655 797 1166 0 89 96 100 160 177 262 274 277 316 320 325 342 342 361 363 385 387 394 395	Archam;Loatomer subunit delta;Delta-coat protein SEC23-interacting protein Sec24 related gene family, member B (S. cerevisiae) Protein Name N-ethylmaleimide-sensitive fusion protein;Suppressor of K(+) transport growth defect 2 Vacuolar proton pump subunit C 1;V-type proton ATPase subunit C 1 Maternal-embryonic 3;Vacuolar protein sorting-associated protein 35;Vesicle protein sorting 35 Gamma-soluble NSF attachment protein;N-ethylmaleimide-sensitive factor attachment protein gamma Ceruloplasmin;Ferroxidase Vacuolar proton pump subunit D;V-ATPase 28 kDa accessory protein;V-type proton ATPase subunit D ADP-ribosylation factor-like protein 10;ADP-ribosylation factor-like protein BA G protein subunit beta-2;Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2 Palmitoyl-protein hydrolase 1;Palmitoyl-protein thioesterase 1 Acid ceramidase;Acid ceramidase subunit alpha;Acid ceramidase subunit beta Defens Protein Name Clathrin, heavy polypeptide (Hc);Clathrin heavy chain 1 Crmp1 protein Calcium-dependent activator protein for secretion 1;Calcium-dependent secretion activator 1 ADP-ribosylation factor 3;ADP-ribosylation factor 1 14-3-3 protein epsilon 14-3-3 protein gamma;14-3-3 protein gamma, N-terminally processed Annexin A7;Annexin VII;Annexin-7;Synexin 14-3-3 protein beta/alpha;14-3-3 protein beta/alpha, N-terminally processed Neuroendocrine-specific protein;Reticulon-1 Arf-GAP domain and FG repeats-containing protein 1;HIV-1 Rev-binding protein homolog ADP-ribosylation factor 5 Dematin 48 kDa subunit;Erythrocyte protein band 4.9;Putative uncharacterized protein GTP-binding protein ASR1b Lysosomal-trafficking regulator 2;Neurobeachin 14-3-3 protein eta Coronin-1A;Coronin-like protein A;Coronin-like protein p57 Dmx-like 2;DmX-like protein 2;Rabconnectin-3 Vacuolar protein sorting-associated protein A Activater of G arobinis insolution action factor 1 Arti-Acte of carobinis insolution action factor 1 Arti-Acte of carobinis insolution action factor 1 Coronin-1A;Coronin-lik	Arcn1;Copd Sec23ip Sec24b Gene Name Nsf;Skd2 Atp6c;Atp6c1;Atp6v1c Mem3;Vps35 Napg;Snapg Cp Atp6v1h Atp6m;Atp6v1d;Vatd Arl10b;Arl8a;Gie2 Gnb2;Gnb4 Ppt;Ppt1;RP23-232N10 Asah;Asah1 C(tc;RP23-366M19.3-0 Cltc;RP23-366M19.3-0 Cltc;RP23-366M19.3-0 Cltc;RP23-366M19.3-0 Cltc;RP23-366M19.3-0 Crmp1 Cadps;Caps;Caps1;Kiaa Arf3;Arf1 Ywhae Ywhag Anx7;Anxa7 Ywhab;RP23-321M14. Nsp;Rtn1 Agfg1;Hrb;Rip Arf5 Epb4.9 Sar1b;Sara1b;Sara2 Lyst2;Nbea Ywhah Coro1;Coro1a Dmxl2;RP24-383M20.: Vps26b Sec23;Sec23a;Sec23r	COPD S23IP Q802X0 Acc. Number NSF VATC1 VPS35 SNAG CERU VATD ARL8A GBB2 5PPT1 ASAH1 ACC. Number (CLH Q6P111 ACAPS1 433E 1433E 1433E 1433B RTN1 AGFG1 ARF5 Q9WVM2 SAR1B NBEA 1433F COR1A COR1A COR1A COR1A COR1A COR1A COC42 IDMXL2 VP26B SC23A	22.43644611 22.44803764 21.75531793 31.08857858 27.25772837 27.08244637 26.18923927 25.13069063 25.04805809 25.04805809 24.46355921 24.29841064 23.99565238 23.24753131 21.12993699 26.3141604 28.66768859 28.59765052 28.54470728 27.49644716 27.30222692 26.3141609 26.24413089 26.21015431 25.9235514 25.9235514 25.9235514 25.567488605 25.5778756 25.56258552 25.54857312 25.39490766 25.39490766 25.39490766 25.39490766 25.39490766

Protein sorting-associated protein 268/Vesice protein sorting 268 Protein transport protein sec23A;SEC23-related protein A Activator of G-protein signaling 3;G-protein-signaling modulator 1;G-protein signalling modulator 1 ADP-ribosylation factor GTPase-activating protein 2;GTPase-activating protein ZNF289 General vesicular transport factor p115;Protein USO1 homolog;Transcytosis-associated protein GDI-1;Rho GDP-dissociation inhibitor 1;Rho-GDI alpha 450 477

496 501

Scaffold protein Pbp1;Syndecan-binding protein 1;Syntenin-1;MCG4375, isoform CRA_b 516

Ags3;Cl0a;Gpsm1;RP2 GPSM1 Arfgap2;Zfp289;Znf285ARFG2 Uso1;Vdp USO1 Arhgdia;C87222;Gdi1 GDIR1 GdbpurGC 4127;UB2;CDCD1

Sdcbp;mCG_4375;RP2:SDCB1

24.89649083

24.80577536 24.7851551

24.70484407

532	Adapter-related protein complex 3 mu-2 subunit;AP-3 complex subunit mu-2	Ap3m2	AP3M2	24.59701819
551	Glycogen synthase kinase-3 beta	Gsk3b	GSK3B	24.4561129
558	Putative uncharacterized protein;RAS-related C3 botulinum substrate 1	mCG_23557;Rac1;Rac3	RAC1	24.43217944
563	Sodium/potassium-dependent ATPase subunit beta-1	Atp1b1;Atp4b	AT1B1	24.4077342
566	Reticulon-3	Rtn3	RTN3	24.38675329
592	Clathrin-coated vesicle/synaptic vesicle proton pump 116 kDa subunit	Atp6n1;Atp6v0a1	VPP1	24.28083948
606	Non-catalytic region of tyrosine kinase adaptor protein 2;SH2/SH3 adaptor protein	mCG_113686;mNck-be	055033	24.22944717
625	Copine VI;Copine-6;Neuronal-copine;Putative uncharacterized protein	Cpne6	CPNE6	24.13946142
630	ADP-ribosylation factor 1 GTPase-activating protein; ADP-ribosylation factor GTPase-activating protein 1	Arf1gap;Arfgap1;RP23-	ARFG1	24.10210792
632	Oculocerebrorenal syndrome of Lowe;Putative uncharacterized protein	Ocrl;RP23-455F14.2-00	Q6NVF0	24.09415996
673	Putative uncharacterized protein;TFG protein;Trk-fused;Trk-fused gene	mCG_130891;RP23-17	Q9Z1A1	23.93559048
699	Putative uncharacterized protein;SAR1 gene homolog A (S. cerevisiae);SAR1 gene homolog A	mCG_15771;Sar1a;Sar	SAR1A	23.828961
706	GABA(A) receptor-associated protein-like 2;Gamma-aminobutyric acid receptor-associated protein-like 2	Gabarapl2	GBRL2	23.79613443
738	ADP-ribosylation factor 4	Arf4	ARF4	23.63680559
807	Apolipoprotein E	Apoe	APOE	23.19516266
811	Esterase 10;Esterase D;S-formylglutathione hydrolase;Sid 478	Es10;Esd;Sid478	ESTD	23.17440985
813	Putative uncharacterized protein Clip1;CAP-Gly domain-containing linker protein 1;Restin	Clip1;Kiaa4046;Rsn	CLIP1	23.16770398
827	MKIAA0079 protein;SEC24 related gene family, member C (S. cerevisiae), isoform CRA_b	mCG_121333;mKIAA00	G3X972	23.10864654
847	Sorcin	Sri	SORCN	23.01545135
891	APG16-like 1;Autophagy-related protein 16-1;Putative uncharacterized protein Atg16l1	Apg16l;Atg16l1	A16L1	22.81170551
911	Bullous pemphigoid antigen 1;Dystonia musculorum protein;Hemidesmosomal plaque protein	Bpag1;Dst;Macf2	DYST	22.71002949
963	Sorting nexin-32;Sorting nexin-6B	Snx32;Snx6b	SNX32	22.4667838
975	Superoxide dismutase [Cu-Zn]	Sod1	SODC	22.40620362
1015	Putative uncharacterized protein	Cdc42	Q3UL78	22.20266551
1049	Autophagy-related protein LC3 B	Map1alc3;Map1lc3;Ma	MLP3B	21.94814809
1067	Coatomer subunit epsilon; Epsilon-coat protein; Putative uncharacterized protein Cope	Cope;Cope1	COPE	21.82772415
1073	33 kDa VAMP-associated protein; Vesicle-associated membrane protein-associated protein A	Vap33;Vapa	VAPA	21.78981486
1078	Psd3 protein;Putative uncharacterized protein	Psd3;Efa6d;Kiaa0942	Q8C0E9	21.78981486
1090	Aftiphilin	Afth;Aftph	AFTIN	21.7133619
1131	Apolipoprotein J;Clusterin;Clusterin alpha chain;Clusterin beta chain;Clustrin;Sulfated glycoprotein 2	Apoj;Clu;Msgp-2	CLUS	21.42789456
1195	Isochorismatase domain-containing protein 1	lsoc1	ISOC1	20.85771984

<u>Table 2</u>

Molecular Motor

Rank Protein Name	Gene Name	Acc. Numbe	er Avrg LFQ
6 150 kDa dynein-associated polypeptide;DAP-150;Dynactin subunit 1;p150-glued	Dctn1	O08788	DCTN1
7 50 kDa dynein-associated polypeptide;Dynactin complex 50 kDa subunit;Dynactin subunit 2	Dctn2	Q99KJ8	DCTN2
17 Cytoplasmic dynein 1 heavy chain 1;Cytoplasmic dynein heavy chain 1;Dynein heavy chain, cytosolic	Dhc1;Dnch1	Q9JHU4	DYHC1
19 Dynactin subunit 4;Dynactin subunit p62	Dctn4	Q8CBY8	DCTN4
27 Actin-related protein 10;Actin-related protein 11	Act11;Actr10	Q9QZB7	ARP10
44 CapZ alpha-2;F-actin-capping protein subunit alpha-2	Cappa2;Capza2	P47754	CAZA2
45 Capping protein (Actin filament) muscle Z-line, beta;CAPZB3	Capzb	P47757	CAPZB
137		E9Q0B6	E9Q0B6
161 Dynactin light chain p24;Dynactin subunit 3	Dctn3	Q9Z0Y1	DCTN3
167 Cytoplasmic dynein 1 intermediate chain 1;Cytoplasmic dynein intermediate chain 1	Dnci1;Dncic1	O88485	DC1I1
172 Dynactin subunit 5;Dynactin subunit p25	Dctn5	Q9QZB9	DCTN5
226 Cytoplasmic dynein 1 light intermediate chain 1;Dynein light chain A	Dncli1;Dnclic1	Q8R1Q8	DC1L1
253 CapZ alpha-1;F-actin-capping protein subunit alpha-1	Cappa1;Capza1	P47753	CAZA1
255 Dynactin subunit 6;Dynactin subunit p27;Protein WS-3;Dynactin 6, isoform CRA_b	Dctn6;Ws3	Q9WUB4	DCTN6
324 Cytoplasmic dynein 1 light intermediate chain 2;Dynein light intermediate chain 2, cytosolic	Dncli2;Dnclic2	Q6PDL0	DC1L2
382 Dynein cytoplasmic 1 intermediate chain 2;Dynein cytoplasmic 1 intermediate chain 2, isoform CRA_a	Dync1i2;mCG_19348	O88487	DC1I2
631 Kinesin-related microtuble-based motor protein; Axonal transporter of synaptic vesicles; Kinesin-like protein KIF	F1A Kif1a;Atsv;Kif1	E9Q9G6	E9Q9G6
649 MKIAA0531 protein; Kinesin heavy chain isoform 5C; Kinesin heavy chain neuron-specific 2	Kif5c;mKIAA0531	P28738	KIF5C
1007 Dynein light chain roadblock-type 1;Dynein light chain 2A, cytoplasmic	Dynlrb1	P62627	DLRB1
1199 Huntingtin;Huntington disease protein homolog	Hd;Hdh;Htt	P42859	HD

Table 3

Small GTPase

Ran	k Protein Name	Gene Name	Acc. Number	Avrg LFQ
	131 Guanosine diphosphate dissociation inhibitor 1;Rab GDP dissociation inhibitor alpha	Gdi1;Rabgdia	GDIA	27.98445519
	232 Ras-related protein Rab-3A	Rab3a	RAB3A	26.61833053
	364 Ras-related protein Rab-1B	Rab1b	RAB1B	25.54102806
	376 DENN domain-containing protein 5B;Rab6IP1-like protein	Dennd5b	DEN5B	25.46297706
	397 GDI-3;Guanosine diphosphate dissociation inhibitor 2;Rab GDP dissociation inhibitor beta	Gdi2;Gdi3	GDIB	25.33513625
	403 Rab3 GTPase-activating protein 150 kDa subunit;Rab3 GTPase-activating protein non-catalytic subunit	Kiaa0839;Rab3gap2	RBGPR	25.30517038
	472 Ras-related protein Rab-7a	Rab7;Rab7a	RAB7A	24.9335359
	547 Putative uncharacterized protein;RAB5C, member RAS oncogene family;Ras-related protein Rab-5C	Rab5c;RP23-390D17.8-00	RAB5C	24.49891119
	553 Ras-related protein Rab-10	Rab10	RAB10	24.45301721
	576 Ras-related protein Rab-2A;Ras-related protein Rab-2B;Putative uncharacterized protein	Rab2;Rab2a;Rab2b	RAB2A	24.34299482
	618 Ras-related protein Rab-11B;Ras-related protein Rab-11A	Rab11b;Rab11;Rab11a	RB11B	24.17008796
	756 Ras-related protein Rab-6A;Putative uncharacterized protein Rab6	MNCb-1660;Rab6;Rab6a	RAB6A	23.52376246
	790 Ras-related protein Rab-5A	Rab5a	RAB5A	23.31285194
	792 Ras-related protein Rab-14;RAB14 protein variant;RAB14, member RAS oncogene family	Rab14;rab14;RP23-186B	RAB14	23.28683861
	888 Ras-related protein Rab-1A;YPT1-related protein;Putative uncharacterized protein Rab1	Rab1;Rab1A	RAB1A	22.84110488
	923 Ras-related protein Rab-6B	D9Bwg0185e;Rab6b	RAB6B	22.63248911
	1012 Ras-related protein Rab-5B	Rab5b	RAB5B	22.21088137
	1028 MKIAA0118 protein;Rab-12;Ras-related protein Rab-21	mKIAA0118;Rab21	RAB21	22.11365697
	1063 Ras-related protein Rab-8B	Rab8b	RAB8B	21.85677049
	1065 Ras-related protein Rab-18	Rab18	RAB18	21.84731891
	1106 Actin-related protein 2/3 complex subunit 5;Actin-related protein 2/3 complex subunit 5-like protein	Arpc5l;RP23-244N6.5-00	ARP5L	21.61178039
	1110 Rab-15;Ras-related protein Rab-23;Putative uncharacterized protein;RAB23, member RAS oncogene family	Rab23;mCG_10410	RAB23	21.59364035
	1112 Ras-related protein Rab-3C;Putative uncharacterized protein;RAB3C, member RAS oncogene family	Rab3c;mCG_117475	RAB3C	21.57433944
	1127 c-Ki-ras;c-K-ras;GTPase KRas;GTPase KRas, N-terminally processed;Ki-Ras;K-Ras 2	Kras;Kras2	RASK	21.46524155
	1150 ADP-ribosylation factor-binding protein GGA2;Gamma-adaptin-related protein 2;Golgi-localized	Gga2;Kiaa1080	GGA2	21.29924715
	1160 Ras-related protein Rab-4B	Rab4;Rab4b	RAB4B	21.16343102
	1167 RAB3 GTPase-activating protein 130 kDa subunit;Rab3 GTPase-activating protein catalytic subunit	Kiaa0066;Rab3gap;Rab3	RB3GP	21.12395214
	1172 Ras-related protein Rab-28	Rab28	RAB28	21.09577559
	1230 Rho GTPase-activating protein 1; Rho-type GTPase-activating protein 1; Putative uncharacterized protein	Arhgap1	RHG01	20.40634205
	1267 Putative uncharacterized protein;Oncogene c-mel;Ras-related protein Rab-8A	Rab8a;Mel;Rab8	RAB8A	19.61433961

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DISCUSSION

8 D. melanogaster as an in vivo model to study HTT function

Abnormalities of axonal transport have been directly and indirectly linked with several neurodegenerative diseases (Hinckelmann et al., 2013; Hirokawa et al., 2010; Millecamps and Julien, 2013). This is the case for Huntington's disease (HD), the disease caused by a mutation in the huntingtin (*HTT*) gene. Mutant HTT leads to defects in axonal transport (Gauthier et al., 2004; Gunawardena et al., 2003), and these abnormalities play a crucial role in HD pathogenesis. Importantly, this decreased efficiency in transport is also observed when HTT is downregulated. Several groups have demonstrated that HTT is a positive regulator of MT based transport. Reduction of HTT impairs vesicle movement to the same extent that the presence of the polyQ HTT (Gauthier et al., 2004; Gunawardena et al., 2003; Szebenyi et al., 2003), suggesting that the abnormalities in transport observed in HD may therefore be a result of a loss of normal function of HTT protein.

HTT has been extensively characterized in cells and mouse models, however, not so many studies have focused on the functional relevance of its homologues in other species. Drosophila melanogaster has proven to be an excellent model to studying neurodegenerative diseases (Ambegaokar et al., 2010; Green and Giorgini, 2012; Moloney et al., 2010). Although fruit flies may seem extremely distant from mammalian biology, there are major similarities in basic cellular processes between them, including gene expression regulation, subcellular trafficking, synaptic transmission, synaptogenesis and cell death. Furthermore, the simplicity in their genetics (only 4 pairs of chromosomes and 12.000 genes, compared to the 23 chromosomes and 20.000 genes in humans) makes it ideal for generating models, which allows in vivo studies (Ambegaokar et al., 2010). Particularly for HD, fly strains have been generated, by overexpressing the full length or N-terminal fragments of human HTT. These models provided a platform to demonstrate that flies expressing human mutant HTT can yield parallel neurodegenerative traits to mammals (Jackson et al., 1998; Lee et al., 2004; Marsh et al., 2003; Romero et al., 2008; Song et al., 2013). Specifically, axonal transport was shown to be altered in HD fly models (Gunawardena et al., 2003; Sinadinos et al., 2009; Zhang et al., 2009). The observation that human HTT fragments causes axonal transport phenotype, along

with previous work showing that transport deficits may be due to a loss normal function of HTT, leads to wonder if the phenotype seen in HD in *D. melanogaster* is due to this as well. In other words, is DmHTT function in transport conserved in *D. melanogaster*?

Our study firmly shows that in the absence of endogenous HTT, fast axonal transport is disturbed in *D. melanogaster*. Previously, three different studies had used the fruit fly as an approach to study this issue. However, their findings were contradictory. Downregulation of DmHTT by expression of transgenic RNAi induces abnormal accumulation of organelles in larval neurons. These accumulations are termed axonal blockages, and their presence correlates well with disruption of axonal transport. Additionally, decreasing the levels of normal DmHTT in the eye causes a rough eye phenotype in adults, which is characteristic of neurodegeneration (Gunawardena and Goldstein, 2003). Differing results come from a study using DmHTT null flies. In this system the distribution of synaptic vesicle markers were normal, reveling no obvious accumulation or axonal swelling (Zhang et al., 2009). Nonetheless, it is important to highlight that in mammals, neither the reduction of HTT nor the presence of mutant HTT results in a complete arrest of axonal transport with axonal blockage, but rather in a reduction of the velocity of moving cargos. Both these studies fail to assess directly whether motility of vesicular cargos is affected. In our study, by direct visualization of synaptotagmin-GFP vesicle dynamics in the fly axons, we could observe that there was a reduction and not a complete seize of movement of vesicles. This was a result of a decrease in the number of fast moving vesicles in the DmHTT KO flies. These results are in agreement with third study previously done, where they observed that Rab11 and not Rab5 vesicles present transport defects when silencing DmHTT in flies (Power et al., 2012). Interestingly, this last observation sustains the hypothesis that HTT is involved in the regulation of transport of specific cargos.

Mammalian HTT has been shown to be involved in the regulation of transport of numerous cargos (Colin et al., 2008; Gauthier et al., 2004; Liot et al., 2013; Power et al., 2012). In our study we demonstrated that DmHTT regulates transport of synaptic vesicles in flies by observing their dynamics in the presence or absence of DmHTT. In mammalian neurons, DmHTT is associated with the small vesicles fraction and

colocalizes with around 70% of BDNF containing vesicles. 30% of BDNF vesicles do not associate with DmHTT. Whether these remaining vesicles are not associated to DmHTT because they are not dynamic or because their transport regulation is independent from HTT, remains to be answered. Our findings also show that there are DmHTT vesicle-like structures that are not associated to BDNF vesicles, suggesting that, as in mammalian HTT, DmHTT could be associated to and regulating transport of different cargos.

In agreement with the hypothesis that the role of HTT is conserved in flies, we were able to observe by videomicroscopy that DmHTT is dynamic, and that it is transported at velocities consistent with FAT. Besides being dynamic, DmHTT is co-transported with BDNF vesicles in axons. Therefore, DmHTT is associated to BDNF vesicles during their transport in neurons. Importantly, DmHTT takes over mammalian HTT function in transport. Transport defects resulting from silencing HTT in neurons, are partially rescued by expression of DmHTT, further demonstrating the close functional homology between these two proteins. Further studies should focus on investigating whether human HTT is able to restore transport in *D. melanogaster*.

Indeed, human and *D. melanogaster* HTT have several similitudes and homologies. For example, in terms of protein size, Drosophila und human HTT are extremely large, containing 3583 and 3144 a.a respectively (Li et al., 1999). The predicted size for DmHTT is 400 kDa. Until now, the lack of an antibody recognizing the endogenous protein was an obstacle for studying DmHTT localization. We generated an antibody that enabled us to confirm that DmHTT is expressed in larvae brain and absent in the DmHTT KO brains. This tool will permit now to further study the expression pattern in different structures and at diverse developmental stages.

Sequence comparison determined that the homologous regions between *Drosophila* and human HTT are mainly located in five different areas. These conserved areas are not only located in the same order in the entire length of the protein, but also share 24% identity and 49% similarity (Li et al., 1999). Particularly, human and *Drosophila* HTT a.a sequence share 30% identity and a similarity of 62% in the dynein interacting domain. In mammals, the relevance of this HTT region has been extensively described. The N-terminal region of mammalian HTT interacts with DIC, Huntingtin associated protein 1 (HAP1), p150^{*Glued*} and Kinesin light chain (KLC).

Overexpression of the N-terminal fragment of HTT enhances transport velocity (Colin et al., 2008; Gauthier et al., 2004; Pardo et al., 2010; Pineda et al., 2009), and when the HAP1 interacting domain or dynein interacting domain is missing in HTT, vesicular transport is defective (Pardo et al., 2010). Additionally, the N-terminal domain of HTT is necessary and sufficient to sustain transport. Our results show that N-terminal 620 a.a of DmHTT also interacts with DIC, as they co-immunoprecipitate. This interaction not only supports the functional homology between mammalian and Drosophila HTT, but also suggests DmHTT implication in other cellular mechanisms as the interaction of HTT with the dynein/dynacting complex extends beyond its role in axonal transport. Silencing HTT alters Golgi organization, spindle orientation in neuronal progenitors and mammary gland basal progenitors, as well as reduces ciliogenesis (Caviston et al., 2007; Elias et al., 2014; Godin et al., 2010; Keryer et al., 2011; Pardo et al., 2010). In mammals these processes depend on the association of HTT with dynein. Specifically, the expression of the N-terminal fragment of DmHTT restores the defect in spindle orientation that occurs as a consequence of the absence of HTT in the neuroblasts of fruit flies. Whether the mechanism underlying this dysfunction is homologous to what occurs in mammals, is still to be known.

Thus, *Drosophila melanogaster* seems to be a relevant model to study HTT function and dysfunction in a non-mammalian model, which will enable to further analyze the many open questions remaining in the field.

9 Motile Vesicles energetic "carry-ons"

We have demonstrated that FAT does not depend on mitochondrial ATP, as previously thought, but it depends on glycolytic ATP produced on vesicles. Perturbing GAPDH or PK, the two ATP generating glycolytic enzymes, slows down vesicular transport. However, knocking down GAPDH does not affect mitochondrial transport. Furthermore, all of the glycolytic enzymes are associated with dynamic vesicles, and are capable of producing their own ATP. Finally, we show that this ATP production is sufficient to sustain their own transport, demonstrating the energetical autonomy of vesicles for transport.

9.1 Local versus global

Fast axonal transport (FAT) is an active process that occurs via the consumption of ATP by molecular motors (Hirokawa et al., 2010). Our results suggest that the ATP is globally constant within axons, therefore being available for all enery-dependent cellular processes. In a cell, this ATP is produced by the breaking-down of glucose by glycolysis, and the Krebs cycle wich then drives the oxidative phosphorylation (OxPhos). Glycolysis is known to occur freely in the cytoplasm and generated a net gain of 2ATP/glucose, whereas mitochondria is where the Krebs cycle and OxPhos happens generating 34 molecules of ATP/glucose.

We showed that vesicular transport slows-down when knocking down either GAPDH or PK. These are the two glycolytic proteins that ensure the production of ATP by this metabolic pathway. The fact that the transport is less efficient, but not completely blocked, suggest that not all transport relies on glycolysis but some of it could depend on mitochondrial ATP. One hypothesis could be that mitochondrial ATP ensures basic transport, whereas glycolysis would be implicated in boosting their velocity. However, we also demonstrated that vesicles are autonomous for transport *in vitro*, proposing that the vesicle-associated glycolysis warrants the ATP needed for molecular motors. Therefore, the effects seen by silencing GAPDH and PK maybe not complete, as the expression of these proteins is only reduced and not completely abolished. Furthermore, pharmacological inhibition of GAPDH, which reduces

GAPDH activity to only 10%, results in a complete arrest of BDNF vesicles without affecting mitochondrial dynamics. Hence, although it has been well established the importance of glycolysis in FAT, further studies need to be performed to understand if this is essential for basic transport or for rendering vesicular transport more efficient. Although inhibition of ATP production by mitochondria is much more efficient in reducing ATP levels than GAPDH inhibition, vesicular transport is only affected when GAPDH is affected. But, why do vesicles prefer a local source from a general one? Although it is generally accepted that small molecules like ATP rapidly diffuse in solution, it has never been proven to be true inside the cell. On the contrary, ATP distribution in erythrocytes is not homogenous. ATP is sequestered in pools within the cytoskeletal-membrane complex (Chu et al., 2012). Our study shows that in neurons, ATP/ADP ratio is globally homogeneous, however there are specific areas where the levels of ATP are higher. Additionally, when depleting mitochondria from axons by expressing Milton-C, leads to a reduction of the ATP: ADP ratio in the axon but not in the cell body. This suggests that the rate of diffusion of ATP is slower than the rate of consumption, and that ATP distribution inside the cell might not be as generally predicted. Therefore, the accessibility of ATP inside the cell for fast highenergetic demanding processes could be restricted. Thus, the proximity of two sequential reactions, ATP production and ATP consumption, could be favorable and more efficient

9.2 Glycolysis local ATP production and cellular functions

This energetic coupling between ATP production and ATP consumption seems to be a mechanism used to energetically sustain not only fast axonal transport.

In spermatozoa, glycolysis and mitochondria are localized in distinct cellular subcompartments. Mitochondria are exclusively in the sperm midpiece, whereas glycolysis takes place mainly in the flagellum where glycolytic enzymes are tightly anchored. The flagellum depends on efficient ATP supply in order to power its motility. It had been proposed that the ATP required for sperm flagellar movement depends predominantly on mitochondrial respiration. Interestingly, inhibition of mitochondrial function was found not to impair motility and to have little effect on intracellular ATP levels (Mukai and Okuno, 2004). In contrast, addition of a non-

metabolizable substrate (2-deoxyglucose) inhibited motility and led to depletion of ATP (Mukai and Okuno, 2004). Complementing these findings were those obtained from a genetic approach, that investigated the phenotype of a mouse model made null for the germ cell-specific isoform of glyceraldehyde 3-phosphate dehydrogenase (GAPDHS). Sperm from male mice null for GAPDHS were infertile, with pronounced defects in motility (Miki et al., 2004). Moreover, they had significantly less ATP even though their oxygen consumption was unchanged (Miki et al., 2004). These finding are in line with our observation that glycolysis energetically sustain specific functions in the cell.

Another example highlighting the importance of ATP production by glycolysis involves red blood cells. Erythrocytes are a particular type of cells, as they lack of nucleus and most organelles, including mitochondria. Therefore, all the energetic requirements of the cell are sustained by glycolytic ATP. Another particularity of red blood cells is their biconcave shape that is maintained by Na⁺/K⁺ and Ca⁺² ATPase pumps. Indeed, the glycolytic enzymes are arranged in the plasma membrane of red blood cells (Campanella et al., 2005; Puchulu-Campanella et al., 2013), thus being able to locally generate ATP to meet the energetic requirement of these pumps. Additionally, it has been shown that ATP is sequestered within a cytoskeletal–membrane complex that includes GAPDH, PGK, PK ankyrin and beta-spectrin. The ATP so entrapped serves as the preferential and proximal substrate for both the Na⁺/K⁺ and Ca²⁺ pumps (Chu et al., 2012).

Finally, the same kind of energetic association has been shown to occur in synaptic vesicles. In this case, synaptic vesicles carry the GAPDH/PGK functional complex and this vesicle bound-complex produces sufficient ATP to fuel the uptake and accumulation of glutamate into the synaptic vesicles (lkemoto et al., 2003). During synaptic transmission, synaptic vesicles release their content, and in order to continuously support normal neurotransmission, they need to be rapidly refilled (Pyle et al., 2000) For this uptake to take place vesicular ATPase H+-pumps consume ATP in order to generate an electrochemical proton gradient that provides the driving force for vesicular glutamate transporters (VGLUT) to reload synaptic vesicles. The ATP generated at the surface of the synaptic vesicle plays an essential role in ensuring rapid glutamate refilling of synaptic vesicles, as it was shown that ATP synthesis by vesicle-bound GAPDH/PGK is more efficient than exogenous ATP for glutamate uptake. Indeed, inhibition of ATP production by mitochondria has little effect on

vesicular glutamate accumulation in synaptosomes, despite the fact that it substantially reduces total synaptosomal ATP. Therefore, mitochondria alone may not entirely be able to meet the energetic requirement for maintaining normal neurotransmition (Ikemoto et al., 2003).

In addition to GAPDH/PGK, the glycolytic ATP generating enzyme pyruvate kinase is also associated to synaptic vesicles and the ATP production by this enzyme supports glutamate uptake. Furthermore, "ATP-trapping" experiments using hexokinase to compete for the ATP with the proton pump, suggests that the ATP produced by vesicle-associated pyruvate kinase is more readily used that the exogenously added ATP, suggests that this locally produced ATP is restricted to the microenvironment around the vesicle where it is more accessible to vesicular ATPase H+-pumps that the exogenously added ATP (Ishida et al., 2009). This further support the notion that for processes with fast high energy demands, the local production of ATP is more efficient that a global source.

We have shown that at HTT works as a scaffold protein that tethers GAPDH to the motile vesicles. This scenario opens the question of whether HTT is implicated in the specific distribution of glycolytic enzymes for the above-mentioned examples. Furthermore, energy demands are required for many other rapid energy-consuming cellular processes including, cell division, cilia beating or intraflagellar transport. However whether the production of ATP is localized and depends on glycolytic enzymes is still unknown.

9.3 Glycolitic enzymes association to motile vesicles

We have shown that all the glycolytic enzymes are associated to motile vesicles. But, how are these proteins targeted to vesicles? Particularly, the recruitment of one of these proteins, GAPDH, is dependent on HTT. Reducing HTT levels both *in* vivo as *in vitro* leads to a reduced localization of GAPDH on vesicles. Indeed, HTT has been described as being associated to vesicles (DiFiglia et al., 1995; Gutekunst et al., 1995; Trottier et al., 1995), and GAPDH is known to interact with HTT (Bae et al., 2006; Kaltenbach et al., 2007; Ratovitski et al., 2012). As HTT functions as a scaffolding protein that regulates transport, recruitment of GAPDH to vesicles could be an alternative mechanism of transport regulation by HTT. However, whether the

remaining glycolytic enzymes are targeted to vesicles through an interaction with HTT is still to be determined. The notion that HTT interacts with 7 of the 9 glycolytic enzymes (Hexokinase, Phosphoglucose Isomerase, Phosphofructose Kinase, Aldolase, Enolase and Pyruvate Kinase) (Kaltenbach et al., 2007; Ratovitski et al., 2012; Shirasaki et al., 2012) could suggest that HTT has a possible role in regulating these associations as well. On the other hand, it has been suggested that HTT is not an essential regulator of all types of vesicles, but just of a specific subtype. For example, Rab11 but not Rab5 vesicles show transport defects in the absence of HTT (Power et al., 2012). We have shown that local glycolysis is required for transport of BDNF, APP, TrKB and synaptophysin, but not for mitochondria as it contains its own local ATP source. Therefore, it would be interesting to investigate if Rab5 transport is energetically dependent of glycolytic enzymes to understand if there is a correlation between HTT regulation of transport and glycolysis-dependence. In other words, is glycolysis fuelling a generalized mechanism for vesicular trafficking?

However, HTT scaffolding might be not the only mechanism involved. For example, our results showed a considerable reduction but not a complete absence of GAPDH on vesicles, suggesting other mechanisms could be involvement of this localization. Therefore, interaction of the different glycolytic components with other scaffolds or alternatively, posttranslational modifications occurring on these enzymes may be other possible mechanisms involved. In agreement with this hypothesis, preliminary results show that the migration profile of cytosolic versus vesicular GAPDH and PK of mouse brain extracts are different. This suggests that different isoforms of these proteins exist, and that they are differentially located in distinct cellular compartments.

9.4 Vesicular Glycolysis in Disease

In HD, the presence of the mutant polyQ stretch leads to transport defects that resemble those of the loss of HTT function (Colin et al., 2008; Gauthier et al., 2004; Gunawardena et al., 2003; Liot et al., 2013; Pla et al., 2013). This defect has been accounted to an alteration of HTT's scaffolding functions: the presence of mutant HTT alters the interaction of the molecular motor complex, which leads to the

detachments of vesicles from MTs (Gauthier et al., 2004). We now showed that HTT is also implicated in the tethering of the glycolytic enzyme GAPDH onto motile vesicles. Interestingly, the absence of GAPDH results in a very similar transport phenotype to the loss of HTT function. Therefore, it would be interesting to assess whether there is a defect in vesicular glycolysis in HD. Does PolyQ HTT alter the distribution of the glycolytic enzymes on motile vesicles in HD? A reduction of this metabolic pathway on dynamic vesicles could be part of the mechanisms leading to a reduced axonal transport in disease.

Moreover, it is interesting to note that we were able to target GAPDH to vesicles by fusing it with a transmembrane domain. This led to an increased amount of GAPDH on vesicles that resulted in an augmentation of basal transport. This particular point could be interesting in HD, as targeting GAPDH to vesicles could restore transport in an HTT-independent manner. If so, it would be important to assess whether restoring axonal transport is sufficient to rescue phenotypes in disease models.

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