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Study of vesicular glycolysis in health and Huntington's Disease

Maximilian Mc Cluskey

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THÈSE

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préparée au sein du **Grenoble Institut des Neurosciences**
dans l'**École Doctorale de Chimie et Sciences du Vivant**

Study of vesicular glycolysis in health and Huntington's disease

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I. ABSTRACT

The axon enables long distance travel of electrical and chemical information between neurons, a process at the basis of neuronal communication and brain function. This information is transported in part by vesicles. Membrane-bound molecular motors propel these vesicles along the axon's cytoskeleton by consuming ATP. The lab has shown previously that the ATP required for this transport is produced by on-board glycolytic enzymes, thus creating an energetically autonomous microenvironment for vesicular transport. What is more, the transport of BDNF is significantly reduced in Huntington's disease (HD), a genetic disease caused by an abnormal CAG repeat expansion in the huntingtin (HTT) gene. This leads to insufficient trophic support of BDNF to the striatum where it plays a crucial role in cell survival. HTT is known to scaffold and mediate molecular motors on vesicles. The objectives of this PhD project were therefore to, design an approach to measure glycolytic activity on vesicles in order to understand the intricacies of glycolytic activity on vesicles as well as to identify the link between vesicular glycolysis and insufficient transport in HD.

To do so, we first wanted to describe the differences in metabolic rates and efficiency between cytosolic and vesicular glycolysis. To measure glycolytic activity, we decided to split the pathway into two segments: the first was determined through the NADH production, the product of GAPDH; and the second segment was measured through the ATP production, produced by PGK and PK. Through these measurements, we found that vesicular glycolysis has a greater affinity for its substrates than the cytosol, making the vesicle more efficient at producing NADH and ATP than cytosolic equivalents.

This then led us to question the importance of NAD⁺ recycling on vesicles. Here we showed, through immunofluorescence and western blot, that LDH, the enzyme responsible for converting pyruvate into lactate and oxidizing NADH in the process, is a vesicle-bound enzyme. Furthermore, we demonstrated that this LDH-dependent NAD⁺ recycling is crucial for overall glycolytic activity on vesicles and required for BDNF transport in cultured cortical neurons. Hence, axonal vesicles produce ATP via aerobic glycolysis, similarly to the Warburg effect in certain cancer cells.

Finally, we studied the link between vesicular glycolysis and BDNF transport in HD. We showed that HTT interacts more strongly on vesicles with at least two glycolytic enzymes, GAPDH and PFK, and that glycolytic enzyme quantity and activity on vesicles is affected in HD. Based on these results we used previously described TM-GAPDH to artificially stimulate glycolysis specifically on vesicles to demonstrate that this approach was sufficient to restore

transport *in vitro*. This provides evidence of the importance of vesicular glycolysis in BDNF transport and HD pathogenesis.

KEY WORDS

Vesicular glycolysis, Huntington's disease, lactate dehydrogenase, axonal transport, huntingtin

II. RÉSUMÉ

L'axone permet le déplacement électriques et chimiques sur de longues distances d'informations entre les neurones, un processus qui est à la base de la communication neuronale et de la fonction cérébrale. Cette information est transportée en partie par des vésicules. Les moteurs moléculaires liés à la membrane propulsent ces vésicules le long du cytosquelette de l'axone en consommant de l'ATP. Le laboratoire de Frédéric Saudou a montré précédemment que l'ATP nécessaire à ce transport est produit par des enzymes glycolytiques liées à ces membranes, créant ainsi un microenvironnement énergétiquement autonome pour le transport vésiculaire. De plus, le transport de BDNF est considérablement réduit dans la maladie de Huntington (MH), une maladie génétique causée par une expansion anormale de répétition de CAG dans le gène de la huntingtine (HTT). Cela conduit à un support trophique insuffisant du BDNF au striatum où il joue normalement un rôle crucial dans la survie cellulaire. La HTT est connu pour être une protéine d'échaffaudage pour les moteurs moléculaires sur les vésicules. Les objectifs de ce projet étaient donc de concevoir une approche permettant la mesure de l'activité glycolytique sur les vésicules afin de comprendre les subtilités de l'activité glycolytique sur les vésicules ainsi que d'identifier le lien entre glycolyse vésiculaire et transport insuffisant caractéristique de la MH.

Pour ce faire, nous avons d'abord voulu décrire les différences de taux métaboliques et d'efficacité entre la glycolyse cytosolique et vésiculaire. Pour mesurer l'activité glycolytique, nous avons décidé de scinder la voie en deux segments: le premier a été déterminé par la production de NADH, le produit de GAPDH; et le deuxième segment a été mesuré par la production d'ATP, produite par PGK et PK. Grâce à ces mesures, nous avons constaté que la glycolyse vésiculaire a une plus grande affinité pour ses substrats que le cytosol, ce qui rend la vésicule plus efficace pour produire du NADH et de l'ATP que ses équivalents cytosoliques.

Cela nous a alors conduit à nous interroger sur l'importance du recyclage de NAD⁺ sur les vésicules. Ici, nous avons montré, par immunofluorescence et western blot, que la LDH, enzyme responsable de la conversion du pyruvate en lactate et de l'oxydation du NADH, est également liée aux vésicules. En outre, nous avons démontré que ce recyclage NAD-dépendant de la LDH est crucial pour l'activité glycolytique globale sur les vésicules et requis pour le transport du BDNF dans des neurones corticaux en culture. Par conséquent, les vésicules axonales produisent de l'ATP par glycolyse aérobie, de manière similaire à l'effet Warburg décrit dans les cellules cancéreuses.

Enfin, nous avons étudié le lien entre la glycolyse vésiculaire et le transport de BDNF dans la MH. Nous avons montré que le HTT interagit plus fortement sur les vésicules avec au moins deux enzymes glycolytiques, GAPDH et PFK, et que la quantité et l'activité de l'enzyme glycolytique sur les vésicules sont affectées dans la MH. Sur la base de ces résultats, nous avons utilisé la construction TM-GAPDH décrite précédemment pour stimuler artificiellement la glycolyse spécifiquement sur les vésicules ce qui nous a permis de démontrer que cette approche était suffisante pour restaurer le transport *in vitro*. Ceci est une preuve de l'importance de la glycolyse vésiculaire dans le transport du BDNF et la pathogenèse de la MH.

MOTS CLÉS

Glycolyse vésiculaire, maladie de Huntington, lactate déshydrogénase, transport axonal, huntingtine

III. ABBREVIATIONS

ΔG : free-energy difference
2-DG: 2-deoxyglucose
3BHM: 3- β -hydroxybutyrate
6-PGDH: 6-phosphogluconate dehydrogenase
6PGL: 6-phosphogluconolactonase
AAV: adeno-associated virus
AD: Alzheimer's disease
ADP: adenosine diphosphate
AMP: adenosine monophosphate
AMPA: α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANLS: astrocyte-neuron lactate shuttle
AR: androgen receptor protein
ASO: antisense oligonucleotide
AST: alanine aminotransferase
Atg: autophagy related protein
ATP: Adenosine triphosphate
A β : amyloid beta
BACHD: Bacterial Artificial Chromosome transgenic model
BDNF: Brain Derived Neurotrophic Factor
BMI: Body Mass Index
bp: base pairs
CBP: CREB-binding protein
CK: creatine kinase
IP: immunoprecipitation
CREB: cAMP Response Element
CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats
DCV: dense-core vesicles
DNA: Deoxyribonucleic acid
Drp: Dynamin-Related Protein
EPSC: excitatory postsynaptic potential
ER: Endoplasmic reticulum
ESCRT: endosomal sorting complex required for transport
FAD: flavin adenine dinucleotide
FAT: Fast axonal transport
FBPase: fructose biphosphatase
FRET: fluorescence resonance energy transfer
G3P: glyceraldehyde-3-phosphate
G6P: glucose-6-phosphate
G6PDH: glucose-6-phosphate dehydrogenase
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
GLT: glutamate transporter
GLUT: glucose transporter
Gpe: external Globus Pallidus
Gpi: internal Globus Pallidus
GPI: phosphoglucose isomerase
GRIK2, also known as GLUR6: Glutamate Receptor Ionotropic Kainate 2
GSH: glutathione
HAP: Huntingtin-Associated Protein

HD: Huntington's Disease
HDAC: deacetylated histone deacetylase
HD-CAB: Huntington's Disease Cognitive Assessment Battery
HEAT: HTT (H), elongation factor 3 (E), the regulatory A subunit of protein phosphatase 2A (A) and TOR1 (T)
HK: hexokinase
HSP: Hereditary spastic paraplegia
HTT: huntingtin
InsP3R: inositol (1,4,5)-triphosphate receptor
KI: knock-in
KIF: kinesin superfamily
LDH: Lactate dehydrogenase
LGN: leucine-glycine-asparagine repeat protein
MAP: Microtubule Associated Protein
MCT: monocarboxylic transporters
MFN: mitochondrial fusion protein mitofusin
mHTT: mutant HTT
MRI: magnetic resonance imaging
mRNA: Messenger RNA
MSN: medium spiny neurons
NAD: nicotinamide adenine dinucleotide
NMDA: N-methyl-D-aspartate
NMR: nuclear magnetic resonance
N-ter: N-terminus
NuMA: Nuclear Mitotic Apparatus protein
P: product
PCM1: pericentriolar material 1 protein
PD: Parkinson's disease
PDH: pyruvate dehydrogenase
PET: Positron emission tomography
PFK: phosphofructokinase
PGAM: phosphoglycerate mutase
PGK: phosphoglycerate kinase
Pi: inorganic phosphate
PK: Pyruvate kinase
polyQ: poly-glutamine
PPP: pentose phosphate pathway
PRD: proline rich domain
PTM: post-translational modification
REST/NRSF: repressor element-1 transcription factor/neuron restrictive silencer factor
RILP: Rab7-interacting lysosomal protein
RNA: Ribonucleic acid
RNAi: RNA interference
ROS: Reactive oxygen species
RPE: Ribulose 5-phosphate epimerase
S: substrate
SAT: Slow axonal transport
SGK: Serum and Glucocorticoid induced Kinase
SMBA: spinal bulbar muscular atrophy
SNP: Single Nucleotide Polymorphism

SNr: Substantia Nigra pars reticulata
SOD: superoxide dismutase
TCA: tricarboxylic acid
TKL: transketolase
TPI: triose phosphate isomerase
UHDRS: Unified Huntington's Disease Rating Scale
ULK: Unc-51 like autophagy activating kinase
UPS: Ubiquitin Proteasome System
VAMP: vesicle-associated membrane protein
VAPB: VAMP-associated protein
VMAT: vesicular monoamine transporter
YAC: Yeast Artificial Chromosome

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V. INTRODUCTION

A. Chapter 1: Huntington's disease

1. General description of Huntington's disease

a) Discovery & history of Huntington's disease

Huntington's disease (HD) is a fatal fully penetrant genetic neurodegenerative disorder caused by a mutation in the huntingtin protein. It was first fully described in 1872 by the physician George Huntington where he outlined the clinical features and autosomal dominant inheritance of this disease initially termed "chorea" (Huntington, 1827). He also stated that this movement disorder was accompanied by personality changes and cognitive decline. The juvenile form of HD was discovered approximately 15 years later by J Hoffman who observed it at very early stages in two daughters aged 4 and 10 years old (Hoffman, 1888).

The gene responsible for this disorder is huntingtin and was not properly identified until much later. In 1983, the discovery of a polymorphic DNA marker had pinpointed the gene to the short arm of the fourth chromosome (Gusella et al., 1983). The development of exon trapping not long after enabled the isolation and identification of the HD gene (initially called IT15, at position 4p16.3) by The Huntington's Disease Collaborative Research Group in 1993 (Marcy E. MacDonald et al., 1993).

b) Prevalence, statistics & heritability of Huntington's disease in humans

HD's prevalence varies according to region. It affects 3 to 7 per 100 000 people of European descent but remains slightly less prevalent in Japanese, Chinese and African cultures (Evans et al., 2013; Pringsheim et al., 2012). Symptoms usually appear between the ages of 40 and 60 years of age and death inevitably ensues 15 to 20 years later. The late-onset nature of the disease renders medical care ethically challenging because patients may have children at the time of diagnosis who may also be carriers of the mutation. The much rarer juvenile form is defined as having an age of onset below 21 years of age.

The huntingtin protein (HTT) contains a polyglutamine stretch near to the N-terminal end of the protein (detailed in Chapter 2) due to a CAG trinucleotide repeat in the gene (detailed

in Chapter 2), the length of which is of great importance for determining age of onset. Healthy patients usually present an average of 17 glutamine repeats. As this number increases, the probability of contracting HD also increases and the age of onset decreases (figure 1). As a general rule, a CAG repeat length above 40 will almost certainly lead to HD by the age of 60 (Langbehn et al., 2004). Some of the first studies on human cohorts, notably in Venezuela, where several cases had been reported, showed that polyglutamine length accounted for around 60% of the variance for age of onset, with the remaining 40% being due to non-HTT related genetic and environmental factors (Wexler et al., 2004).

As mentioned above, HD is an autosomal dominant disorder, meaning that one mutant allele is sufficient to contract the disease, and that it affects men and women equally. As a result the vast majority of cases are heterozygous. Most information concerning homozygous cases comes from the analyses of the Venezuelan kindred which did not take into account the length of CAG and other genetic modifiers (Wexler et al., 1987). It has been shown that although the presence of two mutant HTT (mHTT) alleles in a given individual does not predict an earlier age of onset, it does however induce a more severe clinical evolution of symptoms leading to an earlier death. This suggests that the rate of evolution of HD pathogenesis leading to motor diagnosis is determined by a completely dominant action of the longest expanded allele (J. M. Lee et al., 2012; Squitieri et al., 2003). Indeed, the length of the CAG repeat in the normal HTT gene of a heterozygous patient does not seem to have an impact on disease progression (J. M. Lee et al., 2012). However, the existence of healthy patients with only one intact HTT gene, suggests that HD is not caused by a simple loss of function due to the CAG expansion (Ambrose et al., 1994).

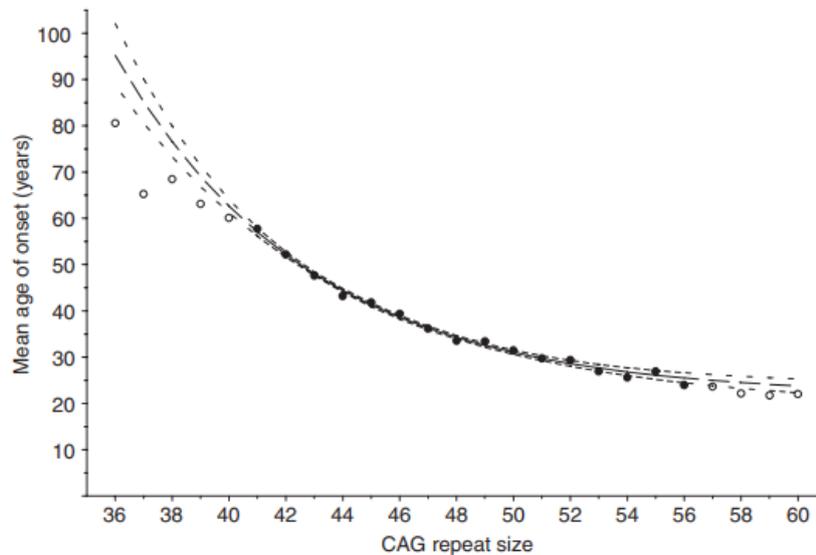


Figure 1: Population estimates of the mean age of onset for CAG repeat lengths 36–60. The solid circles and line indicate the range of data that was used to fit the exponential curves. The blank circles and long dashed lines indicate CAG lengths for which the model’s predictions were extrapolated. Small dashed lines indicate 95% confidence intervals, larger spaces between dashes indicate the region where the model’s predictions were extrapolated (Langbehn et al., Clin Genet, 2004).

Non-HTT related genetic modifiers of HD progression have been greatly investigated as they can be of importance for developing HTT-lowering therapies (developed below). The favored approach for identifying these modifiers has been to search for Single Nucleotide Polymorphisms (SNP) in the surrounding genetic environment of HTT or in genes connected to pathways believed to be involved in HD pathogenesis. Many candidate genes, including Glutamate Receptor Ionotropic Kainate 2 (GRIK2, also known as GLUR6) and the autophagy related protein Atg7, have been associated with earlier age of onset (Gusella & MacDonald, 2009; Rubinsztein et al., 1997). Conversely, other SNPs such as those located in Huntingtin-Associated Protein 1 (HAP1), a protein that interacts with HTT, have been linked to an 8 year delay in age of onset in HD patients (Metzger et al., 2008).

Although HD affects men and women equally, patient gender does have a slight influence on heritability. CAG repeats greater than 28 are unstable during replication leading to small inter-generational variations in CAG length. This instability is known to be stronger in male germline cells (Leeflang et al., 1999; M. E. MacDonald et al., 1993). In association, expansions in CAG repeat size are more common in paternal transmission and large expansions capable of inducing juvenile HD are linked to the father in 75% of cases (Aziz et al., 2011; Kovtun et al., 2000, 2004; Telenius et al., 1993). However, the age of the parent transmitting the disease does not have any effect on CAG instability (Aziz et al., 2011).

c) Symptoms

HD is characterized by three types of symptoms: motor, cognitive and psychiatric. They are assessed using the Unified Huntington's Disease Rating Scale (UHDRS), established in 1996 by the Huntington Study Group (Group, 1996). Motor symptoms are the most characteristic of the disease and are usually the basis for clinical diagnosis. Cognitive and psychiatric symptoms, however, often take a back seat to motor problems, despite being of more concern to patients and care-takers. Indeed, such behavioral changes in HD are more subtle due to the fact that they occur in a range similar to what can be observed in people without any mental issues. Memory loss, depression and mood swings are some of the many abnormalities recorded in HD patients, but even when these become pathological, they cannot be associated with HD alone.

(1) *Motor*

Motor symptoms, in contrast to cognitive and psychiatric symptoms, are more likely to worsen as disease pathogenesis progresses. Chorea and rapid involuntary movements are the most common and characteristic motor symptoms observed in HD patients. They often start in the extremities, such as in the fingers, toes and face, and slowly spread throughout the whole body (Roos et al., 2010). Patients also present dystonia, slow twisting movements of the limbs, bradykinesia, akinesia, and rigidity, but the degree at which these occur is highly dependent on the individual.

The clinical diagnosis of HD in patients is usually concomitant with the appearance of motor symptoms, with UHDRS scores steadily increasing as the disease progresses (Meyer et al., 2012). However, two ongoing large scale studies, TRACK-HD and PREDICT-HD, have identified subtle changes in motor function that occur up to several decades prior to classical disease onset. This may help predict earlier diagnosis in patients (Biglan et al., 2009; Sarah J. Tabrizi et al., 2013).

(2) *Cognitive*

A large array of scaling systems exist to evaluate cognitive capacity, from the initial UHDRS to the more recent HD Cognitive Assessment Battery (HD-CAB) (Stout et al., 2014).

The most predictive cognitive feature in HD is psychomotor slowing, classically demonstrated through the Stroop, Digit symbol substitution and Trail making tests (Maroof et al., 2011; Sarah J. Tabrizi et al., 2012). This is accompanied with difficulties in executive skills such as planning, multi-tasking and attention (Georgiou et al., 1995; Watkins et al., 2000). Similarly to Parkinson's disease (PD), automatic executive tasks seem to require more focused and conscious attention for HD patients.

Learning and memory have long been accepted as being significantly attenuated in HD, especially for visuospatial memory and when personal space manipulation is required (Brouwers et al., 1984; Beatty et al., 1989). Additionally, these features have a tendency to gradually deteriorate as the disease progresses (Davis et al., 2003; Lawrence et al., 1996). However there is still much debate over whether these symptoms may be considered as early biomarkers for HD (Majerová et al., 2012; Stout et al., 2012). This may be due to differences in tests used to assess symptoms stated as being of the same nature, but will only become clear as more precise and accurate tests are set up to assess general cognitive dysfunction.

The ability of patients suffering from HD to recognize emotions is also considerably impaired. Moreover, this is, interestingly, quite specific to negative emotions (S. A. Johnson et al., 2007). The first reports identified the recognition of disgust as particularly affected (Sprenkelmeyer et al., 1996, 1997), but it is now more or less accepted that this disability can be generalized to several other negative emotions, including fear and anger (Henley et al., 2008; Snowden et al., 2008). However, it remains unsure whether this feature is detectable in pre-manifest HD (Adjeroud et al., 2015; Henley et al., 2012) but it is generally agreed that it has a tendency to aggravate as the disease progresses (Labuschagne et al., 2013). Some have observed that impairments in negative emotion recognition were absent when context and other visual cues were presented to patients, suggesting an importance of context in emotion perception (Aviezer et al., 2009).

(3) *Psychiatric*

Neuropsychiatric manifestations are among the most problematic features of HD. Depression is one of the most frequently reported symptoms in patients suffering from HD (Leroi et al., 2002; Paulsen, 2005) and is known to be present early in the prodromal stages of the disease (up to 10 years in some studies) (Duff et al., 2007; Epping & Paulsen, 2011; Julien et al., 2007). However, its evolution throughout a patient's lifetime is still disagreed upon.

Some suggest that depressive symptoms are correlated with the stage of the disease (Dale et al., 2016), whereas others have shown that prevalence of depression does not vary (J. C. Thompson et al., 2012). It has even been reported to decrease over the course of the disease (Paulsen, 2005).

Anxiety and apathy are also common symptoms in HD and also appear at different moments depending on the individual. Indeed, as with depression, anxiety has been shown to appear, even peak, at stages predating motor symptoms (Decruyenaere et al., 2003). Apathy, on the other hand, seems to appear at later more advanced stages of the disease (J. C. Thompson et al., 2012; Van Duijn et al., 2014).

Other symptoms include irritability, mood swings and a higher incidence of suicide but are less common or less well researched (Hubers et al., 2013).

(4) *Other symptoms*

HTT is expressed in virtually all cell types and although HD is associated mainly with brain dysfunction, other bodily functions and organs are also considerably affected. Muscle atrophy is a prime example of this. HD patients show reduced muscle strength by approximately 50% (Busse et al., 2008) associated with several metabolic dysfunctions including reduced mitochondrial adenosine triphosphate (ATP) production during post-exercise recovery (Lodi et al., 2000) and increased lactate concentrations in the plasma in both symptomatic and pre-symptomatic patients (Andrea Ciammola et al., 2011). Muscle cells from mutation carriers studied *in vitro* revealed mitochondrial depolarization, cytochrome C release, increased caspase activities, defective cell differentiation and HTT inclusions in differentiated myotubes (A. Ciammola et al., 2006).

Weight loss is also a major issue in HD. Indeed, patients often have lower Body Mass Indexes (BMI) and increased caloric intake is necessary to maintain minimal body weight as well as compensate for the increased energy expenditure due to their hyper-metabolic state (TeSlaa & Teitell, 2014).

HTT, and therefore mHTT, are most expressed in the brain and testes. Fertility is not affected in men suffering from HD, but they do present lower concentrations of testosterone, reduced numbers of germ cells and abnormal seminiferous tubule morphology (Markianos et al., 2005; Pridmore & Adams, 1991; Van Raamsdonk et al., 2007).

Finally, patients with HD have a higher risk of heart disease (Abildtrup & Shattock, 2013) which contributes to the fact that cardiac arrest is one of the most common causes of death in HD (Sørensen & Fenger, 1992).

2. Animal models of Huntington's disease

a) Mouse models

Three types of mouse models exist: transgenic truncated models, transgenic full-length models, and knock-in (KI) models. Truncated transgenic mouse models express the first exon of the human mHTT protein and full length models express the entire protein. Transgenic models therefore express 3 HTT genes, 2 wild-type forms and 1 mutated form, truncated or not, making the mHTT/HTT ratio different to what is seen in humans.

R6 transgenic mice are the oldest models used to study HD of which two subtypes exist: R6/2 and R6/1. R6/2 mice express the N-terminal portion of the human HTT protein (exon 1) with 150 polyglutamine repeats. They have a very aggressive phenotype with motor symptoms appearing around 1 month of age, as well as a shortened lifespan of 3 to 5 months (Mangiarini et al., 1996). In these mice, striatal volume is reduced (Stack et al., 2005) and mHTT forms intracellular and intranuclear aggregates just as in humans (Turmaine et al., 1997). Motor deficits include tremor, reduced climbing, and clasping (Mangiarini et al., 1996; B. R. Miller et al., 2008) associated with epileptic seizures (Mangiarini et al., 1996) and decreased learning (Murphy et al., 2000). Moreover, these behavioral changes are correlated to neurochemical changes in basal ganglia circuitry (Bibb et al., 2000; M. A. Johnson et al., 2006; B. R. Miller et al., 2008; Ortiz et al., 2010). The R6/1 mouse line expresses 110 to 115 CAG repeats and has a longer lifespan than R6/2 mice. The first symptoms appear around 4-5 months, motor symptoms at 6-7 months and death of the animal occurs at 10-14 months (Mangiarini et al., 1996; Naver et al., 2003). They also present striatal volume decrease and mHTT aggregates (Mangiarini et al., 1996) as well as reduced motor and cognitive capacities associated with neuronal dysfunction (Mangiarini et al., 1996; B. R. Miller et al., 2008).

Full length transgenic models express the whole human mHTT gene including exons, introns and regulatory genetic material located around the gene. Two subtypes exist depending on the nature of the vector used: Yeast Artificial Chromosome (YAC) and Bacterial Artificial Chromosome (BACHD). The advantage of these models compared to the older R6 model, is

that the disease progresses slower and animals live longer allowing for more in depth studies. For YAC mice, several lines have been created depending on the length of the CAG repeat, YAC18 (control), YAC46, YAC72, and YAC128 mice. Only one BACHD mouse model exists and expresses 97 CAA-CAG repeats. Both YAC and BACHD mice suffer from motor deficits associated with cortical and striatal atrophy and dysfunction (Estrada-Sánchez et al., 2015; Gray et al., 2008; Slow et al., 2003; Van Raamsdonk et al., 2005). Additionally, YAC128 is one of the few transgenic models exhibiting both the hyper- and hypoactive behavioral phenotype seen in humans as well as striatal followed by cortical atrophy. By 3 months of age a hyperkinetic phenotype emerges which is then followed by progressive motor deficits. By 9 months of age, motor deficits are apparent and the hypokinetic phenotype is accompanied by striatal atrophy. Finally, when the animal reaches 12 months of age, cortical atrophy starts to occur (Slow et al., 2003; Van Raamsdonk et al., 2005).

The final most recent model for HD is the knock in mouse that carries the first human exon with expanded CAG repeats within the native murine HTT gene, under the mouse HTT promoter. Several models exist including CAG140, zQ175 and HdhQ111. For each of these models, the number in the name corresponds to the number of CAG repeats within the mHTT gene. These mice generally show subtle behavioral, histopathological, and molecular phenotypes compared to the transgenic models that overexpress mHTT (Chang et al., 2015), such as characteristic nuclear aggregates and decreased striatal volume in CAG140 mice (Menalled et al., 2003; Hickey et al., 2009), as well as decreased body weight, tremor and abnormal gait in combination with decreased cognitive ability for zQ175 mice (Heikkinen et al., 2012; L. B. Menalled et al., 2012). Moreover, these phenotypes are detectable in heterozygous mice as well which renders this model more resembling to what can be seen in human patients. This is not the case for transgenic models.

b) [Other models](#)

Although mice remain the most common models to study HD, several other mHTT expressing animals have been generated since the discovery of the gene. Similarly to mice, a few transgenic rat models exist, such as HD51 rats that express a truncated form of human HTT with 51 CAG repeats under the control of the rat's endogenous HTT gene promoter. mHTT was mainly expressed in the basal ganglia, hippocampus and several areas in the cortex, as well as in the cerebellum and spinal cord but at lower levels. Interestingly, these rats exhibit

hyperactive behavior in early stages before the onset of weight loss and HD-like motor abnormalities such as gait and dyskinesia of the head at around 10 to 15 months of age (H. P. Nguyen et al., 2006; von Hörsten et al., 2003). Transgenic rats expressing full length mHTT have also been generated. BACHD rats express 97 CAG repeats, capable of accumulating in a similar manner to what is seen in humans, associated with reduced dopamine levels and motor deficits (Yu-Taeger et al., 2012).

Modeling HD in bigger animals has also been attempted. Transgenic rhesus monkeys expressing exon 1 of mHTT with 84Q under the control of the human ubiquitin promoter were created in 2001 by injecting lentiviruses into fertilized oocytes (Chan et al., 2010; Yang et al., 2009). They developed key clinical HD features including dystonia, chorea, and quite uniquely, seizures, seen in no other HD animals other than humans (Yan et al., 2018; Shang-hsun Yang et al., 2009). Unfortunately, postnatal death was very common in these animals and those that survived past birth died prematurely (A. W. S. Chan et al., 2010). A similar outcome was seen in transgenic pigs that expressed 105 CAG repeats in an N-terminal portion of HTT consisting of the first 208 amino acids (D. Yang et al., 2010). Conversely, full length transgenic sheep do not present any overtly aggressive phenotypes and have normal lifespans, showing only a decrease in DARPP-32 expression (an MSN marker) (Jacobsen et al., 2010).

Smaller models of HD have also been studied. *Caenorhabditis elegans* neurons expressing human full length expanded polyglutamine HTT contain protein aggregates that induce neurodegeneration and mechano-sensory dysfunction (Faber et al., 1999; J. A. Parker et al., 2001). Expressing poly-glutamine HTT fragments in zebrafish has demonstrated that neurodegeneration was initiated before aggregate formation, suggesting that neurotoxicity is due to oligomeric mHTT (Schiffer et al., 2007). Finally, truncated and full length mHTT expressed in different drosophila cell types leads to motor and cellular dysfunction associated with lower lifespan in these animals (Jackson et al., 1998; W. C. M. Lee et al., 2004; Steffan et al., 2001).

3. [Neurodegeneration and cellular dysfunction](#)

a) [Neurodegeneration](#)

Neurodegeneration of the brain is a hallmark of HD, in particular that of the striatum which is the most pronounced (figure 2). This deep brain structure atrophies long before clinical

diagnosis and progressively decreases in size throughout the patient's life. Evidence of this has been observed up to 15 years before patients develop any clear motor symptoms (Aylward et al., 2011; Hobbs et al., 2010). Interestingly, striatal volume is reduced by a third at the time of diagnosis, such significant reductions are not observed for other brain structures (Aylward et al., 2004; Coppen et al., 2018). As a result, neurodegeneration of the striatum specifically remains one of the best biomarkers of HD as it correlates strongly with age of onset, CAG length, UHDRS scores and age of death (Bohanna et al., 2011; Dogan et al., 2013; A. E. Hendricks et al., 2012).

A close second to striatal atrophy is loss of cortical grey and white matter, especially cortico-striatal projecting neurons (Yi Hong et al., 2018). Although degeneration in this area has not always been correlated with age of onset and CAG length as it is with the striatum (Ruocco et al., 2006), it tends to appear early in the disease, even during prodromal stages according to some reports (Yi Hong et al., 2018). Nonetheless, cortical atrophy occurs later and slower than the striatum and mainly in layers 3, 5 and 6 of the cortex (Passani et al., 1997; Jean Paul Vonsattel et al., 1985). Subsequently, it has been shown that remaining neurons in layers 3 and 5 of the prefrontal cortex show dendritic augmentation as a possible compensatory mechanism for overall cortical atrophy (Sotrel et al., 1993).

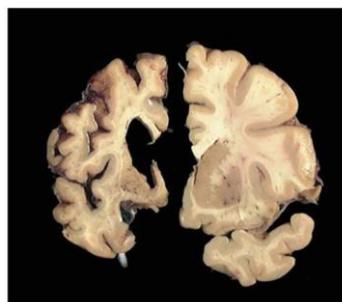


Figure 2: The HD brain degenerates. Images showing a normal human brain slice on the right and an advanced stage 4 HD brain slice on the left. At late stages of the disease, cortical and striatal atrophy is evident. Image courtesy of the Harvard Brain Tissue Resource Center.

Other brain regions also atrophy throughout the disease but to a lesser extent or later than their cortical and striatal counterparts. Among them, neurodegeneration in prodromal HD has been observed in the thalamus, of which the atrophy correlates and contributes to cognitive impairment (Kassubek et al., 2004). The hypothalamus, amygdala and globus pallidus also present volumetric reductions (Dogan et al., 2013; Douaud et al., 2006; Kassubek et al., 2004). Finally, magnetic resonance imaging (MRI) studies have shown that the cerebellum is reduced

late in the disease but its degeneration may be due to disease duration rather than a direct consequence of cellular dysfunction (Ruocco et al., 2006).

b) Cellular features and dysfunctions

(1) *Inclusion bodies*

As striatal atrophy is a hallmark of HD on the macroscopic level, inclusion bodies are a staple on the microscopic level. They are formed through aggregation of mHTT in cells, particularly neurons (DiFiglia et al., 1997; Turmaine et al., 1997) where they accumulate in nuclei as well as the cytoplasm, dendrites and axon terminals (DePamphilis et al., 2007). Additionally, these inclusions have been reported to accumulate more frequently in neurons located in the cortex than in the striatum and other brain structures (Sapp et al., 1999). First mHTT monomers, specifically N-terminal fragments, following proteolysis (see chapter 2) (Landles et al., 2010; Ratovitski et al., 2009; Schilling et al., 2007), assemble into a variety of oligomeric formations and gradually form inclusion bodies as they accumulate. This process depends on the amino acid sequences flanking the polyglutamine stretch, post-translational modifications of mHTT, and levels of molecular chaperones (Gu et al., 2009; Jeong et al., 2009; Tam et al., 2010; Thakur et al., 2009).

The reason inclusion bodies accumulate is not yet fully understood but has been suggested to be linked to inefficient degradation mechanisms since several other proteins have been found sequestered within these aggregates, including those important for transcription and protein quality control, thus contributing to the loss of function phenotype of the disease (Soto et al., 2003). It is also believed that the progressive accumulation of mHTT to form aggregates may be a neuroprotective process put in place by the cell to compensate for degradation deficiencies, and that the oligomeric form of mHTT is more dangerous for cellular function than the aggregates (Legleiter et al., 2010; Olshina et al., 2010; Saudou et al., 1998).

(2) *Cortico-striatal imbalance*

The cortex and striatum are important structures within the basal ganglia circuitry responsible for motor coordination and mood, two phenotypes that are particularly affected in HD. The cortex is a laminar structure made up of six cortical layers that project neurons

throughout the brain, including deep brain structures such as the basal ganglia. In HD, pyramidal neurons from layers 3, 5 and 6 are the most affected and reduced by 30% by time of death (Cudkowicz & Kowall, 1990; Hedreen et al., 1991; Sotrel et al., 1991; Heinsen et al., 1994). Neurons in layers 3 and 5 project to the basal ganglia, brain stem, and spinal cord, neurons from layer 6 project to the thalamus (Estrada-Sánchez & Rebec, 2013; McGeorge & Faull, 1989). Recently, it has been shown that cortical neurons have increased activity in very early pre-symptomatic stages before progressively decreasing as the disease unfolds (Burgold et al., 2019).

The striatum is composed of GABAergic medium spiny neurons (MSN) (at 95%) and interneurons. Two subtypes of MSN exist and are of interest in HD: striato-nigral MSN that express substance P, dynorphin, and D1-like dopamine receptors and that project to the internal Globus Pallidus (GPi) and Substantia Nigra pars reticulata (SNr) forming the direct pathway, and striato-pallidal MSN that contain enkephalin and express D2-like dopamine receptors and that project to the external Globus Pallidus (GPe) forming the indirect pathway. Both types of MSN integrate glutamatergic cortical input and relay this information to downstream basal ganglia (Kreitzer and Malenka, Neuron, 2008). The direct and indirect pathways serve to fine tune motor function. The direct pathway is grossly considered to initiate movement whereas the indirect pathway supposedly inhibits it (Alexander & Crutcher, 1990; DeLong, 1990; Albin et al., 1989).

While the presence of mHTT is deleterious to many neuronal sub-types, medium spiny neurons of the striatum exhibit enhanced vulnerability. Indeed, striato-pallidal MSN of the indirect pathway are the most affected in the early stages of the disease leading to insufficient movement inhibition, which explains why chorea and involuntary movements are the first motor symptoms seen in patients. MSN in the direct pathway are lost much later in advanced stages of the disease resulting in rigidity and bradykinesia (Albin et al., 1992; Galvan et al., 2012; L. Menalled et al., 2000; E. Sapp et al., 1995; J P Vonsattel & DiFiglia, 1998). The reason for this selective degeneration of neurons in the striatum is not yet fully understood but some studies have suggested that differential cortical inputs to these two types of neurons may be responsible (Berretta et al., 1997; Reiner et al., 2003).

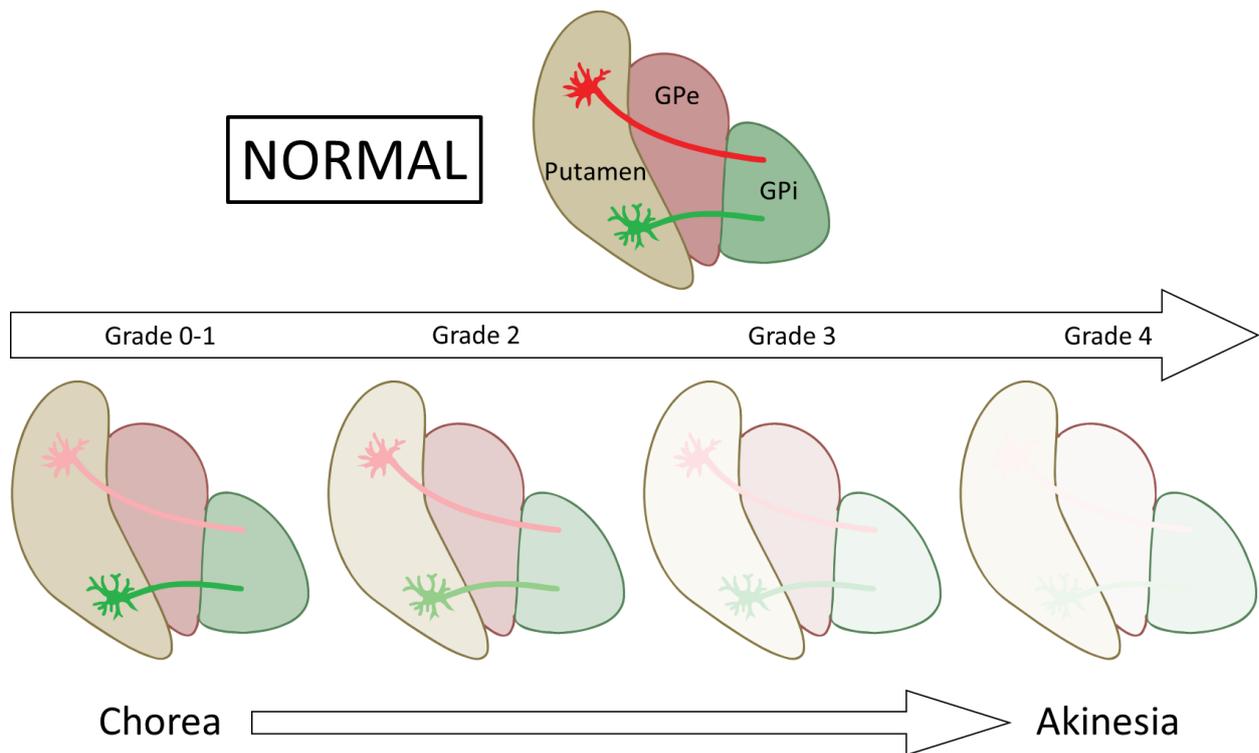


Figure 3: Evolution of medium spiny neuron degeneration. Illustration of the initial loss of enkephalin-positive MSNs (in red) at the beginning of the disease which leads to involuntary movements, followed by the gradual degeneration of substance P-positive neurons (in green) leading to rigidity and akinesia.

(3) Axonal transport

A major cellular dysfunction in HD is axonal transport and has been linked to striatal degeneration. Striatal neurons require Brain Derived Neurotrophic Factor (BDNF) for survival which they do not produce themselves, rendering them reliant on extra-striatal input. Of the many structures that project onto the striatum, cortical input is its most valuable source of BDNF. Indeed, a lack of BDNF causes dysfunction and neurodegeneration in both striatal and cortical neurons (Baquet et al., 2004), and its concentration has been reported to be reduced in the striatum in patients and mouse models of HD (Apostol et al., 2008; Gharami et al., 2008). BDNF is transported in vesicles from cortical somas to cortico-striatal synapses. This mechanism has been shown to be significantly reduced in HD mouse lines such as CAG140 and zQ175, and may be a direct cause of striatal followed by cortical degeneration (Gauthier et al., 2004; Virlogeux et al., 2018). Many other organelles show decreases in transport including autophagosomes (Y. C. Wong & Holzbaur, 2014) and mitochondria, that are known to associate with mHTT (Orr et al., 2008). Finally, glutamate release at cortico-striatal synapses is altered in HD, which has also been linked to defective transport, suggesting that transport of

different types of vesicles such as synaptic vesicles is modified in the disease (Gunawardena & Goldstein, 2005; Virlogeux et al., 2018).

(4) *Energy metabolism*

Defective glucose uptake and mitochondrial function are both strong contributors to metabolic dysregulation in HD. Several studies have shown reduction of glucose transporters GLUT1 and GLUT3 in both cortical and striatal neurons from animal models such as CAG140 mice (McClory et al., 2014) as well as in *post mortem* tissue samples from HD patients (Gamberino & Brennan, 1994). This reduction in transporter expression can be linked to reduced glucose uptake in neurons (Adanyeguh et al., 2015; Lalić et al., 2008; Powers et al., 2007). Pre-manifest HD subjects show a metabolic decrease in the striatum, combined with frontal, temporal and thalamic hyper-metabolism, as a possible compensatory reaction to progressive decrease of glucose uptake in the caudate (Ciarmiello et al., 2006, 2012; Feigin et al., 2007). Additionally, glucose hypometabolism and reduced GLUT3 expression on neuronal membranes in HD are caused, at least in part, by deficient Rab11 activity (X. Li et al., 2012), a protein that regulates trafficking of numerous receptors to cell membranes, including GLUT3 (McClory et al., 2014). Glucose metabolism is decreased in some regions of the cerebral cortex and throughout the whole striatum in HD patients, and the level of lactate is increased in the striatum, possibly linked to deficiencies in aerobic energy metabolism (mitochondria) (L. Harms et al., 1997).

Mitochondria are a central problem in HD, so much so that treatment of animals with 3-nitropropionic acid, a mitochondrial toxin, causes selective death of medium sized spiny neurons of the striatum, similar to the pathology observed in HD (Beal et al., 1993). Several mitochondrial mechanisms are altered in both patients and animal models, including modification of mitochondrial gene expression, DNA damage (Reddy & Shirendeb, 2012) and a decrease in enzymatic activity of multiple complexes of the oxidative respiratory chain (Johri et al., 2013; S. J. Tabrizi et al., 1999), resulting in calcium dysregulation and low ATP production (Seong et al., 2005). From a physical point of view, mitochondria are structurally damaged with broken cristae, appearing smaller and in reduced numbers in post mortem brain tissue (Costa et al., 2010; J. Kim et al., 2010; Song et al., 2011). To explain this phenomenon, some have shown that mHTT interacts with Dynamin-Related Protein 1 (Drp1), responsible for mitochondrial fission, resulting in elevated GTPase activity of Drp1 and thus an imbalance

between mitochondrial fragmentation and fusion (Shirendeb et al., 2012; Song et al., 2011). This finally leads to oxidative damage due to the accumulation of impaired mitochondria which is highly detrimental to overall neuronal function (Martinez–Vicente et al., 2010; Twig & Shirihai, 2011).

(5) *Synaptic transmission*

Synaptic disconnection, especially between cortical and striatal neurons, is also a prominent feature of HD pathogenesis. This is linked to several neuronal functions that are affected by the presence of mHTT throughout the cortico-striatal network. Synaptic vesicle transport, docking and recycling lead to disrupted glutamate and dopamine release which in turn results in altered neuronal activity (Cepeda et al., 2003). This is associated with downregulation of proteins involved in synaptic function, endocytosis and exocytosis (Burgold et al., 2019; Diprospero et al., 2004; Modregger et al., 2002; Morton et al., 2001; Smith et al., 2005).

Upon release into the synaptic cleft and binding to the various pre and post synaptic receptors, glutamate is quickly recycled by the pre-synaptic cell and astrocytes that are tightly bound to the synapse. In HD, the expression of glutamate transporters is reduced and decreases with disease severity (Arzberger et al., 1997; Behrens et al., 2002; Liévens et al., 2001) resulting in overly present glutamate in the synaptic cleft and therefore excitotoxicity. Additionally, increased glutamate release has been seen in 1 month old YAC128 mice that then decreased with age (Joshi et al., 2009) which can be linked to the cortical hyperactivity noted in early stages of the disease.

Downstream targets of glutamate are also altered in HD, including ionotropic and metabotropic receptors. Two types of N-methyl-D-aspartate receptors (NMDA receptors) are known to locate differently along the membrane of the post-synaptic neuron: NR1/NR2A receptors are located synaptically and tend to mediate pro-survival signals, and NR1/NR2B receptors are found extra-synaptically and play a role in cell death (Gouix et al., 2009; Hardingham et al., 2002; Papadia et al., 2008). However, mHTT specifically increases extra-synaptic NMDA receptor expression and current, with increased EPSC charge and elevated NMDA peak currents in pre-symptomatic (1 month) YAC128 MSN (Milnerwood et al., 2010) and overexpression of NR2B subunits in homozygous CAG150 knock-in mice results in exacerbated striatal neurodegeneration (Heng et al., 2009). Although the manner in which

mHTT increases NR2B activity is not well known, several studies have shown that selective inhibition of extra-synaptic receptors through low dose memantine or ifenprodil, an NR2B-type selective antagonist, have neuroprotective effects on striatal neurons both *in vitro* and *in vivo* (Fan et al., 2009; Zeron et al., 2002).

Metabotropic receptors are G-protein coupled receptors that regulate downstream signaling pathways such as calcium release. Several of these receptors have been shown to be decreased in the striatal cells of symptomatic R6/2 mice (Cha et al., 1998). mHTT interacts with the inositol (1,4,5)-triphosphate receptor (InsP3R), of which the activation depends on group 1 metabotropic receptors. This interaction increases InsP3R sensitivity which induces increased calcium release in striatal cells (Tang et al., 2003).

Neuronal activity at the synapse malfunctions also due to α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. Analysis of AMPA-mediated excitatory postsynaptic potential (EPSC) and evoked receptor activity revealed subtle but significant changes in young pre-symptomatic YAC128 mice (Milnerwood & Raymond, 2007). Additionally, a clear disease progression-dependent reduction in EPSC frequencies of AMPA responses is found in the more aggressive R6/2 HD model at onset of motor symptoms (Cepeda et al., 2003).

As mentioned previously, dopamine plays a fundamental role in HD. Reduced dopamine receptor labeling occurs in pre-symptomatic HD patients and correlates with disease progression, cell loss and cognitive impairment (Piccini, 2004). Equally, diminished receptor expression has also been noted before and after behavioral onset in several mouse models (Bibb et al., 2000; Jarabek et al., 2004; Kennedy et al., 2005). To date, the only commercialized drug for treating the disease is Tetrabenazine, a vesicular monoamine transporter type 2 (VMAT2) inhibitor used to increase basal dopamine in the striatum to compensate for decreased receptor expression (Feigin et al., 2007; Hongyu Wang et al., 2010).

(6) *Glia*

Although a lot of focus is centered around neurons in HD, glial cells also express mHTT and are also affected in the disease. Astrocytes are star shaped cells that envelop synapses and are an integral part of the blood brain barrier. Several studies have shown that mHTT expression and aggregation in astrocytes (although less than in neurons (Bradford et al., 2009)) contribute to neuronal glutamate excitotoxicity via downregulation of the glutamate transporter

EAAT2/GLT1 located on the astrocytic membrane resulting in impaired glutamate recycling (Arzberger et al., 1997; Ellrichmann et al., 2013; Estrada-Sánchez & Rebec, 2012; Faideau et al., 2010; Khakh et al., 2017; Liévens et al., 2001; J. Y. Shin et al., 2005). Others have demonstrated that symptom onset in R6/2 and Q175 HD mouse models is associated with decreased Kir4.1 potassium channel expression on astrocytes, leading to elevated striatal extracellular potassium, which increased MSN excitability (Tong et al., 2014). MHTT has equally been shown to reduce astrocytic release of BDNF (Yan Hong et al., 2016; Linhui Wang et al., 2012) and contribute to astrocyte-mediated pericytic cell death along cerebral blood vessels (Hsiao et al., 2015).

In the brain, microglia represent approximately 5-10% of cells and are considered the resident immune cells (Frost & Schafer, 2016). Experiments on human HD brain tissue identified the presence of reactive microglia in the neostriatum, cortex and globus pallidus which were absent in control brain tissue (E. Sapp et al., 2001; J. P. G. Vonsattel et al., 2011; Jean Paul Vonsattel et al., 1985). However, the number of activated microglia in the striatum and cortex showed a direct correlation with degree of neuronal loss and microglia were closely associated with pyramidal neurons, suggesting that neuroinflammatory changes might simply be induced by the degenerating neurons (E. Sapp et al., 2001). Moreover mHTT causes an increase in basal pro-inflammatory gene expression. This in turn leads to higher levels of pro-inflammatory cytokines production (Björkqvist et al., 2008; Crotti et al., 2014; H. M. Yang et al., 2017) and neuroinflammatory mediators specifically in regions such as the cortex and striatum (Silvestroni et al., 2009) perpetuating inflammation and tissue damage. Conversely, depleting mHTT in all other cells except microglia seems to rescue behavioral performance and neuropathology (Petkau et al., 2019).

Finally, oligodendrocytes, responsible for myelin production and encapsulation of axons in the brain, also malfunction in HD. Broad abnormalities in superficial white matter are present before any clinical symptoms in human HD brains (Phillips et al., 2016) and transcript levels of myelin-related genes in striatal and cortical tissues are significantly lower in YAC128 mice from 2 weeks of age (Yi Teo et al., 2016). Strikingly, transgenic mice that selectively express mHTT in oligodendrocytes show progressive neurological symptoms and early death, as well as age-dependent demyelination and reduced myelin gene expression (Huang et al., 2015).

(7) *Other dysfunctions*

Many other physiological mechanisms are at risk in HD, including protein degrading systems, neurogenesis, embryogenesis and transcription.

mHTT is believed to be degraded by both the Ubiquitin Proteasome System (UPS), given that the protein is ubiquitinated (X. J. Li & Li, 2011), as well as autophagy since inclusion bodies sequester autophagy related proteins such as mTOR (Ravikumar et al., 2004). It is still very much debated whether mHTT truly affects the UPS. Some suggest that the proteasome is simply saturated by the overwhelming amount of mHTT (Bennett et al., 2005), whereas others believe that this cannot be possible having shown that proteasome malfunction occurs before aggregate formation (Bennett et al., 2005; Venkatraman et al., 2004). A final hypothesis is that proteasome activity deficiency may be an indirect consequence of mitochondrial dysfunction leading to less ATP available for protein degradation (Hipp et al., 2012). MHTT's effect on autophagy is also complex. It has been shown that treatment with an autophagy activator attenuates huntingtin accumulation and cell death *in vitro* in CAG140 cells (Ravikumar et al., 2004), yet basal autophagic activity is maintained in HD cells (Baldo et al., 2013). To explain this, it has demonstrated that autophagic vacuoles form at normal rates in HD cells and are adequately eliminated by lysosomes, but fail to efficiently trap cytosolic cargo in their lumen, suggesting that inefficient engulfment of cytosolic components by autophagosomes is responsible for their slower turnover, functional decay and accumulation inside HD cells (Martinez–Vicente et al., 2010).

HD is also considered by some as a developmental disorder based on the fact that silencing HTT in mice causes embryonic death (Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995) and that conditional knock-in mice expressing mHTT 97Q until postnatal day 21, develop a HD-like phenotype including neuropathology and motor deficits (Molero et al., 2016). Nonetheless, this phenotype is not as severe as in the mice with lifelong expression of mHTT. In conditional knock downs for HTT and knock-in zQ175 mice, corticostriatal connectivity is altered and excitatory synapse formation is increased in the developing striatum which suggests HTT loss of function in the development of corticostriatal synaptic connectivity (McKinstry et al., 2014). It has recently been demonstrated that several neurodevelopmental processes in the cortex are affected in both human and mouse embryos (Barnat et al., 2020).

Finally, mHTT interacts with major components of the general transcriptional machinery, affecting both general promoter accessibility and recruitment of RNA polymerase

II (Seredenina & Luthi-Carter, 2012) which leads to aberrant expression of genes involved in pro-survival pathways including BDNF (Zuccato et al., 2003).

4. Treating Huntington's disease

Management options at this time are limited, and there is still no therapy to slow the neurodegeneration or the overall rate of function loss. No drug has proven to be effective in any clinical tests on humans (Mason & Barker, 2009). Over the past two decades, 99 clinical trials have been performed in HD investigating 41 different compounds. However, the success rate remains low with only 3.5% of trials progressing to the next stage (Travessa et al., 2017).

a) Symptomatic treatment

The only drug specifically licensed to treat chorea is tetrabenazine (Huntington-Study Group, 2006), a synaptic dopamine transport inhibitor, which provides a sustained anti-choreic effect at doses in the range 50–75 mg per day. Unfortunately, it has many side effects including sleep problems, depression, anxiety and restlessness (Coppen & Roos, 2017). Deutetrabenazine is a modified version of tetrabenazine that contains deuterium molecules and therefore has a prolonged half-life. The FIRST-HD study revealed that deutetrabenazine significantly reduced chorea (Huntington-Study Group, 2016) and it is possible that it may result in fewer side effects than its predecessor, despite no head-to-head comparison (Rodrigues et al., 2017). Pharmacological intervention to treat psychiatric symptoms include selective serotonin uptake inhibitors such as citalopram, fluoxetine, and mirtazepine, which have serotonergic and noradrenergic effects. Neuroleptics can be useful in treating aggression and psychosis. A number of medications including methylphenidate, atomoxetine, modafinil, amantadine, bromocriptine and bupropion have been used to treat apathy (Eddy et al., 2016). Anti-cholinesterase inhibitors and coping mechanisms for the patient's work environment, for example, are used to treat cognition in HD (Y. Li et al., 2013).

b) Genetic therapy

Two main genetic strategies are currently being tested: lowering of overall HTT expression, thus targeting both wild-type (WT) and mutant forms, or specific reduction of

mHTT. Each of these is faced with certain limitations. The functional impact of lowering levels of WT HTT in the adult human brain has yet to be fully elucidated. Conditional inactivation of HTT in mice leads to severe behavioral abnormalities and progressive brain atrophy (Dietrich et al., 2017). By contrast, others have shown that depletion of HTT in adult neuronal cells does not cause neurodegeneration or any other detectable phenotypes (Tanaka et al., 2016), and that transient, partial reduction of WT HTT is safe and well tolerated (Boudreau et al., 2009; Drouet et al., 2009; Grondin et al., 2012; Kordasiewicz et al., 2012; McBride et al., 2011). In fact, humans expressing a single functional copy of HTT (expressing 50% of normal HTT levels) do not display any overt behavioral abnormalities (Ambrose et al., 1994). An approach based on allele specificity has a certain appeal therefore, given that it avoids the unknowns of removing WT HTT. Allele selectivity can be achieved by either targeting the expanded CAG tract on the mutant allele (Datson et al., 2017; Evers et al., 2011) or by targeting other HTT polymorphisms, such as SNPs and nucleotide insertions or deletions associated with the CAG expansion. Several studies have demonstrated the efficiency of such techniques in both patients and animal models (Carroll et al., 2011; M. A. Kay, 2015; Pfister et al., 2009; Skotte et al., 2014; Y. Zhang et al., 2009). Multiple SNPs have been identified in diverse populations of patients with HD (Bilsen et al., 2008; Carroll et al., 2011; M. A. Kay, 2015; Pfister et al., 2009), one study has even demonstrated that targeting individual SNPs on the three most common HD haplotypes would provide a cumulative therapeutic option for nearly 80% of patients with HD (M. A. Kay, 2015).

Lowering HTT expression, whether it be all HTT or mHTT alone, is mainly achieved by targeting RNA with the use of antisense oligonucleotides (ASOs), RNA interference (RNAi) or small molecule splicing inhibitors. ASOs are synthetic nucleic acid molecules that hybridize with complementary RNA sequences to promote gene silencing, alter transcript processing (Evers et al., 2014) or prevent transcript translation (Gagnon et al., 2010). However, ASOs can only transiently lower mHTT because they are degraded over time (Southwell et al., 2014). The IONIS HTTRx compound is a non-allele specific ASO that targets human HTT through the formation of an ASO/RNA complex signaled for RNase H degradation (Macdonald et al., 2015). RNAi delivery is more invasive than ASOs requiring intracranial injection into the striatum. However, a single treatment may provide permanent HTT lowering (Wild & Tabrizi, 2017). For instance, delivery of RNAi reagents using adeno-associated virus (AAV) and lentiviral vectors have been used to achieve long-term HTT lowering in the CNS (Boudreau et al., 2009; Cambon et al., 2017; McBride et al., 2011).

Targeting the DNA of mHTT can be achieved using two approaches, zinc finger proteins and the clustered inter-spaced short palindromic repeats system (CRISPR/Cas9). Zinc fingers proteins form a structural motif that bind to DNA. Zinc fingers form a structural motif that bind to the expanded CAG DNA and have been used to reduce levels of mHTT protein in animal models. However, as they create non-native proteins, they have the potential to cause immune reactions thus further work is needed (Agustín-Pavón et al., 2016). CRISPR/Cas9 technology has been used in fibroblasts of an HD patient to excise the promoter regions, transcription start site and the CAG mutation expansion of the mHTT gene. This resulted in permanent and mutant allele-specific inactivation of the mHTT gene (J. W. Shin et al., 2016). Recently the method was successfully tested in an HD rodent model (Su Yang et al., 2017). This affirms the feasibility of this approach but much work is still needed, especially given recent concerns about unexpected off-target mutations with CRISPR/Cas9 gene editing (Schaefer et al., 2017).

c) [Non-genetic therapy](#)

Therapeutic strategies that do not target mHTT expression are limited by the fact that they are usually employed to compensate a specific deficiency caused by the presence of mHTT, but, as presented above, many independent deficiencies occur in several subcellular compartments in HD. Nevertheless, these types of strategies remain important given that many patients do not know that they are ill until late in life, by which time genetic therapies are practically useless.

Great efforts have been made to alleviate cellular degeneration through cell replacement therapy. Many clinical trials have adopted the transplantation of fetal-derived cells into the striatum of patients. However, it has been suggested that implanting cells into the striatum alone is limited by the fact that other regions of the HD brain degenerate over the course of the disease, and that these regions continue to degenerate despite the cell graft (Bachoud-Lévi et al., 2006). Nonetheless, it has been reported that implanted cells establish functional connections with host tissue and lead to motor improvements in several animal models (Campbell et al., 1993; Hantraye et al., 1992; Nakao et al., 1999). Other approaches include stem-cell transplantation, which has a lower risk of being rejected by the host (Fricker et al., 1999), and targeting of neurogenesis (Cho et al., 2007).

Several molecules and drugs that act on mitochondria have shown beneficial effects in cell and animal models. Creatine and Coenzyme Q10 have shown decreased HD pathology in R6/2 and N171-82Q lines of HD mice by increasing ATP levels and mitochondrial function (Andreassen et al., 2001; Ferrante et al., 2000; J. A. Parker et al., 2005; Schilling et al., 2001). Unfortunately, treatment of patients with such drugs did not have any beneficial effect (Verbessem et al., 2003). Other drugs include cystamine and cysteamine. These molecules were used to treat HD before the discovery of HTT and enable the decrease of abnormally high transglutaminase activity in the brain. Indeed, transglutaminase inhibition has been shown to increase BDNF release which in turn promotes striatal survival (Borrell-Pagès et al., 2006; Pinto et al., 2009).

HTT's role in transcription is also a valid therapeutic target. For instance inhibition of histone deacetylase has beneficial effects and slows neurodegeneration in a worm model of HD (Steffan et al., 2001). Moreover, several studies have shown that similar results can be obtained through up-regulation of trophic support such as increased BDNF expression (Gharami et al., 2008; Simmons et al., 2009). Overall, the complexity of HD renders the development of viable therapeutic strategies challenging.

B. Chapter 2: Huntingtin protein and function

1. From huntingtin gene to huntingtin protein

a) The huntingtin gene and transcription

The human HTT gene is located on the short arm of chromosome 4 at position 16.3 (4p16.3) and encodes a 348 kDa protein, containing 3144 amino acids spanning 67 exons. The promoter region is rich in G and C nucleic acids and is highly conserved between species, which is also the case for certain regions of the HTT gene, notably between humans and mice for example (around 80%) suggesting potential shared function (F. Lin & Worman, 1995). Several regulatory elements have been identified within the promoter region including multiple Sp1 regions and transcription factor binding sites such as STAT1, capable of influencing HTT expression (Coles et al., 1998; De Souza et al., 2018).

Initially, two messenger RNA (mRNA) transcripts were discovered in humans, one short (10366 base pairs (bp)) enriched in the heart, muscle, liver and lungs, and one long (13711 bp) found in the brain (B. Lin et al., 1993). The number of transcripts is now believed to be much higher, one study having found up to 22 HTT splicing variants. The existence of these splice variants is of potential significance to the expression pattern of HTT, since loss of nuclear localization signals and alterations to sites of posttranslational modification can have an impact on HTT function, cleavage and localization (Hughes et al., 2014; Mort et al., 2015; Ruzo et al., 2015).

b) Huntingtin localization

HTT is a widespread protein expressed in all tissues and cell types around the body. Transcripts have been found in the brain, heart, placenta, lung, liver, muscle, kidney, pancreas and testes (S. H. Li et al., 1993) in the following cell types: neurons, glial cells, hematopoietic cells (bone marrow, white and red pulp in the spleen), glandular epithelium (fallopian tube, colon, breast, salivary gland, pancreas, and uterus), squamous epithelium (skin), macrophages and pneumocytes in the lung, seminiferous ducts in the testis and glomeruli or tubule cells in the kidney (Marques Sousa & Humbert, 2013). It is weakly expressed in cell types that present

mesenchymal properties such as fibroblasts of the skin, adipocytes in the breast, stromal cells in the uterus, myocytes and smooth muscle cells (Marques Sousa & Humbert, 2013).

c) [HTT protein structure](#)

Several domains exist within the HTT protein. The N-terminal (N-ter) end is composed of a set of 17 highly conserved amino acids that play an important role in subcellular localization (Atwal et al., 2007; Rockabrand et al., 2007). They are believed to enable HTT's nuclear export via interaction with nuclear pores (Cornett et al., 2005), targeting to mitochondria, endoplasmic reticulum (ER) and Golgi apparatus, as well as regulation of the cell's response to stress and DNA damage (T. Maiuri et al., 2013; Tamara Maiuri et al., 2017). One study has demonstrated that the deletion of these 17 amino acids induces learning and synaptic deficiencies in mice but that subcellular localization of HTT was unaffected (André et al., 2017). This may possibly be due to a nuclear export sequence on the C-terminal end of the protein (Xia et al., 2003). This N-ter region is also subject to several post-translational modifications (PTMs) important for HTT functions and localization (Figure 4) (Atwal et al., 2007, 2011; Atwal & Truant, 2008; Rockabrand et al., 2007; Zuccato et al., 2010).

On the 18th amino acid begins the poly-glutamine (polyQ) stretch, famously expanded in HD (Figure 4). On one hand, this sequence has been conserved throughout several vertebrates dating back to the sea urchins where it contained only 4 glutamine repeats. Invertebrates, on the other hand, are not known to express a polyQ sequence (Tartari et al., 2008; Zuccato et al., 2010). Normal HTT contains different sizes of polyQ and the reason for this is not well understood. Nevertheless, shorter CAG length is associated with enhanced autophagy and longevity in mice (Zheng et al., 2010), and mammals, humans especially, express the largest polyQ stretches.

The polyQ sequence is followed by a proline rich domain (PRD), only present in mammals, that is thought to play a role in protein-protein interaction. Conversely though, deletion of this domain in mice does not have any effect on behavior (André et al., 2017; Tartari et al., 2008).

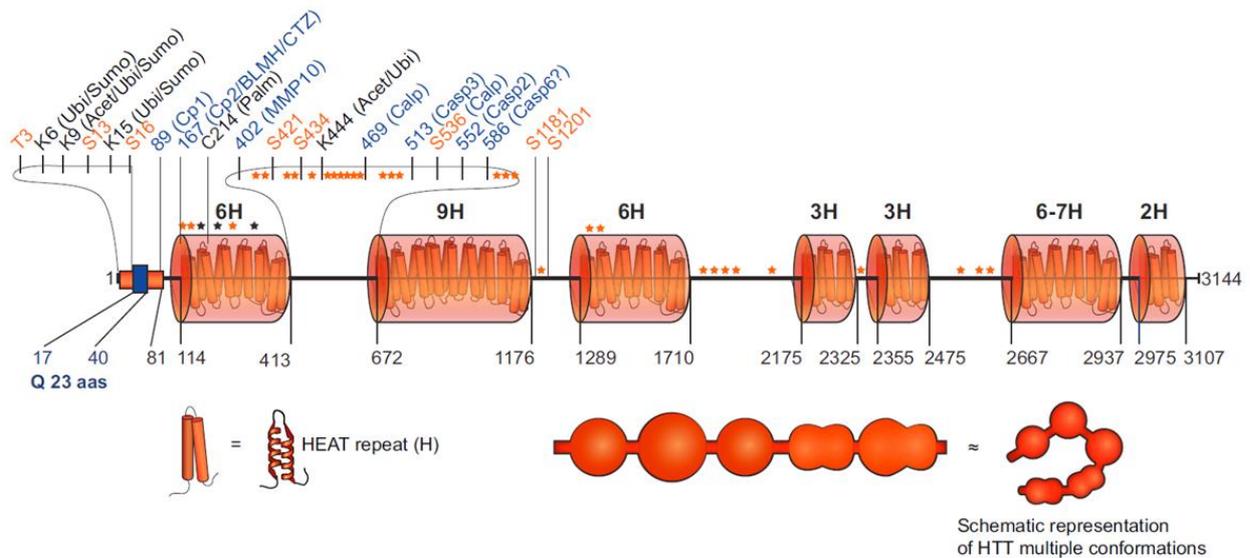


Figure 4: Schematic representation of the HTT protein structure and PTM sites. The amino acids listed correspond to PTM sites within the HTT sequence, in orange are phosphorylation sites identified by mass spectrometry, and in blue are cleavage sites. Orange and black stars indicate, respectively, phosphorylation and acetylation sites identified by mass spectrometry only with no further confirmations. Image taken from Saudou & Humbert, 2016.

Very little is known about what follows the PRD domain since most of the scientific focus has been centered around the first exon given that it contains the HD mutation. This C-terminal portion, that essentially represents the vast majority of the protein, contains several HEAT repeats, formed of antiparallel alpha-helices separated by non-helical regions. They are named after the proteins in which they were first identified: HTT (H), elongation factor 3 (E), the regulatory A subunit of protein phosphatase 2A (A) and TOR1 (T). HTT is made of 16 to 36 of these HEAT repeats regrouped into 7 larger clusters (Palidwor et al., 2009; Takano & Gusella, 2002; Tartari et al., 2008). They are believed to be important for HTT's protein-protein interactions (Andrade-Navarro & Bork, 1995; Neuwald & Hirano, 2000; Palidwor et al., 2009).

Concerning HTT's 3-dimensional structure, the first 17 amino acids may form an amphipathic alpha helix able to attach to membranes, followed by a flexible polyQ (Arndt et al., 2015). The PRD is a straight rigid alpha helix that may serve to stabilize its polyQ neighbor, a function that may influence aggregation (M. W. Kim et al., 2009). Moreover, HTT is highly dynamic and can bend in various ways to adopt different 3-dimensional conformations. Some have shown that the protein's various intramolecular interactions have enabled the protein to be found in around 100 different conformations (Seong et al., 2009). HTT can also dimerize through interaction of its middle portion, the N-ter parts of HTT can bind to different C-terminal regions of HTT all of which can be disrupted upon proteolysis (El-Daher et al., 2015;

Ochaba et al., 2014). One breakthrough study was able to characterize, by cryo-electron microscopy, certain aspects of HTT's interaction with one of its partners, HAP40, where they found 3 important domains: N-HEAT made of 21 HEAT domains organized into an alpha solenoid structure, C-HEAT composed of 12 HEAT repeats in a spiral, and a series of tandem alpha helices bridging the two other domains (Q. Guo et al., 2018). Finally, the length of the polyQ stretch is known to influence several features of HTT's conformation, such as solubility through its interaction with other soluble domains (Darnell et al., 2007; Harjes & Wanker, 2003; Shi Hua Li & Li, 2004; Perutz et al., 1994).

d) [HTT interactors](#)

HTT is thought to have hundreds of interactors (Tourette et al., 2014). One study suggested the existence of 747 candidate interacting partners, identifying many proteins involved in cell signaling, microtubule-based transport, and proteostasis (Shirasaki et al., 2012). Proteins containing Src homology region 3 (SH3) or tryptophan (WW) domains are able to interact with HTT through its PRD. These include proteins such as SH3GL3/endophilin3 (Sittler et al., 2001), protein kinase C and casein kinase substrate in neurons 1 (PACSIN1; syndapin) (Modregger et al., 2002), PSD-95 (Sun et al., 2001) and p53 (Steffan et al., 2000). Most potential interacting regions of HTT are sought out in the N-ter region, because of the large quantities of PTMs located in this area of the protein. However, other proteins including huntingtin-interacting protein (HIP) 1, HIP14, and HAP1 are known to interact with the HEAT repeat domains located in the middle and C-terminal half of the protein (Kalchman et al., 1997; Singaraja et al., 2002; Wanker et al., 1997). HTT's ability to bind interactors at different domains may also serve to combine cellular functions, as exemplified by the trimeric complex formed by HTT with the autophagy-initiating kinase Unc-51 like autophagy activating kinase (ULK) 1 and the autophagy receptor p62/SQSTM1 that couples the induction of autophagy and the selective recruitment of cargo into autophagosomes (Rui et al., 2015). The most important interactors of HTT will be developed through the description of HTT function in part C.

e) [HTT proteolysis](#)

The HTT protein also contains multiple cleavage sites and is a substrate to many different proteases such as caspases, metalloproteinases and cathepsins (Gafni & Ellerby, 2002;

Goldberg et al., 1996; Hermel et al., 2004; Y. J. Kim et al., 2006; Lunkes et al., 2002; J. P. Miller et al., 2010). These cleavage sites are located in proline, glutamate, aspartate, serine and threonine rich domains (PEST) (Warby et al., 2008). A lot of focus has been centered around the cleavage of polyQ HTT. The longer the polyQ expansion, the more likely the protein is to be cleaved by a protease (Goldberg et al., 1996), leading to greater amounts of cleavage products: first the N-ter fragment that is known to translocate to the nucleus and aggregate (Benn et al., 2005; Graham et al., 2006; Frederic Saudou et al., 1999) and second the non-polyQ-containing C-ter fragments that also have a role in cellular toxicity through interaction with dynamin causing ER vacuolation (El-Daher et al., 2015). WT HTT cleavage can also occur but is not well understood. It is believed to have a role in HTT function modification, inactivation and apoptosis. For example, dual cleavage at positions 586 and 552 generates fragments that induce an increase in autophagy (Martin et al., 2014).

2. Huntingtin post-translational modifications

a) Acetylation

The HTT protein becomes even more complex when one considers the amount of PTMs it is capable of undergoing. Several of these have been identified over the years and have often been implicated in HD pathogenesis. Some of the most studied are summarized here.

Acetylation can occur at several lysine residues within HTT's sequence. The lysine residue at position 444 (L444) is acetylated by the acetyltransferase cAMP Response Element (CREB) Binding Protein (CBP) and deacetylated histone deacetylase 1 (HDAC1) (Jeong et al., 2009). Acetylation at this position of HTT is known to promote autophagic clearance of mHTT (Y. Fu et al., 2017). Indeed, HTT acetylation prompts the protein's degradation via the autophagosome, and CBP's affinity for mHTT is higher than the WT form (Y. Fu et al., 2017; Jeong et al., 2009). A more recent study has demonstrated that acetylation of HTT at positions 6, 9 and 15 reduces HTT's capacity to aggregate and increases the protein's interaction with lipids (Chaibva et al., 2016) (Figure 4).

b) [SUMOylation and ubiquitination](#)

Intriguingly, small ubiquitin-like modifiers (SUMO)-ylating and ubiquitylating enzymes target the same HTT residues as those described above: lysines 6, 9 and 15 (Figure 4) (Steffan et al., 2004). The ligase E3, also known as Rhes, is implicated in SUMOylation of mHTT for which it has a higher affinity compared to WT HTT. This enzyme is enriched in the striatum, pointing towards a potential pathogenic mechanism in HD (Subramaniam et al., 2009). Indeed, SUMOylation of these sites stabilizes mHTT and increases nuclear localization of the protein where it may play a role in transcriptional dysfunction. It also increases mHTT aggregation by inhibiting proteasomal degradation (Steffan et al., 2004).

On the other hand, ubiquitination of HTT at these same lysine residues, mediated by ligase E2-25K (Kalchman et al., 1996), contributes to lowering mHTT toxicity by targeting the protein to the Ubiquitin-Proteasome System (UPS) (Jana et al., 2005; Kalchman et al., 1996).

c) [Phosphorylation](#)

Several serine (S) and threonine (T) amino acids on the HTT protein are able to be phosphorylated of which many of the responsible kinases and phosphatases have been identified (Figure 4). For all of these sites, phosphorylation is considered neuroprotective and is decreased on mHTT.

Serine 421 is phosphorylated by protein kinase A (AKT) and Serum and Glucocorticoid induced Kinase (SGK), and dephosphorylated by calcineurin (Humbert et al., 2002; Pardo et al., 2006; Rangone et al., 2004). The state of phosphorylation at this site has an impact on vesicular transport of cargo such as BDNF within the cortico-striatal network: S421-phosphorylated HTT promotes anterograde vesicular transport, through an increase in speed and distance traveled, whereas S421-dephosphorylated HTT induces retrograde transport. This is due to S421-phosphorylated HTT's increased affinity for anterograde molecular motor, kinesin 1 (Colin et al., 2008). Moreover, S421 phosphorylation causes a decrease in caspase 6 activity which reduces N-ter fragment formation that normally translocate to the nucleus (Havel et al., 2011; Warby et al., 2009). Interestingly, S421 phosphorylation is lowest in the striatum and cortex (Warby et al., 2005), evidence of its potential role in HD. In fact, mHTT phosphorylation at S421 is reduced compared to WT HTT, possibly due to reduced AKT activity (Warby et al., 2005). Furthermore, this PTM is known to decrease aggregate formation

and neurotoxicity of HTT both *in vitro* and *in vivo* (Humbert et al., 2002; Pardo et al., 2006). Indeed, induction of S421 phosphorylation reduces behavioral dysfunction and striatal neurodegeneration in BACHD mice (Kratzer et al., 2016).

Phosphorylation at S434, S1181 and S1201 is modulated by cyclin-dependent kinase (CDK) 5, a widespread kinase for which the activator, p39, is particularly enriched in neurons (Humbert et al., 2000) Phosphorylation at these sites is known to be neuroprotective. The cellular implications and consequences regarding phosphorylation at serines 1181 and 1201 are unclear at this time, some have shown that dephosphorylated HTT promotes vesicular transport of BDNF which is beneficial for neurogenesis (Ben M'Barek et al., 2013), whereas others have demonstrated that WT HTT dephosphorylation renders the protein more toxic (Anne et al., 2007). Phosphorylation at serine 434 contributes to reducing HTT cleavage by caspase 3 at amino acid 513 (Luo et al., 2005).

The kinase complex IKK phosphorylates HTT at serines 13 and 16, a process that has been associated with increased HTT degradation by the UPS and autophagy. In the case of mHTT, phosphorylation and therefore degradation are diminished (L. M. Thompson et al., 2009). Also, BACHD mice expressing phosphomimetic mHTT at S13 and S16 show a reduction of several neuropathological and abnormal behavioral phenotypes (Gu et al., 2009).

Finally, HTT is also phosphorylated at threonine 3, of which the lowest levels of HTT phosphorylation are found in the striatum and cortex, similarly to S421. Interestingly, the length of the polyQ within HTT is inversely correlated with the amount of phosphorylation at T3. Additionally, similarly to other phosphorylation sites, T3 phosphorylation is also associated with reduced mHTT toxicity (Aiken et al., 2009).

d) Palmitoylation and methylation

Lesser known modifications such as palmitoylation and methylation may also influence HTT. Concerning palmitoylation, there is only one known site in the HTT protein sequence, located at cysteine 214. This modification does not supposedly last very long, given that its half-life is only of a couple of hours. Moreover, overexpression of HIP14, a HTT interactor, increases HTT palmitoylation and relocates the protein to the Golgi apparatus (Yanai et al., 2006). Although this PTM has not been greatly studied, its implication in HD may be of interest since inhibition of HTT palmitoylation can induce HTT aggregation and synaptic dysfunction in a similar manner to what is seen in HD (Yanai et al., 2006).

For methylation, it is known that HTT interacts with the methylating enzyme Protein arginine N-methyltransferase (PRMT) 5 but does not seem to be itself methylated (Ratovitski et al., 2015).

3. Huntingtin functions

a) Axonal transport

HTT has numerous interactors, which makes the variety of possible functions and roles of this protein equally vast. As mentioned above, axonal transport is mediated by HTT. Indeed, HTT interacts with several elements of the molecular motor machinery including dynein and HAP1 that itself interacts with p150, a subunit of dynactin, and kinesin 1 (Figure 5) (Caviston et al., 2007; Colin et al., 2008; Gauthier et al., 2004; Gunawardena et al., 2003; McGuire et al., 2006; Twelvetrees et al., 2010). Through these interactions, HTT mediates transport of several organelles in the anterograde as well as retrograde direction. In neurons, this transport is present in both axons and dendrites. Although all HTT-mediated cargoes are not known, those that have been identified are mainly of vesicular nature. These include synaptic vesicles, endosomes containing TrkB, internalized upon BDNF binding, lysosomes, autophagosomes, and dense core vesicles that transport BDNF, amyloid precursor protein (APP) and synaptic receptors (Colin et al., 2008; Gauthier et al., 2004; Liot et al., 2013; Twelvetrees et al., 2010; Y. C. Wong & Holzbaaur, 2014; Zala, Hinckelmann, & Saudou, 2013). It has been reported that overexpression of HTT promotes transport of each of these cargoes, whereas silencing causes a decrease. This suggests that HTT may play the role of transport facilitator (Gunawardena et al., 2003; Zala, Hinckelmann, & Saudou, 2013). Moreover, as described above, phosphorylation of HTT at S421 controls transport directionality (Bruyère et al., 2020; Colin et al., 2008; Ehinger et al., 2020).

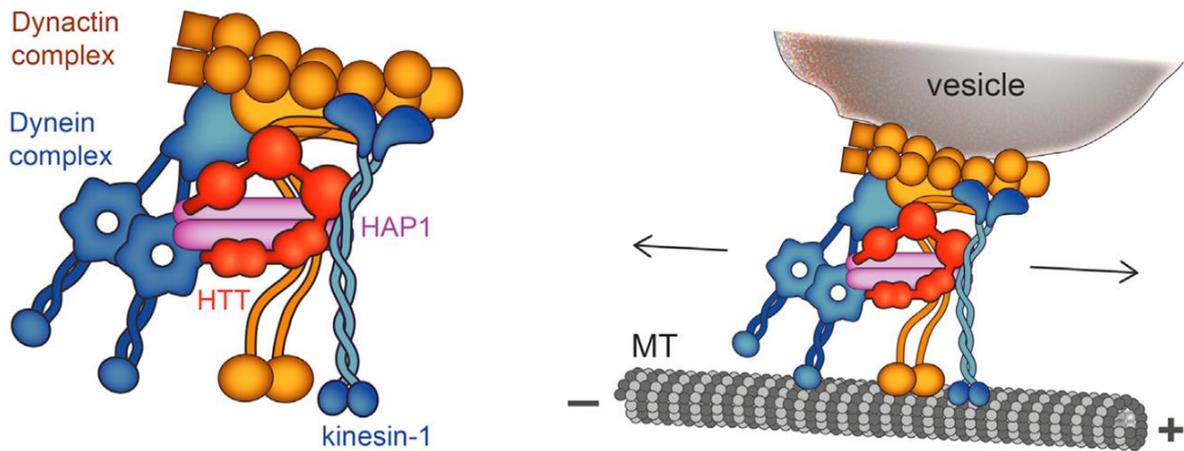


Figure 5: HTT interacts with molecular motors and mediates vesicular transport. HTT interacts specifically with the dynactin subunit, HAP1, and dynein within the molecular motor complex which enables HTT to control transport in both retrograde and anterograde directions. Image taken from Saudou & Humbert, neuron, 2016.

HTT may also play a role in mitochondrial transport, since mitochondria are known to be dysfunctional in HD (Chiang et al., 2012). However, it is unclear whether this is due to direct influence of the WT HTT protein on the motor machinery responsible for transporting mitochondria, or simply a new function gained by the mutant form (Orr et al., 2008). HTT may also indirectly affect transport via c-Jun N-terminal kinase (JNK) 3 activation. This leads to kinesin 1 phosphorylation and therefore it's detachment from microtubules (G. A. Morfini et al., 2009).

b) Endocytosis

HTT interacts with clathrin mediated endocytosis proteins such as HIP1 and HIP12 (Engqvist-Goldstein et al., 2001; Legendre-Guillemain et al., 2002; Waelter et al., 2001). Upon proteolysis, HTT's C-terminal domains can associate with dynamin 1, a protein that is involved in vesicular membrane invagination, which leads to ER vacuolation and deficient endocytosis (El-Daher et al., 2015). HTT may also be involved in vesicular recycling given that it is able to interact with endocytic proteins such as endophilin A3 (Sittler et al., 1998) and Ras-related protein (RAB) 11, a protein necessary for endocytosis and recycling (X. Li et al., 2008). Indeed, HTT downregulation causes RAB11 membrane detachment and inactivation. Finally, HTT also regulates endosome motility through its interaction with RAB5 and HAP40 (Pal et al., 2006).

c) [Ciliogenesis](#)

HTT is found in a variety of cilia including non-motile sensory cilia of neurons, photoreceptor cilia and motile cilia of trachea and ependymal cells where it plays a crucial role during tissue development and homeostasis. In photoreceptors, HTT is present in all compartments of the cilia, from the base to the adjacent centriole to the tip of the axoneme (Karam et al., 2015). HTT, through its interaction with HAP1, was also colocalized with pericentriolar material 1 protein (PCM1) where it regulates ciliogenesis. Indeed, HTT silencing in mouse cells impairs the retrograde trafficking of PCM1 which leads to reduced primary cilia formation through PCM1 mislocalization and alteration of the cilia layer (Keryer et al., 2011). Another study investigated cilia formation in neurons during embryogenesis of the *Xenopus*. They found that HTT silencing affected cilia polarity and function, leading to abnormal development of trigeminal and motor neurons (Haremaki et al., 2015).

d) [Cell division](#)

HTT also plays a role in mitosis where it can be found at the mitotic spindle pole and along astral microtubules that radiate from the pole to the edges of the cell (Elias et al., 2014; Godin et al., 2010; Gutekunst et al., 1995). Studies involving silencing of HTT have been able to highlight its role during mitotic phases. One study showed that HTT was responsible for mitotic spindle orientation through the protein's interaction with dynein, which promoted the accumulation of necessary mitotic proteins, Nuclear Mitotic Apparatus protein (NuMA) and the leucine-glycine-asparagine repeat protein (LGN) in neurons (Godin et al., 2010). Another found that in dividing mammary epithelial cells HTT enabled cortical accumulation of the mitotic complex formed of dynein, dynactin, NuMA and LGN through kinesin 1 based transport along astral microtubules (Elias et al., 2014). This role of HTT has justified its involvement in embryonic development and may be linked to its dysfunction in HD.

e) [Protein degradation](#)

HTT is implicated in autophagy. This was suggested based on the fact that polyQ HTT causes autophagic cargo loading deficiencies which induces reduction in the cell's capacity to degrade aggregated HTT (Martinez-Vicente et al., 2010). This may partly be linked to HTT's

role in vesicular transport (Y. C. Wong & Holzbaur, 2014). Further proof of HTT's implication in autophagy lies within the protein's amino acid sequence of which regions are similar to those found in autophagy related proteins such as Atg23 and Atg11. Moreover, HTT is capable of interacting with LC3, ULK1 and p62, key proteins for initiating selective autophagy (Ochaba et al., 2014). For example, ULK1, a kinase responsible for autophagy initiation, is normally bound to mTOR which suppresses its activity. HTT is able to bind ULK1 and break the bond between ULK1 and mTOR thus initiating autophagy (Rui et al., 2015).

f) Transcription

HTT has been identified to interact with a vast quantity of transcription factors including, p53, CBP, and the repressor element-1 transcription factor/neuron restrictive silencer factor (REST/NRSF) (Steffan et al., 2000; Zuccato et al., 2003). Some suggest that itself might be a transcription factor as it is able to bind DNA directly (Benn et al., 2008). For all of these proteins, HTT may have an activating or repressing role in transcription of a wide variety of proteins involved in a great number of cellular functions. Of note, HTT promotes transcription of BDNF through its interaction with REST/NRSF in the cytoplasm, thus preventing the complex from entering the nucleus where it normally represses transcription of this trophic factor (Zuccato et al., 2003). Finally, HTT is also involved in chromatin remodeling (Seong et al., 2009).

Other functions of HTT include calcium signaling, maintenance of cell morphology and survival, as over-expression of HTT in neuronal as well as non-neuronal cells protects against apoptosis (Ho et al., 2001; Leavitt et al., 2006; Rigamonti et al., 2001).

C. Chapter 3: Axonal transport

1. Basics of axonal transport

a) General description of axonal transport

Most proteins required for neuronal function are produced in the cell body (soma) and then distributed around the cell's various extensions through transport. Cytoskeletal based transport is responsible for this targeted distribution of proteins and organelles within the neuron's axon and dendrites. Axonal transport was discovered by using radioactive tracers in squid and mouse nerves in the 1960s (Droz & Leblond, 1962). Since then, many different organelles have been found to require transport to be directed from one end of the axon to the other. This is enabled by molecular motors that bridge these organelles to the cell's cytoskeleton that generate movement through consumption of cellular energy. In the axon, two directions are possible for the transported cargo: anterograde transport, moving from the soma to the axon extremity, often a synapse, and retrograde transport, in the opposite direction.

Axonal transport can be split into 2 types: fast and slow (McEwen & Grafstein, 1968). Fast axonal transport (FAT) moves cargo at a rate between 0.5 and 5 $\mu\text{m/s}$, this is the speed of cargo such as vesicles, endosomes, and mRNA. Slow axonal transport (SAT) is associated with speeds inferior to 0.5 $\mu\text{m/s}$, corresponding to the movement of cytoskeletal proteins, enzymes and molecular motors (Brown, 2003). Intriguingly, both transport types are enabled by the same molecular motors (Roy, 2014), but SAT related motors are considered slower due to the increased numbers of pauses (Brown, 2003; H. Wang & Oster, 2002). This distinction between FAT and SAT can be explained by the differences in numbers of molecular motors present on a given vesicle, where more motors move cargo along the axon faster than those with less. The size of the cargo has an influence on transport as well, given that larger cargos pause more frequently than smaller ones (R. H. Lee & Mitchell, 2015). Finally, molecular motor speed can also be regulated by other proteins associated present within the vicinity of the motor, for example, kinesin 1, when associated to Hsc70, ensures SAT, whereas, when it isn't, it moves within the speed range of FAT (Terada et al., 2010).

Actin and microtubules are the 2 main cytoskeletal components of the axon. Microtubules are highly dynamic structures, formed of alpha and beta tubulin dimers that associate to form oligomers, 13 of which on average, will then associate to form the final tube-

like structures that are microtubules. In the axon, directionality is strictly dictated by the orientation of microtubules. The plus end is more dynamic and always oriented towards the axon terminal, whereas the minus end, more stable, is always located in the axon initiation segment at the soma (P. W. Baas et al., 1988; Peter W. Baas & Lin, 2011; Tas et al., 2017). This directionality of microtubules is central to determining transport orientation in the axon.

Actin is also found in neurons and their axons and is also highly dynamic. For long it was assumed that actin was only located at specific areas of neuronal branches such as dendritic spines where it serves to scaffold spine morphology or and the axon terminal. But more recently, super-resolution microscopy has revealed the presence of actin rings located below the plasma membrane regularly interspaced throughout the axon by spectrin (Lukinavičius et al., 2014; Xu et al., 2013).

b) Molecular motors

Transport of all cargo throughout the axon is dependent on molecular motor proteins that allow the cargo to move along the cell's cytoskeleton. Interestingly, the variety of molecular motors is more vast in neurons compared to other cell types, which is telling of the importance of transport in these cell types (Kuta et al., 2010; M. A. Silverman et al., 2010). Three types of motors are found in neurons: kinesins, dyneins and myosins. Structurally speaking, all possess a head able to bind to the cytoskeleton and capable of hydrolyzing ATP in order to initiate movement, a tail that binds to the cargo and a middle region, linking the tail to the head (Hirokawa et al., 2010). These motors are also associated with other scaffolding proteins able to modulate motor activity (M. meng Fu & Holzbaaur, 2014).

Most kinesin molecular motors form dimers and move towards the plus end of microtubules making them responsible for anterograde transport in the axon. 45 genes in humans make up the kinesin superfamily (KIF), of which 38 have been found in the brain (Miki et al., 2001). They are subdivided into three groups: kinesins N, C and M depending on where the motor region is located within the protein (Figure 6). Of these kinesins, three superfamilies are implicated in axonal transport: kinesins 1, 2 and 3 (Miki et al., 2001). Their speed is approximately 0.5 to 1 $\mu\text{m/s}$ and they are involved in the transport of various cargos including vesicles, organelles, proteins and RNA (Hirokawa et al., 2010).

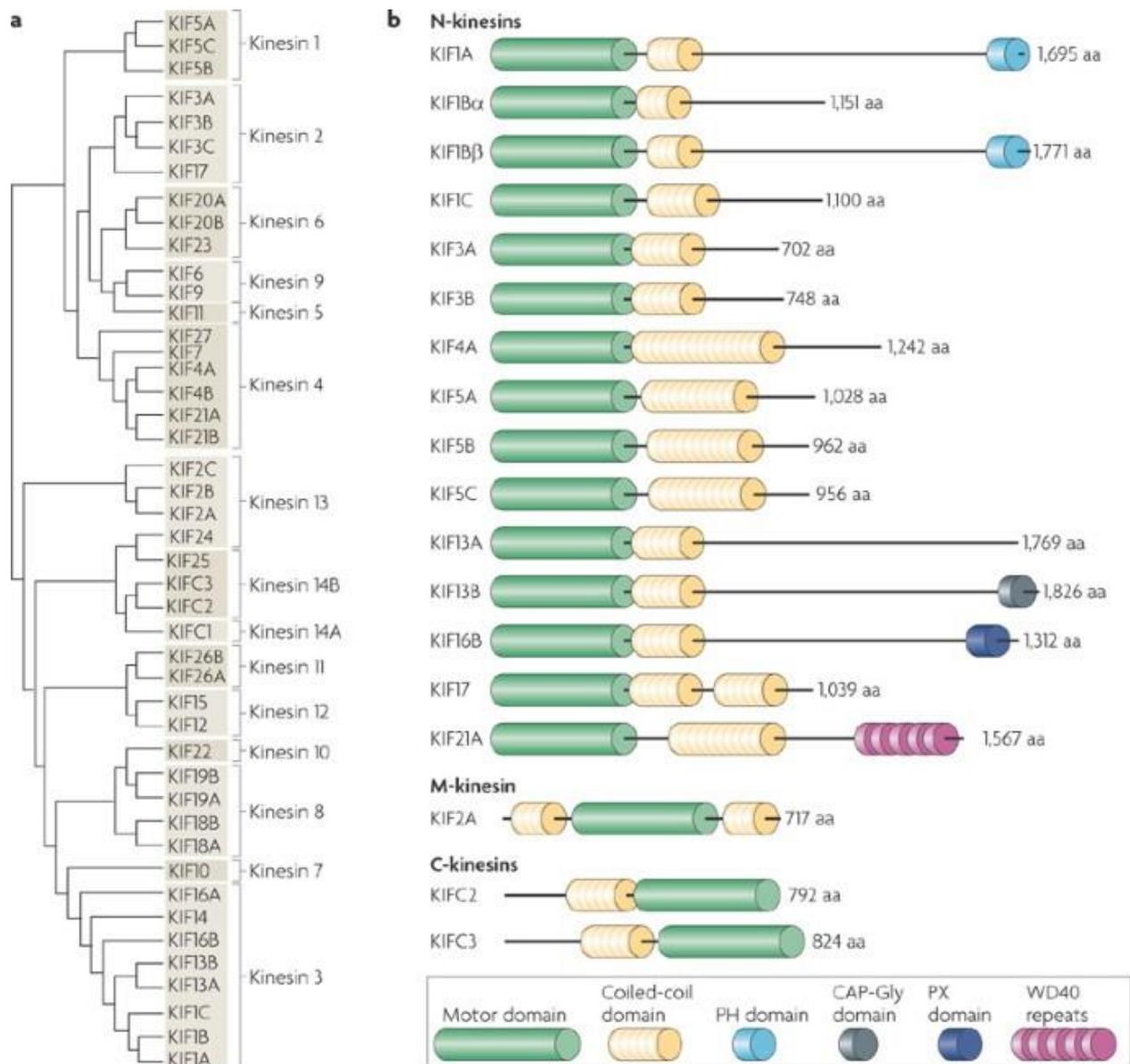


Figure 6: The kinesin superfamily. In a) is represented the phylogenetic analysis of all 45 kinesin genes in mice and in b) the three subcategories based on motor region location (Hirokawa et al., Nat Rev Mol Cell Biol, 2009).

Kinesin 1 was the first to be purified in 1985 (Vale et al., 1985) and it has three isoforms: KIF5A, KIF5B and KIF5C, all capable of forming homo- and heterodimers with one another. The strength of its head is approximately 5 to 6 pN (Svoboda & Block, 1994). It is highly processive meaning it is able to move over a large distance without detaching from the cytoskeleton, and thus is often found to be the molecular motor required for vesicular transport along microtubules in the axon. It also binds other proteins such as FEZ1, JIP1 and JIP3 which increases its affinity for vesicular proteins (Byrd et al., 2001; Toda et al., 2008; Verhey et al., 2001). KIF5 is implicated in the transport of secretory vesicles containing APP and BDNF, through its interaction with HAP1 which is itself bound to HTT (Gauthier et al., 2004; McGuire

et al., 2006). It also mediates transport of mitochondria and lysosomes (Jellali et al., 1994; Maday et al., 2012). Additionally it participates in SAT through its interaction with neurofilaments to transport cytoskeletal proteins (Magrané et al., 2014a; Terada et al., 2000). Outside of the axon, it ensures transport between the ER and Golgi apparatus (Hirokawa et al., 2009).

Similarly to kinesin 1, kinesin 2 transports cargo along microtubules and forms polymers (homodimers and heterotrimers, (Scholey, 2013)). It has a similar strength to kinesin 1, around 5 pN, but is less processive and therefore detaches more often from microtubules (Schroeder et al., 2012). KIF17, a kinesin 2 subtype, is not present in the axon but is instead involved in transport of AMPA and NMDA receptors in dendrites (Franker et al., 2016; Yin et al., 2011). Other kinesin 2 subtypes, KIF3A and KIF3B, have been implicated in the transport of vesicles necessary for neurite elongation (Takeda et al., 2000), as well as Rab4, Rab5 and Rab11 positive endosomes, and lysosomes (Brown, 2003; Castle et al., 2014). Kinesin 2 also mediates transport between the ER and Golgi (Hirokawa et al., 2009).

Eight isoforms of kinesin 3 exist. KIF1A and 1Bbeta are the only monomeric isoforms and a fairly low processivity compared to other kinesins, which is increased, however, upon dimerization (Soppina et al., 2014). They possess sequence regions similar to pleckstrin that enable attachment to vesicular membranes (Klopfenstein & Vale, 2004). Their interaction with cargos is mediated by surrounding adaptor proteins such as DENN/MADD and liprin alpha (Niwa et al., 2008; H. Shin et al., 2003).

Dynein motors also bind to microtubules but move towards the minus end enabling retrograde transport. There are two types: axonemal dynein located in cilia and flagella, and cytoplasmic dynein found in the axon. Cytoplasmic dynein is made of two heavy chains, responsible for ATPase activity and cargo interaction, two intermediate chains and four light chains that stabilize the motor-cargo complex (Reck-Peterson et al., 2018). Of the two genes that code for the heavy chains of dynein, only one is found in axons and dendrites, meaning it must be able to interact with many different cargos. This is ensured by the intermediate chains (Kikkawa, 2013). Dynein transports many cargo including vesicles of BDNF and neurofilaments (Gauthier et al., 2004; Wagner et al., 2004). The strength of this motor (around 1 pN) as well as its processivity are much smaller than kinesin (Mallik & Gross, 2004). Although dynein motors are able to obtain high velocities around 0.5 to 1 $\mu\text{m/s}$, their overall speed is slightly lower than kinesin. This is due to the fact that dynein motors pause far more frequently and side-step more often than kinesin (Mallik et al., 2005). Conversely to kinesin however, dynein molecules are able to cooperate in order to generate more strength (Mallik et

al., 2013). This also makes them less likely to detach from the microtubule upon encountering an obstacle (Dixit et al., 2008; Vershinin et al., 2006). Dynactin is a protein that associates with dynein to regulate its activity, its attachment to cargo and its processivity. Of the 23 subunits that make dynactin, p150 interacts directly with dynein and microtubules (Culver-Hanlon et al., 2006; Vaughan & Vallee, 1995; Waterman-Storer et al., 1995). Other regulators of dynein activity and attachment include BICD2 (protein bicaudal D homolog 2) and HOOK3 (McKenney et al., 2014; Urnavicius et al., 2015, 2018).

The third and final type of molecular motor is myosin, of which several subtypes exist and have been found in neurons. Their structure is similar to other molecular motors, but they tend to bind the actin cytoskeleton (although they have been reported to interact with microtubules as well, Beaven et al., 2015) and are dependent on calcium and therefore neuronal activity. Myosin II is involved in neurotransmitter release and synaptic vesicle recycling at presynaptic sites (Mochida et al., 1994). It also plays an architectural role in neurons, indeed its phosphorylation induces actin polymerization in spines and axon initiation segment destabilization by detaching from actin rings. Myosin V has been found to transport several cargos including vesicles and mitochondria (Araujo et al., 2019; Gramlich & Klyachko, 2017; Prekeris & Terrian, 1997). All myosin motors travel towards the plus end of actin, except for myosin VI (Wells et al., 1999) which plays a role in exocytosis at the axon terminal and endocytosis of AMPA receptors at postsynaptic sites (Osterweil et al., 2005; W. Wagner et al., 2019).

c) [Axonal cargo dynamics and motors](#)

(1) *Fast axonal transport*

FAT is responsible for the trafficking of a wide panel of different organelles within the axon, the majority of which are vesicular in nature. Synaptic and dense-core vesicles are secreted from the Golgi apparatus, encapsulating different cargos such as neurotransmitters, trophic factors and other proteins required for synaptic transmission. Both will then travel along the cytoskeleton towards the distal part of the axon. Synaptic vesicles are able to accumulate at the axon terminal where they form a pool of reserve vesicles awaiting release. This release is modulated by synaptic activity and local calcium concentrations. Dense-core vesicles (due to the dark appearance in electron microscopy) on the other hand are not able to accumulate

and require large amounts of calcium in order to undergo exocytosis (Hartmann et al., 2001). Synaptic vesicles and their content are also recycled, which is not the case for dense-core vesicles. Nevertheless both are transported by the same kinesin 3 motor machinery, which is dimerized on these types of vesicles in order to increase their processivity (Lo et al., 2011). BDNF-containing dense core vesicles are an exception to this rule, since their transport is ensured by kinesin 1 and not kinesin 3 (Gauthier et al., 2004). As mentioned in chapter 2, HTT serves as a scaffolding protein and mediates directionality of BDNF transport through its phosphorylation at S421 (Colin et al., 2008; Gauthier et al., 2004; Zala et al., 2008). Additionally, it has been shown that S421 phosphorylation also induces kinesin 1 recruitment (Colin et al., 2008).

APP-positive vesicles are also transported by kinesin-1 (Kamal et al., 2000), enabling them to travel at high speeds within the axon, around 1 $\mu\text{m/s}$. Their transport is anterograde and retrograde, meaning dynein is bound to their surface as well (Falzone et al., 2009; Kaether et al., 2000). JIP1, a scaffolding protein, serves as a bridge between APP, kinesin 1, via its heavy chain, and dynactin, via its p150 subunit (M. meng Fu & Holzbaur, 2014). Through this interaction as well as its phosphorylation, JIP1 is able to regulate both kinesin and dynein activity (Blasius et al., 2007; Verhey et al., 2001). Indeed JNK-dependent phosphorylation at S421 serves as a molecular switch that dictates anterograde and retrograde transport of APP-positive vesicles, similarly to what is seen with HTT on BDNF vesicles (Bruyère et al., 2020; M. meng Fu & Holzbaur, 2014).

Endosomes and lysosomes are enriched in the cell body but may also be found dispersed heterogeneously throughout the axon (S. Lee et al., 2011). However, they are less mature than those in the soma, being less acid and containing lower amounts of proteolytic enzymes (Gowrishankar et al., 2015). Neurotrophins in the synaptic cleft can bind to presynaptic receptors which undergo endocytosis and travel back to the soma as signaling endosomes (Chowdary et al., 2012; Harrington & Ginty, 2013). These start their journey along the axon as early Rab5-positive endosomes which then mature into Rab7-positive endosomes (Cui et al., 2007; Deinhardt et al., 2006; Sandow et al., 2000). Returning endosomes require robust retrograde transport, one study demonstrated that nerve growth factor (NGF) containing endosomes were specifically retrograde with speeds situated between 0.2 and 3 $\mu\text{m/s}$ (Cui et al., 2007). This transport is mediated by the dynein/dynactin complex (Heerssen et al., 2004) and is enabled by dynactin's interaction with the Rab7-interacting lysosomal protein (RILP) that recruits dynein to the vesicular membrane (Johansson et al., 2007; Jordens et al., 2001). Additional regulatory proteins include ORP1L, DLIC and snapin (Cai et al., 2010; Holleran et

al., 2001) as well as HTT (Liot et al., 2013). Lysosomal transport, however, is bidirectional and their anterograde transport is mediated by kinesins 1 and 2 (Brown, 2003; Castle et al., 2014; A. G. Hendricks et al., 2010; Rosa-Ferreira & Munro, 2011; Sadowski et al., 2009).

Autophagosomes are formed mainly in the soma and distal portions of the axon (Maday & Holzbaur, 2014). In this area, they are bidirectional, transported by kinesin 1 and dynein motors, but they quickly switch to an exclusively retrograde transport and make their way back towards the soma (Maday et al., 2012). This switch is mediated by scaffolding proteins such as JIP1 (Fu & Holzbaur, 2014; Wong & Holzbaur, 2014) which binds to LC3 and has an inhibitory effect on kinesin 1 leading to retrograde transport of the autophagosome (Fu & Holzbaur, 2014). As they travel closer to the soma, autophagosomes will fuse with late endosomes to become autolysosomes (S. Lee et al., 2011; Maday et al., 2012). The fusion events that lead to the acidification of autolysosomes are dependent on efficient retrograde transport (Fu & Holzbaur, 2014; Wong & Holzbaur, 2014). This efficient clearance of degraded material is important for protein recycling in neurons and to compensate for the continuous anterograde transport in the axon (Maday & Holzbaur, 2014).

Mitochondria navigate throughout the axon, but are particularly enriched in areas of high energy demand such as the presynaptic terminal (Mandal & Drerup, 2019). Ribosomes have also been found in proximity of mitochondria, further proving its importance as an energy supplier (Cioni et al., 2019). Mitochondria also participate in calcium dynamics. The Mitochondrial Calcium Uniporter (MCU) is located in the internal membrane of the mitochondria and allows calcium entry when its cytosolic concentration is too high, a mechanism important for neurotransmitter release at the synapse (Vaccaro et al., 2017). *In vitro*, 20 to 30 % of mitochondria are motile and move in both directions in hippocampal axons depending on the maturation stage of the neurons in culture (Hollenbeck & Saxton, 2005). *In vivo* however, only 10% of mitochondria are motile and have a tendency to move in the anterograde direction (Misgeld et al., 2007). This transport of mitochondria is mediated by neuronal activity which will induce a local calcium concentration increase causing mitochondrial transport to stop (MacAskill, Rinholm, et al., 2009; X. Wang & Schwarz, 2009). Calcium binds the Mitochondrial Rho GTPase (Miro) that itself interacts with kinesin 1 via adaptors TRAK1 and TRAK2, upon which it has a regulatory effect causing mitochondrial transport to stop (Fransson et al., 2003, 2006; X. Guo et al., 2005; MacAskill, Brickley, et al., 2009). The exact mechanism by which calcium-bound Miro inhibits kinesin 1 and dynein is not yet fully understood (MacAskill, Brickley, et al., 2009; X. Wang & Schwarz, 2009). Several other scaffolding proteins such as syntabulin and FEZ1 regulate kinesin 1 recruitment (Cai et

al., 2005; Ikuta et al., 2007). The same scaffolding proteins may also regulate recruitment and activation of the dynein/dynactin complex (Russo et al., 2009; van Spronsen et al., 2013). However, kinesin 1 does not seem to be the only motor responsible for anterograde transport, as a portion of mitochondria remain mobile in kinesin 1 knockout neurons (Pilling et al., 2006).

Finally, the smooth endoplasmic reticulum is also transported in the axon. It forms a continuum of thin and dense tubules and its role is to produce lipids and regulate calcium dynamics in the axon via its calcium channels (IP3R and RyR) and pumps (Ca²⁺/ATPase SERCA) (Luarte et al., 2018). These calcium dynamics are important for neurotransmitter release (de Juan-Sanz et al., 2017). Additionally, enzymes located at the surface of the ER have been found responsible for lipid formation such as cholesterol (Posse de Chaves et al., 1997). The ER also interacts with other organelles within the axon such as vesicles, mitochondria and endosomes (Friedman et al., 2013; Rocha et al., 2009; Y. Wu et al., 2017).

(2) *Slow axonal transport*

Organelles travel at high speed up and down the axon, but cytosolic and cytoskeletal proteins move along the axon far more slowly. Neurofilaments are transported as pre-assembled oligomers by kinesin 1 (specifically KIF5A) and dynein but the regulation of this transport remains unknown (Uchida et al., 2009; Lina Wang & Brown, 2010; Yabe et al., 1999). Transport of actin and tubulin is less characterized, possibly due to the unstable polymerization and depolymerization cycles of these polymers. Microtubule fragments as well as tubulin dimers have been found to be transported by kinesin motors (Terada et al., 2000; Lei Wang & Brown, 2002). Actin however, seems to reach the axon terminal in a wave-like manner during neurite outgrowth, its replenishment once connections are established is not yet known (Flynn et al., 2009). Many cytosolic proteins are also transported in the axon (Roy, 2014), of which some travel as complexes (Scott et al., 2011). Intriguingly SAT utilizes the same molecular motors as its faster counterpart. This has been explained by the much lower processivity of motors involved in SAT that are marked by long pausing times and short bursts of mobility (Lei Wang et al., 2000). Even more surprising is the fact that far more SAT proteins accumulate at the axon terminal compared to FAT, despite the slower system (Garner & Mahler, 1987; McEwen & Grafstein, 1968; Roy, 2014).

2. Regulating and powering axonal transport

a) Regulation mechanisms of axonal transport

Axonal cargoes often bind several opposing molecular motors despite a unidirectional movement, for example late endosomes bind kinesin 1, kinesin 2 and dynein simultaneously (A. G. Hendricks et al., 2010), or autophagosomes that co-immunoprecipitate with kinesin 1 and dynein (Encalada et al., 2011). Molecular motors are also able to combine their efforts to increase processivity. Some suggest that 1 to 2 kinesins and 6 to 12 dynein motors can interact on a given organelle associated to microtubules (A. E. Hendricks et al., 2012; A. G. Hendricks et al., 2010; Mallik et al., 2013).

Models vary when it comes to explaining how opposing motors on a same organelle contribute to efficient axonal transport. Some suggest that a “tug-of-war” between motors dictates transport directionality (Figure 7), whereas others believe that each motor is highly regulated by the scaffolding proteins in its vicinity. An intermediate model exists whereby only kinesin motors may be regulated (Fu & Holzbaaur, 2014; Müller et al., 2008). The tug-of-war model seems fitting for cargoes that navigate equally in both retrograde and anterograde directions, such as autophagosomes and lysosomes, but the regulated motor model is more applicable to endosomes or secretory vesicles that show robust retrograde and anterograde transports respectively (Fu & Holzbaaur, 2014; A. G. Hendricks et al., 2010; Maday et al., 2012; Müller et al., 2008).

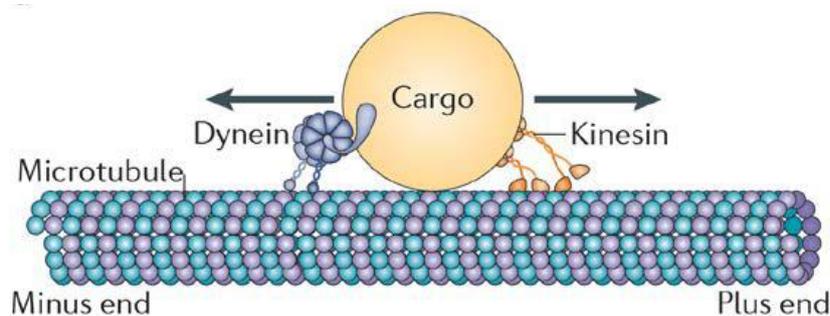


Figure 7: Tug-of-war model of transport. Based on the tug-of-war model, transport directionality is determined by the force ratio set by the molecular motors bound to their surface, here kinesin and dynein (Hancock, 2014).

Kinesin 1 has the capacity to auto inhibit itself through interaction between its tail and motor regions (Kaan et al., 2011). Additionally, scaffolding proteins such as JIP1 and JIP3 bind

and activate kinesin 1 (Blasius et al., 2007; M. M. Fu & Holzbaaur, 2013). As mentioned in chapter 2, HTT, through its phosphorylation at S421, stabilizes kinesin 1's attachment to the p150 subunit of dynactin which promotes anterograde transport. Its dephosphorylation has the opposite effect (Colin et al., 2008).

Dynein motors also have regulating partners including Lis1 which is able to stop transport by blocking ATP consumption, or BICD1 and BICD2 that potentiate dynein-directed transport of endosomes (McKenney & Ori-Mckenney, 2014; Schlager et al., 2014; Terenzio et al., 2014). In any case, the tight regulation of molecular motors suggests that the diversity of transport patterns observed is likely determined at the level of the organelle and the environment in which it is being transported, rather than a general rule for a given motor.

There is a wide variety of microtubule structures in the axon. This is due to the ability of tubulin to undergo PTMs and associate with various Microtubule Associated Proteins (MAPs), all able to modify molecular motor attachment and activity (Atherton et al., 2018). Molecular motors target specific regions of the axon based on its cytoskeletal properties, for instance, kinesin 1 has a tendency to bind to microtubule bundles. This was demonstrated by artificially destabilizing microtubules through pharmacological treatment which leads to an absence of kinesin 1 in the axon (Balabanian et al., 2017; Kapitein et al., 2010; Tas et al., 2017). However, most kinesin motors do not seem to differentiate between the axon and dendrites of a given neuron, but the majority is directed towards the axon nonetheless ((Lipka et al., 2016). Once they reach the axon terminal, kinesins detach from microtubules because of their instability and lower quantities in this area, this has been reported for kinesin 3 (Guedes-Dias et al., 2019). Dynein on the other hand preferentially transports in dendrites in a bidirectional manner given the mixed polarity of microtubules in this area of the cell (Kapitein et al., 2010). The dynactin-dynein complex is also enriched in the axon terminal and has increased affinity for tyrosinated microtubules, also particularly concentrated in this location (Moughamian & Holzbaaur, 2012; Nirschl et al., 2016).

In addition to molecular motors, the cell's cytoskeleton may also participate in directing transport to and from the axon. Notably, the actin cytoskeleton around the axon initiation segment serves as a barrier or filter for cargo moving in and out of the axon. This is enabled in part by myosin V that binds organelles to inhibit their entrance into the axon when these are destined for somatodendritic compartments (Janssen et al., 2017; Lewis et al., 2009; Watanabe et al., 2012).

b) [Energy for transport](#)

Molecular motors hydrolyze ATP to generate force and movement. For example, kinesin 1 consumes 1 ATP molecule every 8 nm step (Hackney, 1994). This means that the number of ATP molecules consumed by kinesin 1 travelling in a single direction reaches millions in the average rat cortical axon and nearly billions in human motor neurons. This is even greater for retrograde transport by dynein molecules that take similar step sizes but require to be present in far greater numbers on a given organelle. These motors also take back and side steps making the total number steps and therefore the amount of ATP potentially even bigger (A. G. Hendricks et al., 2010; Rai et al., 2013; Soppina et al., 2009).

It has been reported that both glycolysis and mitochondria provide energy at the synapse. As mentioned above, mitochondrial arrest in the presynaptic area is required for correct synaptic vesicle release. Interestingly, despite only providing around 20% of total ATP at the synapse, inhibition of glycolysis alone resulted in a 50% decrease in synaptic endocytosis, and full blockage of synaptic currents required the combined inhibition of both glycolysis and mitochondria (Rangaraju et al., 2014; Sobieski et al., 2017). Additionally, neuronal activity has been shown to recruit GLUT4 transporters to the plasma membrane further suggesting glycolysis plays a crucial role at the synapse (Ashrafi et al., 2017).

The energy required for transport of mitochondria comes from oxidative phosphorylation of the given transported mitochondrion (Zala, Hinckelmann, Yu, et al., 2013). Surrounding energy levels can also influence mitochondrial transport. For example, an increase in ADP levels causes mitochondrial transport to stop (Mironov, 2007). Mitochondria are also able to sense local glucose concentrations in the axon. High glucose concentrations activate O-GlcNAc Transferase (OGT) which itself O-GlcNAcylates serine residues on Milton resulting in mitochondrial arrest (Pekkurnaz et al., 2014).

The energy required for vesicular transport, however, is provided by glycolysis, and not by mitochondria, nor the diffusion of ATP produced in the soma. The Saudou laboratory identified most enzymes of the glycolytic chain on membranes of motile vesicles and showed that these are sufficient to provide the energy necessary for transport (Figure 8). On-board glycolysis has been found responsible for providing energy to several cargo types, including dense-core vesicles, synaptic vesicles and signaling endosomes. Indeed, selective silencing of several glycolytic enzymes induced a significant reduction in BDNF-containing dense core vesicles in cultured neurons. Moreover, isolated vesicles are able to self-propel in presence of microtubules and a range of glycolytic substrates including glucose (Hinckelmann et al., 2016;

Zala, Hinckelmann, Yu, et al., 2013). Additionally, BDNF transport can be artificially rescued by expressing a specific vesicle-targeted form of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the sixth glycolytic enzyme, following GAPDH silencing in neurons (Zala, Hinckelmann, Yu, et al., 2013).

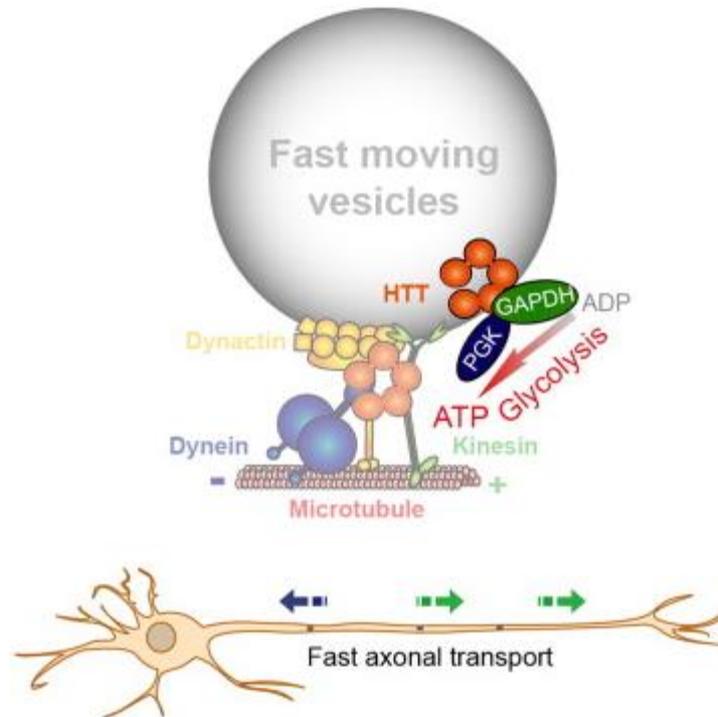


Figure 8: Glycolysis provides energy for vesicular transport. Glycolytic enzymes produce ATP to be consumed by the molecular motor complex which powers vesicular transport along microtubules. The attachment of glycolysis and molecular motors to vesicles is mediated by HTT (Zala, Hinckelmann, Yu, et al., 2013).

Much debate surrounds the potential contribution oligodendrocytes might have on energy metabolism in neurons. Oligodendrocytes are responsible for myelinating axons in the central nervous system (CNS) to enable isolation of the axon and saltatory propagation of the electrical signal (FRANKENHAEUSER, 1952; Hartline & Colman, 2007; McDougall et al., 2018). In various mouse models deficient for oligodendrocytic proteins, myelination proceeds normally, but axons develop swellings that contain arrested mitochondria and dense bodies, suggesting a role of oligodendrocytes in transport of several axonal organelles (Garbern et al., 2002; Griffiths et al., 1998; Lappe-Siefke et al., 2003).

3. Visualizing transport *in vitro* and *in vivo*

a) *In vitro* methods for visualizing transport

Different levels of complexity are available for measuring transport. The simplest system consists in *in vitro* reconstruction of microtubules on a plate that enable one to visualize functional aspects of a single molecular motor or cargo. The system is easily manipulated to study specific behaviors and regulations of transport in an isolated manner. Primary neurons in culture have been frequently used to assess transport in a more physiological manner than simple *in vitro* reconstruction given that, as has been developed above, many environmental factors influence transport in the axon. Indeed, in culture, neurons grow axons and establish synaptic connections with surrounding cells, similarly to what happens *in vivo*. This is combined with high resolution microscopy, such as the spinning-disk confocal microscope, which facilitates high spatial and temporal resolution. Individual cargoes can be tracked in *in vitro* and *ex vivo* tissues but some criticize these techniques for not correctly reconstructing the physiological environment of the cell (Gibbs et al., 2016; Lewis et al., 2016; Takihara et al., 2015). Indeed neurons in culture are not myelinated meaning the lack the potential influence these may have on neuronal and axonal function. Equally, the artificial environment of neurons in culture is often different than what is found *in vivo*, especially in terms of energy demand (Schwarz, 2013).

The development of microfluidic devices is a step up from basic cell cultures. Usually these devices consist of a series of chambers and microgrooves that essentially shape the neurons in culture, thus isolating different compartments of the cell to facilitate their observation. Moreover, the complexity of brain networks cannot be recreated in cell cultures, but microfluidic devices bring order by enabling one to separate different cell types on a given dish and manipulate specific compartments (Moutaux et al., 2018). This has been of particular interest for studying axonal versus dendritic transport in health and disease (Virlogeux et al., 2018; M. Zhang et al., 2010), the influence of neuronal activity (Moutaux et al., 2018) as well as axonal injury and regeneration (Taylor et al., 2005).

b) [In vivo methods for visualizing transport](#)

In vivo monitoring of transport has always been considered ideal but naturally more complex. Nevertheless, more straightforward approaches to express fluorescently tagged proteins are made possible by the development of gene editing techniques such as CRISPR, and the development of two-photon microscopy has allowed the observation of organelle dynamics in neurons *in vivo*. Certain animal models facilitate the observation of transport because of their translucent properties. This is the case for the wing of drosophila, making it is possible to monitor transport of fluorescently tagged organelles in mechano-sensory neurons. This has been done for dense core vesicles (DCVs) and mitochondria (Vagnoni et al., 2016). Zebrafish have the advantage of being entirely translucent at certain stages of development, which has enabled the study of mitochondria, endosomes and lysosomes (Drerup & Nechiporuk, 2013, 2016; Ponomareva et al., 2014, 2016) in a broader variety of neuronal cell types than drosophila (Auer et al., 2015). On a larger scale, mitochondrial transport has been assessed in a mouse model expressing fluorescent mitochondria (named MitoMouse) in several neuronal subtypes (Misgeld et al., 2007). These MitoMice have since been crossed with other mouse lines to measure transport deficiencies in amyotrophic lateral sclerosis (ALS) for example (Bilsland et al., 2010; Magrané et al., 2014b). Others have developed a way to monitor pyramidal axonal transport in live behaving mice through a surgically fitted window (Lewis et al., 2016; Smit-Rigter et al., 2016). More recently, some have managed to record axonal transport in ex vivo brain slices through in utero electroporation (Turchetto et al., 2020).

4. [Axonal transport in neurodegenerative diseases](#)

Motor proteins are expressed throughout the body but dysfunctions and mutations linked to the motor machinery mainly result in neurological phenotypes, which is evidence of the importance of axonal transport in the brain. Many neurodegenerative disorders present defects in axonal transport but some suggest that these may be secondary to upstream dysfunctions such as insufficient energy supply. Nevertheless, the strong link between the brain and a cell's dependence on transport suggests that it may be a valid target for treating neurodegenerative disorders.

a) [Amyotrophic Lateral Sclerosis](#)

ALS is characterized by cortical, bulbar and spinal motor neuron degeneration leading to progressive muscle atrophy, paralysis and spasticity. Regarding transport, swellings containing immobile vesicles, lysosomes, mitochondria and neurofilaments occur in the initial segment of motor axon (Sasaki et al., 1990), and there is evidence of heightened neurofilament phosphorylation in ALS which results in increased neurofilament pausing (Ackerley et al., 2000, 2003). Mutations in superoxide dismutase 1 (SOD1) have been known to cause ALS, of which three have been replicated in mouse models: G93A, G37R and G85R (Bruijn et al., 1997; Gurney et al., 1994; P. C. Wong et al., 1995). In G37R and G93A mice, SAT is impaired before mice develop clinical ALS-like symptoms possibly due to over activation of p38 and CDK5-p25 kinases (M. D. Nguyen et al., 2001; Tortarolo et al., 2003). Video microscopy revealed that anterograde transport of mitochondria was reduced in cultured G93A mouse neurons whereas retrograde transport was increased, which caused a 50% reduction of axonal mitochondria (De Vos et al., 2007). This may be a result of over active p38 that phosphorylates kinesin light chains (De Vos et al., 2000). However, the link between degeneration of motor neurons in ALS and deficient axonal transport of mitochondria is still debated given that some have found that no axonal transport deficiencies were seen in the G85R model (Marinković et al., 2012). Other mutations, such as those found in alsin, a guanine nucleotide exchange factor for Rab5, and vesicle-associated membrane protein (VAMP)-associated protein (VAPB), an ER membrane protein that anchors lipids to the ER, have also been reported to cause ALS (Devon et al., 2006; Gros-Louis et al., 2008; Otomo et al., 2003; Teuling et al., 2007).

b) [Alzheimer's disease](#)

Alzheimer's disease (AD) is the most common form of dementia and is characterized by the presence of amyloid plaques and neurofibrillary tangles in brain tissue. Amyloid plaques are formed by the abnormally high cleavage of APP into toxic amyloid beta (A β). APP undergoes anterograde vesicular transport and is known to interact with kinesin motors (Kamal et al., 2001). Some suggest that APP proteolysis may occur during the axonal transit of the protein down the axon, since depleting kinesin motors increases the chance of A β forming (Kamal et al., 2001). However, this is debated based on the fact that the enzymes necessary for APP cleavage travel separately from APP-positive vesicles (Goldsbury et al., 2006). Moreover,

A β itself may inhibit organelle transport through its promotion of actin polymerization (Hiruma et al., 2003).

The neurofibrillary tangles are formed by aggregation of tau, a microtubule associated stabilizing protein. In AD, tau is hyper phosphorylated which causes tau to detach and the cytoskeleton to break down, leading to inefficient transport (Patrick et al., 1999; U. Wagner et al., 1996). Indeed, NGF signaling endosomes show inefficient retrograde transport in the hippocampus of transgenic mice overexpressing mutant APP (Salehi et al., 2006).

c) [Parkinson's disease](#)

PD is marked by dopaminergic neuron degeneration in the substantia nigra. Similarly to HD, inclusion bodies are a hallmark of the disease and are composed mainly of highly phosphorylated alpha-synuclein. In *post mortem* tissue isolated from PD patient brains, lower levels of kinesin and dynein expression have been found in early stages of the disorder (Y. Chu et al., 2012). This has been linked to mitochondrial transport for which several associated genes, such as α -synuclein, parkin, DJ1 (Parkinson's disease (autosomal recessive, early onset) 7), PINK1 (phosphatase and tensin homologue (PTEN)-induced kinase 1), and LRRK2 (leucine-rich repeat kinase 2), have been reported as mutated in certain familial forms of PD (Abou-Sleiman et al., 2006).

Exposure to neurotoxins such as MPTP, MPP⁺ and rotenone can induce PD-like degeneration in animal models. MPP⁺ increases retrograde and decreases anterograde vesicular transport through activation of caspase 3 and PKC γ (G. Morfini et al., 2007). With rotenone, mitochondria travelling in the retrograde direction pause less leading to an increase in global mitochondrial retrograde transport (Arnold et al., 2011).

d) [Charcot-Marie-Tooth disease](#)

Charcot–Marie–Tooth disease is characterized by sensory loss and atrophy of distal muscles. Many subtypes exist but can be categorized into four groups: CMT1, 3 and 4, characterized by reduction of motor neuron conduction due to degeneration of myelin sheaths, and CMT2, characterized by axonal degeneration of motor neurons (Züchner & Vance, 2006) Several studies have identified axonal transport defects in CMT2. Indeed, many of the mutations responsible for CMT2 type A are located in or around the gene coding for

mitochondrial fusion protein mitofusin 2 (MFN2), a GTPase positioned on the outer membrane of mitochondria that regulates mitochondrial fusion and architecture (Züchner et al., 2004). Mitochondria are highly aggregated and form clusters in the distal portions of motor neuron axons in transgenic CMT2 mice (Cartoni et al., 2010; Detmer et al., 2008). This is linked to MFN2's ability to interact with adaptor proteins that regulate mitochondria's interaction with kinesin motors leading to deficient mitochondrial transport in transgenic cultured neurons (Baloh et al., 2007; Misko et al., 2010). Certain cases of CMT2 type B present mutations in Rab7, a regulatory adaptor protein of retrograde endosomal transport in response to NGF receptor internalization (28). Mutations in the Rab7 gene lead to accumulation of NGF activated endosomes in the axon terminal that are unable to return to the cell body. This lack of trophic support leads to cell death in NGF dependent neurons (29). Additionally, some CMT2 patients show mutations in kinesin 3 and dynein motors, for which the latter is unable to dimerize correctly (Weedon et al., 2011; Zhao et al., 2001).

e) [Spinal bulbar muscular atrophy](#)

PolyQ disorders such as HD and spinal bulbar muscular atrophy (SMBA) have been shown to be associated with axonal transport deficits. HD's axonal transport deficits have been covered in the previous chapters. SMBA is characterized by lower motor neuron degeneration and is caused by a polyQ expansion in the gene coding for androgen receptor protein (AR). Normally, androgen binds to its receptor and is translocated to the nucleus where it serves as a transcription factor. However, similarly to mHTT, polyQ AR obtains new functions and affects cellular function in a manner that is independent of its transcriptional function. Indeed, it has been suggested that polyQ AR may interfere with mitochondrial and kinesin distribution by inducing kinesin heavy chain phosphorylation, a modification that facilitates kinesin detachment from microtubules (Gerardo Morfini et al., 2006; Piccioni et al., 2002; Szebenyi et al., 2003). Dynein is also mutated in its heavy chain which affects the motor's stability and function (M. B. Harms et al., 2012). Adaptor proteins have equally been found mutated in SMBA. A mutation in dynactin, specifically in its p150 subunit, was found in patients, affecting its ability to bind to microtubules causing dynein and dynactin accumulation (Puls et al., 2003).

f) Hereditary spastic paraplegia

Hereditary spastic paraplegia (HSP) consists in upper motor neuron dysfunction leading to spasticity and sometimes neurological symptoms (infrequent). Mutations in several genes are responsible for HSPs of which many have been linked to axonal transport (Dion et al., 2009; Stevanin et al., 2008). 40% of cases are due to a mutation in the SPAST, the gene coding for spastin (Beetz et al., 2006). Spastin's physiological role is to destabilize microtubules by dissociating tubulin-tubulin interactions (Roll-Mecak & Vale, 2008). Mutant spastin is unable to do this correctly leading to mitochondrial transport defects (McDermott et al., 2003). The KIF5A motor is also mutated in some forms of HSPs and shows decreased affinity for microtubules and decreased velocity in transporting its known cargo, neurofilaments and GABAA receptor (Ebbing et al., 2008; E. Reid, 2003; Twelvetrees et al., 2010). Other forms of HSP have been linked to mutations known to affect mitochondrial ATP synthesis (REEP1, HSP60 and SPG7) which leads to defective transport of mitochondria but also of other organelles such as neurofilaments (Ferreirinha et al., 2004), as well as mutations linked to molecular motor partners involved in endosomal trafficking in the axon (CHMP1B, a protein associated with the ESCRT (endosomal sorting complex required for transport)–III complex) (Evan Reid et al., 2005; Stevanin et al., 2008).

D. Chapter 4: Energy in the brain

1. Sources of energy for the brain

a) Glucose and its byproducts

The human brain is an extremely energy-demanding organ. Although it represents only 2% of the body's weight, it consumes approximately 20% of its overall available energy. Early studies on brain metabolism revealed that glucose was the obligatory substrate for producing energy (Kety & Schmidt, 1948). Since then, analyses on a cellular level have shown that energy metabolism in the brain is more complex and varies according to the cell type, suggesting the existence of specific metabolic profiles that contribute differently to the various steps of energy production (Sokoloff, 1981). Nevertheless, the main currency for energy in all brain cells is ATP and glucose provides the brain with at least 95% of this currency (Magistretti & Allaman, 2018). Glucose is actively pulled into the brain, meaning that variations in blood sugar levels do not influence brain glucose concentrations, except when it is very low. Glucose uptake is triggered by brain activity and enabled by a series of GLUTs on the membranes of various cell types that mediate the transfer of glucose from the blood to neurons. The cells involved in this transfer form the neurovascular unit (Figure 9) (Lecrux & Hamel, 2011). GLUT4 is expressed by neurons and will be upregulated in case of synaptic activity, a mechanism that is both AMPK and insulin dependent (Ashrafi et al., 2017; Pearson-Leary et al., 2018).

Like many other cellular tissues, the brain is able to store glucose in the form of glycogen through glycogenesis. This storage is not homogenous throughout brain cells nor throughout brain regions. In the adult brain, glycogen is primarily found in astrocytes, given that they contain glycogen metabolizing enzymes such as glycogen synthase and glycogen phosphorylase (Cataldo & Broadwell, 1986; Pfeiffer-Guglielmi et al., 2003). Examination of whole brain distribution of glycogen reveals that certain domains are more concentrated than others, such as the cortex, striatum and hypothalamus (Sagar & Ferriero, 1987). Moreover, grey matter tends to contain twice as much glycogen than white matter (Kong et al., 2002).

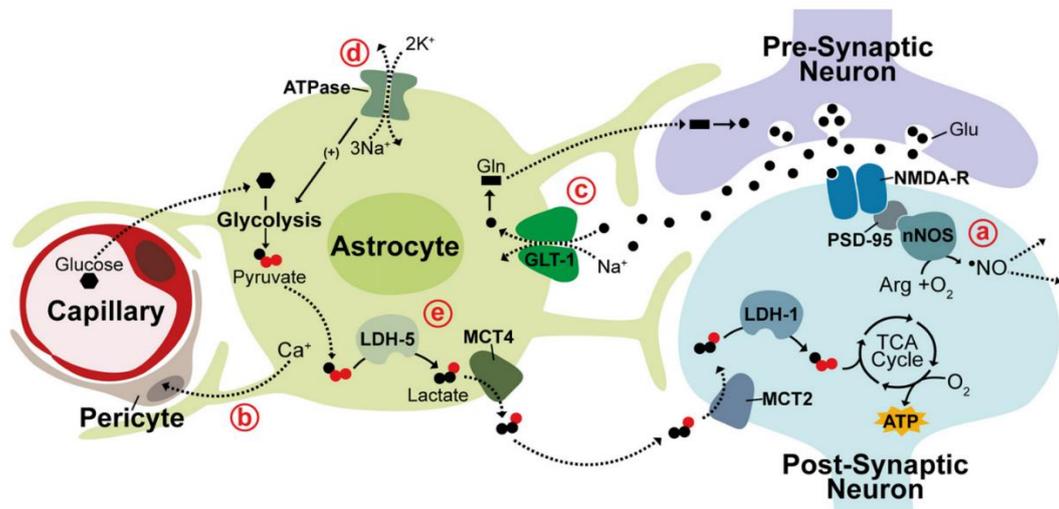


Figure 9: The neurovascular unit. Schematic representation of the neurovascular unit where neuronal activity triggers glucose uptake from the blood by astrocytes, which is then broken down by glycolysis to form lactate. Lactate is then transported from astrocytes to neurons, converted back into pyruvate and further broken down by the mitochondria and Krebs cycle to produce ATP for neurons (Watts et al., 2018).

During hypoglycemia, glycogen is the first emergency source of energy for the brain. At resting state, with normal blood glucose concentrations, this storage is very slowly metabolized by brain cells. Glycogen breakdown will then drastically increase as blood sugar decreases, but this will only satisfy the brain's energy demands for a few minutes (Bak & Walls, 2018a; Choi et al., 2003; Öz et al., 2009; Wender et al., 2000).

The cells metabolism is a very complex process involving a great number of enzymatic reactions, of which two major pathways contribute to the actual production of ATP: glycolysis and oxidative respiration (these will be detailed further in Chapter 5). Glucose is broken down through glycolysis and its end product, pyruvate, fuels oxidative respiration. In the brain, there is a different reliance on one or the other depending on the cell type. Astrocytes and oligodendrocytes produce most of their ATP via glycolysis whereas neurons rely more on mitochondria. This means that, although the brain's primary fuel is glucose, a given cell type does not necessarily import glucose only as its main source of ATP. Glucose is known to be stored and utilized by astrocytes, whereas, lactate, a byproduct of glycolysis, serves as an important energy source for neurons where it feeds directly into the Krebs cycle in mitochondria.

The sources of lactate for neurons are quite numerous, for instance, the high glycolytic activity in astrocytes and oligodendrocytes enables production and transfer of lactate across membranes via monocarboxylic transporters (MCTs) (this will be developed below). Lactate

may also access neurons directly from the blood. During exercise, blood lactate concentration increases, which is followed by a rise in its uptake by neurons (Ide & Secher, 2000).

b) Ketones

The brain cannot store and use fatty acids for energy. Ketones produced by fatty acid breakdown in the liver, however, can serve as an alternative energy source for brain function. The main ketones utilized by the brain include 3- β -hydroxybutyrate (3BHM), acetoacetate and acetone and are all able to move across cell membranes through MCTs (Laffel, 1999). Once inside the cell, acetoacetate is converted to Acetyl Coenzyme A which is able to enter the tricarboxylic acid (TCA) cycle and produce ATP in mitochondria.

Although they do not represent a large source of energy in adults, ketone bodies do play a more significant role in the developing brain, accounting for lipid synthesis as well as 30-70% of energy production (Cunnane & Crawford, 2014; Nehlig, 2004). As the brain matures, ketones are used in neurons to produce neurotransmitters such as glutamate (Laffel, 1999) or opportunistically as an important energy source during periods of prolonged fasting, once glycogen reserves are depleted (Morris, 2005). However, ketones are not metabolized glycolytically, only through the Krebs cycle can they be used to produce energy.

2. Lactate shuttling between glia and neurons

Neurons are responsible for 80-90% of all energy consumption in the brain (Howarth et al., 2012). Yet at resting state, both neurons and astrocytes take up similar amounts of glucose from the blood stream (Nehlig et al., 2004). Conversely, astrocytes but not neurons increase their glucose uptake upon neuronal activation (Chuquet et al., 2010), suggesting a cross talk between astrocytes and neurons regarding glucose metabolism upon functional activation (Voutsinos-Porche et al., 2003). This has been linked to the different metabolic profiles in these cell types. Indeed, it is thought that glial cells, such as astrocytes and oligodendrocytes, use mainly glycolysis for ATP production, whereas neurons rely mainly on mitochondria.

Neurons produce much higher quantities of CO₂ compared to astrocytes, suggesting a greater dependence on the TCA cycle in mitochondria (Hamberger & Hyden, 1963). On the other hand, glycolysis and lactate production remain the astrocyte's favored pathways for ATP production (Figure 10). This is possibly due to inefficient coupling between glycolysis and

mitochondria in these cells. Indeed, pyruvate dehydrogenase (PDH), the enzyme responsible for transforming pyruvate into Acetyl-CoA, is highly phosphorylated which has the consequence of lowering its enzymatic activity (Figure 10) (Itoh et al., 2004). Additionally, within the mitochondria, the complexes that form the respiratory chain (complexes 1 to 4) are organized differently rendering them less efficient in astrocytes compared to neurons. Complex 1 is dissociated from the rest in astrocytes, whereas all complexes associate to form a supercomplex in neurons (Lopez-Fabuel et al., 2016). Moreover, astrocyte specific reduction of cytochrome oxidase activity has no phenotypical consequences for up to a year in mice (Supplie et al., 2017).

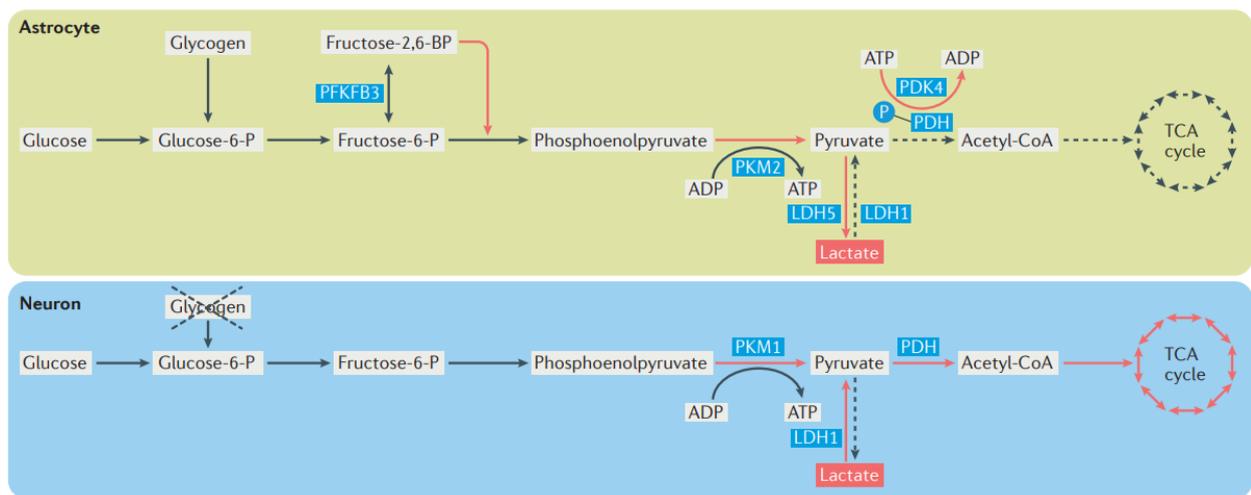


Figure 10: Metabolic differences between astrocytes and neurons. Illustration of certain metabolic differences in neurons and astrocytes accounting for their variable reliance on glycolysis and mitochondria for energy production. In astrocytes, glycolysis and lactate-producing lactate dehydrogenase (LDH) are particularly active, whereas in neurons, pyruvate-producing LDH and the TCA cycle are more dynamic. Red arrows show reactions that are notably active (Magistretti & Allaman, 2018).

Further evidence of differences in metabolic mechanisms is seen in the expression profiles for LDH between neurons and astrocytes. Neurons mostly express LDH1, an isoform of the enzyme that favors the conversion of lactate into pyruvate, whereas astrocytes tend to express LDH5, the isoform that facilitates the opposite reaction (Bittar et al., 1996). These observations suggesting two different metabolic profiles between neurons and astrocytes have given rise to the astrocyte-neuron lactate shuttle (ANLS) model (Figure 9). It suggests that glutamate released at the synapse upon neuronal activation would be taken up by astrocytes along with Na^+ ions. This would lead to an increase in astrocytic sodium concentration that would in turn activate $\text{Na}^+\text{-K}^+$ ATPase pumps, a process that would then, through ATP

consumption, trigger glycolysis and glucose uptake in astrocytes. Glycolysis and LDH would then breakdown glucose to form lactate which would then be shuttled to neurons and fed into the mitochondrial respiration chain to produce ATP (Cholet et al., 2001; Pellerin & Magistretti, 1994; Voutsinos-Porche et al., 2003). However, this model remains controversial and is not unanimously agreed upon in the scientific community (Bak & Walls, 2018b; Dienel, 2017).

More recently, the ANLS model has been extended to other cell types. Indeed, neuronal exchange of lactate has been found in oligodendrocytes as well (Figure 11). These cells are responsible for myelinating and electrically isolating axons in the central nervous system. During the cell's development, ATP is primarily generated via mitochondria and the lactate required for this process is transferred from astrocytes (Rinholm et al., 2011; Sánchez-Abarca et al., 2001). However, once the oligodendrocyte is fully mature, similarly to astrocytes, it will rely mainly on glycolysis for ATP production. As a result, they will also produce lactate which can then be transported into the myelinated axon through MCT1. Interestingly, genetic inhibition of this transfer leads to axonal dysregulation (Fünfschilling et al., 2012; Saab et al., 2013).

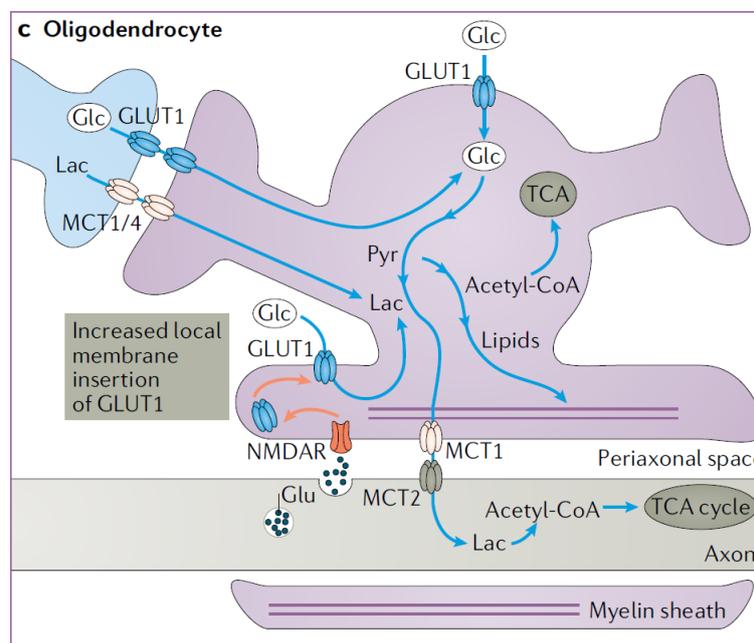


Figure 11: Energy transfer between oligodendrocytes and neurons. Illustration of lactate production in oligodendrocytes and its transfer to myelinated axons for ATP production through the TCA cycle in mitochondria (Cunnane et al., 2020).

Microglia, on the other hand, do not provide energy for neurons, since they produce ATP through the Krebs cycle under normal physiological conditions. Once activated during

neuroinflammation, however, ATP production in these cells shifts from a mitochondrial based process to a glycolytic process (Aldana, 2019).

3. Energy in neurodegenerative disorders

Despite being affected in many neurodegenerative disorders, metabolic rate in the brain decreases progressively with age. It is often observed in early stages of several diseases and therefore contributes to and aggravates the degenerative processes that are already naturally occurring (Cunnane et al., 2016). Impaired glucose metabolism is known to have consequences on several highly demanding cellular functions such as ion transport, vesicle recycling and synaptic transmission. As mentioned above, glucose uptake is insulin dependent. Intriguingly, insulin resistance associated with reduced glucose uptake is a frequently observed phenotype in neurodegenerative disorders (Pearson-Leary et al., 2018). Moreover, as these diseases develop, neuroinflammation triggers microglial activation associated with increased GLUT expression and energy consumption, therefore pumping glucose away from neurons (Deczkowska et al., 2018).

a) Huntington's disease

Energy is a central element to the development of Huntington's disease. However, an exact understanding of the pathological events that occur in HD metabolism is difficult to obtain, given the complexity of the cell's energy generating pathways and the multiple areas of said pathways that have been reported to be affected in HD. This puzzle becomes more complex when considering the ability of the cell to maintain homeostasis when certain of its functions become compromised. Indeed, a great number of enzymes contribute to ATP production and changes in expression or activity of one of these enzymes will most likely lead to antagonist changes among the rest in order to maintain normal energy production. It is therefore difficult to predict whether a metabolic difference observed at a given time-point in HD is a damaging consequence of the disease, or simply the cell's metabolic compensation to modification elsewhere. For instance, ATP consumption is increased in 6 week old knock-in Q111 mice and then decreased at 13 weeks (Tkac et al., 2012).

Nevertheless, certain differences remain constant throughout the disease. For example, several studies have documented the decrease in glucose uptake observed in the cortex and

striatum of presymptomatic and symptomatic patients (Ciarmiello et al., 2006; Feigin et al., 2007). This is associated with decreased GLUT expression in several cell types in both humans and animal models (Gamberino & Brennan, 1994; McClory et al., 2014; Vittori et al., 2014). Reduced glucose uptake causes astrocytes to rely on fatty acids as an alternative energy source, at the expense of producing much larger amounts of toxic ROS (Polyzos et al., 2019).

Downstream pathways such as glycolysis and the Krebs cycle are also affected in HD. Positron emission tomography (PET) scans reveal that glycolytic ATP production is significantly higher at rest in HD patients, which limits the brain's capacity to increase its production during activity (Mochel et al., 2012; Powers et al., 2007). This can be linked to their high caloric intake despite unchanged body mass index (TeSlaa & Teitell, 2014). Others have observed that glycolytic enzyme quantity is modified in post-mortem tissue, such as decreased phosphofruktokinase (S. J. Tabrizi et al., 1999), as well as changes in the TCA cycle in mitochondria (Butterworth et al., 1983, 1985; Sorolla et al., 2010). Moreover, inhibition of succinate dehydrogenase, an enzyme of the Krebs cycle and mitochondrial transport chain, with 3-nitropropionic acid is sufficient to initiate striatal degeneration in rat and has been used as a HD model in the past (Brouillet et al., 1998).

b) [Alzheimer's disease](#)

AD is one of the most common neurodegenerative disorders and is characterized by several metabolic symptoms, such as weight loss and poor appetite. This disease is also characterized by reduced glucose uptake (Oliveira et al., 2015; Ryu et al., 2019) which leads to regional hypometabolism in the brain, a phenomenon that precedes hippocampal atrophy and plaque formation (D. H. S. Silverman et al., 2001; Small et al., 1995). Reduced vascularization and blood flow contribute to this hypometabolic state by limiting nutrient supply (Hamel et al., 2008; Kumar-Singh et al., 2005; Shibata et al., 2000). Nevertheless, oxygen, lactate and ketone metabolism remain relatively unchanged (Ryu et al., 2019; Toppala et al., 2019). This insufficient glucose uptake in oligodendrocytes causes white matter atrophy which in turn disrupts mitochondrial transport and function leading to overall axonal dysfunction (Bartzokis, 2011; Duarte et al., 2018; Klosinski et al., 2015).

Many reports have observed defective enzymatic activity due to A β accumulation in mitochondria, for instance, cytochrome C oxidase activity in complex IV isolated from AD patients is reduced (Parker et al., 1990). Important TCA cycle enzymes, PDH and α -

ketoglutarate dehydrogenase, are also reduced in AD, features that are also observed early in the disease (Casley et al., 2002; Wirths et al., 2001). Finally, these enzymatic issues are associated with increased reactive oxygen species (ROS) production from the mitochondria (Calingasan et al., 1999; Gabbita et al., 1998; Greilberger et al., 2008; Hensley et al., 1999; Pratico et al., 1998).

c) [Parkinson's disease](#)

Metabolic problems arise in Parkinson's disease (PD) as well, as patients suffer from severe weight loss and reduced glucose metabolism that correlates with motor and cognitive decline (J. S. Chu et al., 2019; Matthews et al., 2018). The substantia nigra pars compacta, a particularly affected brain area in PD, shows decreased glycolysis and TCA cycle activity associated with mitochondrial fragmentation increased oxidative stress (Gibson et al., 2003; Klivenyi et al., 2004; Warby et al., 2008). Mitochondria are of significant interest in PD because mutations in several mitochondrial genes or even treatment with mitochondrial toxins such as MPP⁺ lead to PD (Langston et al., 1983; W. D. Parker et al., 2008; Plun-Favreau et al., 2007).

E. Chapter 5: Glycolysis and associated pathways

1. Glucose is broken down through glycolysis

Eukaryotic cells rely mainly on glucose as their external source of energy. First, glucose is transported from the blood flow across the plasma membrane of the cell via passive bidirectional transporters named GLUT (1 to 5), following which it will be broken down via different metabolic pathways. These glucose transporters have different properties and expression patterns throughout the body. GLUT1 and GLUT3 are expressed in all cell types and have a low K_M value (1 mM, explained below) which is much lower than blood concentration. These transporters ensure a steady continuous flow of glucose for each cell of the body. GLUT2 is expressed in the liver and pancreas and possesses a much higher K_M of around 15 to 20 mM which means that glucose will only be transported through these receptors when at high concentration. This enables the liver to sense high blood sugar and regulate glycogen synthesis and causes the pancreas to secrete insulin in response. This spike in insulin will induce GLUT4, found in muscles, adipocytes and neurons, to increase its expression. Finally, GLUT5 is expressed in the small intestine and transports fructose mainly.

Within the cell, a series of catabolic reactions convert and breakdown glucose into various byproducts in order to produce ATP, the cell's universal energy currency. A very large number of enzymes and cofactors are responsible for this catabolism of glucose forming an extremely complex metabolic network. Glycolysis is one of the most conserved cellular functions in life on earth, from bacteria to humans, and corresponds to the first set of metabolic steps responsible for glucose breakdown within the cell. It is composed of ten enzymes that function one after the other and include hexokinase (HK), phosphoglucose isomerase (PGI), phosphofructokinase (PFK), aldolase, triose phosphate isomerase (TPI), glyceraldehyde 3 phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK), phosphoglycerate mutase (PGAM), enolase, and pyruvate kinase (PK). These enzymes convert 1 molecule of glucose into 2 molecules of pyruvate, all whilst reducing 1 molecule of NAD^+ into NADH and phosphorylating 2 molecules of ADP into ATP. The overall reaction of glycolysis is: $\text{Glucose} + 2 \text{ADP} + 2 \text{Pi} + 2\text{NAD}^+ \rightarrow 2 \text{Pyruvate} + 2 \text{ATP} + 2\text{H}_2\text{O} + 2\text{NADH} + 2\text{H}^+$ (Figure 12).

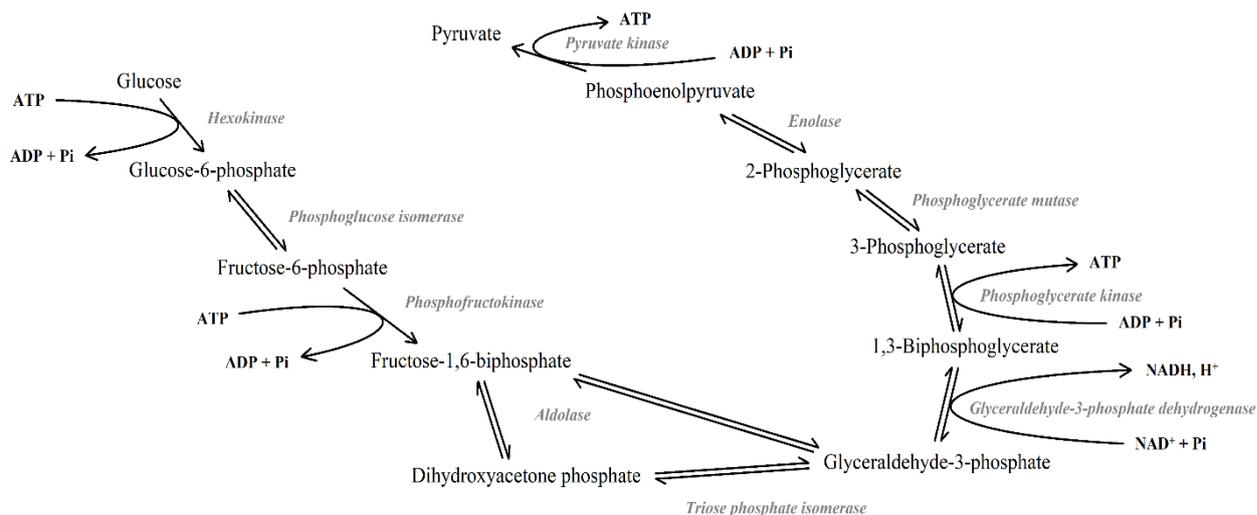


Figure 12: Glycolysis. The glycolytic pathway contains ten enzymatic steps that ensure the production of pyruvate from glucose. It also produces a net quantity of two ATP molecules per glucose and reduces one NAD^+ . The enzymes responsible for each reaction are noted in grey. Full arrows represent irreversible reactions and double half arrows represent reversible reactions.

Glycolysis contains two parts: the preparatory phase and the payoff phase. The preparatory phase is comprised of the first five glycolytic enzymes and converts glucose into glyceraldehyde-3-phosphate (G3P). Here glucose, a six carbon ring, is broken down into two 3-carbon molecules (G3P) and is referred to as preparatory because it consumes two molecules of ATP at steps 1 (HK) and 3 (PFK). The payoff phase transforms glyceraldehyde-3-phosphate into pyruvate and is made up of the five remaining glycolytic enzymes. This phase is responsible for reducing NAD^+ into NADH (GAPDH, step 6) and phosphorylating two molecules of ADP into ATP at steps 7 (PGK) and 10 (PK). Given that the preparatory phase produces 2 G3P molecules for every glucose molecule, the payoff phase essentially occurs twice which is why the net production of ATP by glycolysis is +2 (Figure 12).

2. Glycolytic regulation

Glycolytic activity is tightly regulated by energy availability within and outside the cell, and will therefore depend primarily on the cell's ATP requirements and the amount of glucose that it will import. Within the glycolytic chain, three steps are irreversible and therefore dictate the overall flux of this pathway (Figure 12). These are HK, PFK and PK, the activities of which

are regulated in different ways, whether it be through the reversible binding of allosteric modifiers, post-translational modification or transcriptional adjustments.

Hexokinase catalyzes the first glycolytic reaction which consists in the phosphorylation of glucose into glucose-6-phosphate (G6P). Given the exergonic nature of this reaction, glucose is rapidly transformed by HK which accounts for the low glucose concentration within cells. This enzyme is sensitive to the concentration of its product, G6P. In most cell types, when G6P accumulates, HK will stop glucose phosphorylation. This is not the case in liver cells however, that express a HK isoenzyme called glucokinase that is not inhibited by G6P and that will redirect it to form glycogen.

HK's product G6P is a substrate for glycolysis and the pentose phosphate pathway, making HK a regulatory step for two different pathways. PFK however is a commitment step for glycolysis only, which is why it is often considered the most important regulatory step of this pathway. PFK uses ATP to phosphorylate fructose-6-phosphate into fructose-1,6-biphosphate and is regulated by several metabolites. When in excess, ATP is able to allosterically inhibit PFK by binding a region distinct from its catalytic site, causing the enzyme to lose its affinity for fructose-6-phosphate. This inhibition can be enhanced by citrate, an early Krebs cycle intermediate product. A drop in pH will also inhibit PFK, in order to avoid excess lactate production and release into the blood stream. Adenosine monophosphate (AMP) has the opposite effect of ATP. The explanation for which AMP and not ADP has an activating effect on PFK is because when ATP is scarce, ADP will be converted into ATP and AMP by adenylate kinase. AMP is therefore the true final indicator of energy depletion in the cell and will stimulate glycolysis through PFK as a result. Another PFK activator is fructose-2,6-biphosphate, the concentration of which is mediated by PFK2, an enzyme with kinase and phosphatase functions that is itself dependent on the cellular concentration of glucose.

PK is the third and final regulatory step of glycolysis and catalyzes the conversion of phosphoenolpyruvate into pyruvate, combined with the phosphorylation of ADP to form ATP. There are two genes that code for PK, PKL, found mainly in the liver, and PKM, mainly expressed in muscle tissue. Both isoenzymes are inhibited when downstream products accumulate, such as ATP, similarly to PFK, and alanine, a product of the Cahill cycle and amino acid formation in hepatocytes. However when blood glucose concentration is low, PKL, the liver-specific isoenzyme, and not PKM, is phosphorylated by protein kinase A and subsequently inhibited. This will diminish glycolysis in the liver only, so that the remaining blood quantities of glucose can be metabolized by organs that require it the most, such as the brain and muscles (Biochemistry, fifth edition, Berg, Tymoczko and Stryer).

3. Pathways associated to glycolysis

a) The Krebs cycle and oxidative respiration

Once glycolysis occurs, the classical fate of pyruvate under normal physiological conditions is to be transferred to mitochondria. Porins and transporters at the surface allow passive and active transport, respectively, of pyruvate across the outer and then inner mitochondrial membranes. Once in the matrix, pyruvate is converted into Acetyl-CoA, the initial substrate for ATP production in mitochondria. Here, two metabolic pathways function in tandem to produce ATP: the Krebs cycle, also known as the TCA or citric acid cycle, that occurs in the mitochondrial matrix, and the inner transmembrane oxidative respiratory chain. The Krebs cycle corresponds to the series of closed loop enzymes that gradually oxidize Acetyl-CoA into CO₂. The result of this loop with regards to ATP production is to generate electrons for the respiratory chain, located within the inner membrane of mitochondria. These electrons are shuttled from the Krebs cycle to the respiratory chain via NADH and FADH₂ molecules. Next, the various transmembrane complexes of the respiratory chain will exchange electrons in order to create a proton gradient between the matrix and intermembrane space. The energy from this gradient will be harnessed by the final complex of the chain (ATP synthase) in order to phosphorylate ADP into ATP (Figure 13). When compared to glycolysis, mitochondria are much slower but produce far greater amounts of ATP, with approximately 36 to 38 ATP molecules for each molecule of glucose. They also require much more oxygen than glycolysis in order to function.

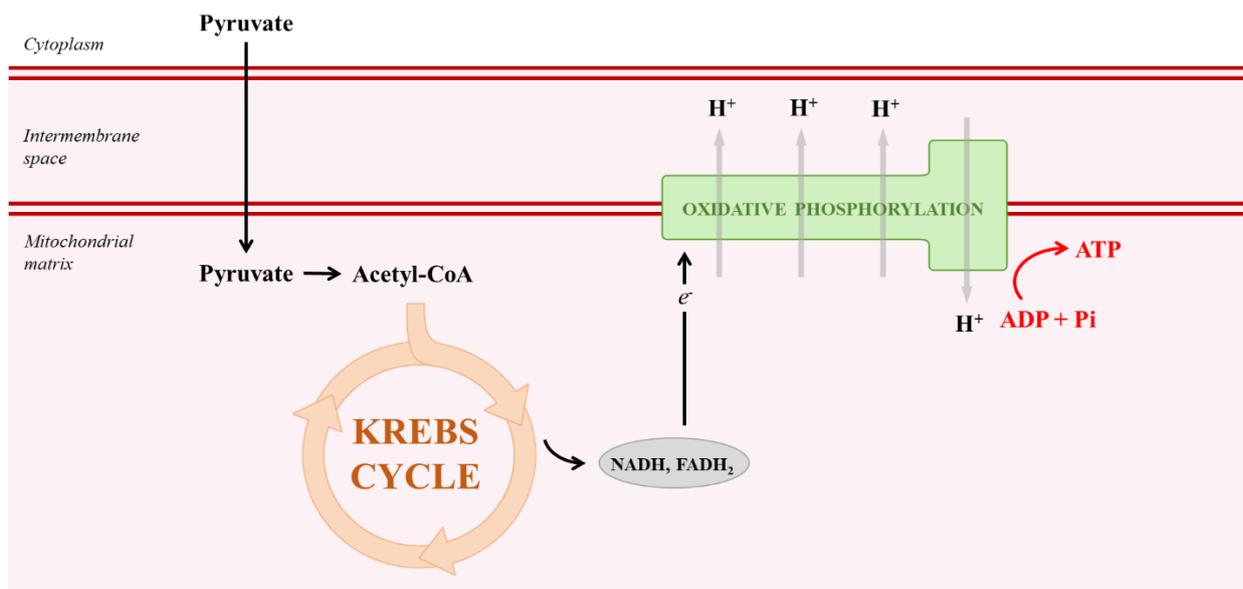


Figure 13: Mitochondrial ATP production. Pyruvate is imported to the mitochondrial matrix where it is converted to Acetyl-CoA and cyclically broken down through the Krebs cycle. Reduced NADH and FADH₂ serve as electron shuttles for oxidative phosphorylation. The respiratory chain will then generate a proton gradient across the inner membrane that will then be used to phosphorylate ADP molecules into ATP.

Glucose is not the sole supplier in Acetyl-CoA to mitochondria, it may also be obtained from amino acid, fatty acid and lipid degradation. Mitochondrial metabolic activity is tightly regulated to ensure a steady and adjusted flow of ATP production. In excess, NADH inhibits most Krebs cycle enzymes, and so when the respiratory chain is blocked or malfunctioning, NADH will accumulate and shut down the Krebs cycle as a consequence. ATP is also an inhibitor of PDH activity, the enzyme responsible for converting pyruvate into Acetyl-CoA.

b) [Lactate dehydrogenase](#)

GAPDH catalyzes the dehydrogenation and concomitant phosphorylation of glyceraldehyde-3-phosphate into 1,3-bis-phosphoglycerate. This step also reduces one molecule of NAD⁺ to NADH, which can be used for electron transfer in the mitochondrial respiratory chain under physiological conditions. However, during hypoxia or when O₂ is limited, the mitochondrion is unavailable and given the low cytosolic concentration of NAD⁺/NADH, it must be recycled nonetheless so that glycolysis can continue functioning. LDH, often considered the eleventh enzyme of glycolysis, is responsible for this NAD⁺ recycling. It converts pyruvate into lactate, thus diverting it from mitochondria, and oxidizing one NADH molecule in the process. This is referred to as anaerobic glycolysis (Figure 14).

Lactate is then excreted from the cell where it is able to serve as a substrate for different pathways (detailed below).

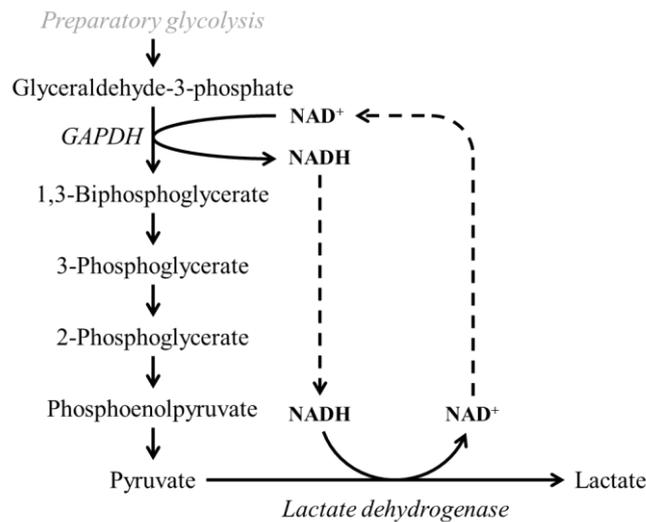


Figure 14: Anaerobic glycolysis and NAD⁺ recycling by lactate dehydrogenase. LDH catalyzes the conversion of pyruvate into lactate when mitochondria are unavailable. This serves to recycle NAD⁺ enabling glycolysis to continue functioning.

Regarding its structure, LDH monomers associate to form a tetramer of approximately 140 kDa. 4 LDH genes have been described: LDHA, LDHB, LDHC and LDHD. The LDHA, B and C isoforms use or produce L-lactate whereas LDHD uses or produces the other stereoisomer, D-lactate, which, when found in humans, is usually of microbiological origin. LDHA is expressed mainly in muscle tissue and is therefore often referred to as LDH-M, LDHB is found in the heart and is called LDH-H. LDHC is only present in the testes. The A (M) and B (H) isoforms can associate into 5 possible homo- and hetero-tetramers (isoenzymes): LDH-1 (4 H isoforms, H₄), LDH-2 (H₃M₁), LDH-3 (H₂M₂), LDH-4 (H₁M₃) and LDH-5 (M₄). Although they are structurally very similar, the presence of one isoform or the other will confer different catalytic properties to the final LDH tetramer. LDHA has a net charge of -6 which gives it a preferential affinity for pyruvate, and therefore a higher chance of converting pyruvate into lactate, whereas LDHB's net charge is of +1, giving it a greater affinity for lactate and thus preferentially converts lactate into pyruvate (Read et al., 2001). Furthermore, the LDHA:LDHB expression ratio is correlated to lactate release and absorption in various tissues. For example, muscle tissue expresses mainly LDHA compared to LDHB and is therefore the largest lactate producing tissue in the body, whereas the liver and kidneys express more LDHB than LDHA and thus absorb large amounts of lactate for glucose production (neoglucogenesis).

Brain expression of LDHA and LDHB is variable according to cell type and time point during development. According to some studies, LDHA expression would be highest during early stages of neurodevelopment and then gradually decrease with age, whereas LDHB would follow an opposite trend (Goyal et al., 2014). LDHA and LDHB are also expressed differently according to brain region, for instance, some have shown that although LDHB was expressed equally throughout the brain, LDHA was present in larger concentrations in the hippocampus, hypothalamus, and cerebral cortex (Laughton et al., 2000; Ross et al., 2010). Others have demonstrated that elevated glycolysis, which is linked to a higher LDHA expression, was found preferentially in cerebral regions specialized in synapse formation, suggesting that glycolysis and therefore LDHA may be required for growing and differentiating neurons (Goyal et al., 2014). Finally the variable expression of LDHA and LDHB has also been linked to cell type, whereby astrocytes and oligodendrocytes would be more reliant on glycolysis and would express LDHA thus producing lactate that would then be transferred to neurons, transformed back to pyruvate by neuronal LDHB which would then fuel the mitochondria (Kimelberg, 2004; Pellerin & Magistretti, 1994).

LDH is also at the center of tumor cell metabolism. In the 1920s, Otto Warburg observed that cancer cells showed increased glycolytic rate associated with higher glucose consumption and lactate production (Warburg et al., 1927). Thus, despite the presence of sufficient oxygen and functioning mitochondria, cancer cells generate ATP through glycolysis and LDH, a process Warburg termed aerobic glycolysis and that is now also referred to as the Warburg effect. This important implication of LDH in tumorigenesis has made it an attractive candidate for treatment development. Indeed, abnormal LDHA upregulation and LDHB downregulation are a common marker of several types of cancers and it has been shown that attenuating LDHA activity in cancer cells reduces their tumorigenicity and ability to proliferate (Fantin et al., 2006). However, the effect of LDHA inhibition on malignancy depends on the metabolic profile of the cancer type, as those that rely on glutaminolysis and the Krebs cycle, rather than glycolysis and the pentose phosphate pathway, do not seem affected (Billiard et al., 2013).

Many LDH inhibitors have been developed as a result. Oxamate used in combination with phenformin, a mitochondrial inhibitor, was able to reduce tumor size, glucose uptake and ATP generation and increase tumor apoptosis *in vivo* (Miskimins et al., 2014). Unfortunately, oxamate has a low cellular penetrance and needed to be used in high concentrations to have an effect. On the other hand Galloflavin, a synthetic LDH inhibitor, requires much lower doses and has shown promising results for breast cancer treatment but still required doses as high as

250 μM which remains more concentrated than what is commonly used in chemotherapy (Farabegoli et al., 2012). Some groups have designed inhibitors specifically aimed at LDHA but still require development (Granchi et al., 2011; Maftouh et al., 2014). Therefore, it is this lack of specificity of current LDH inhibitors, combined with the necessity to have to use high doses in order to generate an effect on tumorigenicity, that are the main limiting factors for the development of cancer therapeutic strategies based on LDH inhibition.

c) [The pentose phosphate pathway](#)

The pentose phosphate pathway (PPP) is tightly linked to glycolysis as many of the reactions and enzymes of this pathway use or produce several glycolytic intermediates. The PPP can be divided into two subparts: the oxidative and non-oxidative branches. The oxidative PPP transforms glucose-6-phosphate, product of HK, into 6-phosphogluconolactone then 6-phosphogluconate and finally ribulose-5-phosphate. This process reduces two NADP^+ molecules. Ribulose-5-phosphate will then enter the non-oxidative PPP and become ribose-5-phosphate or xylulose-5-phosphate that can then be reshuffled to form either fructose-6-phosphate or G3P, linking the pathway back to glycolysis. Ribose-5-phosphate is also an important building block for DNA and RNA (Figure 15).

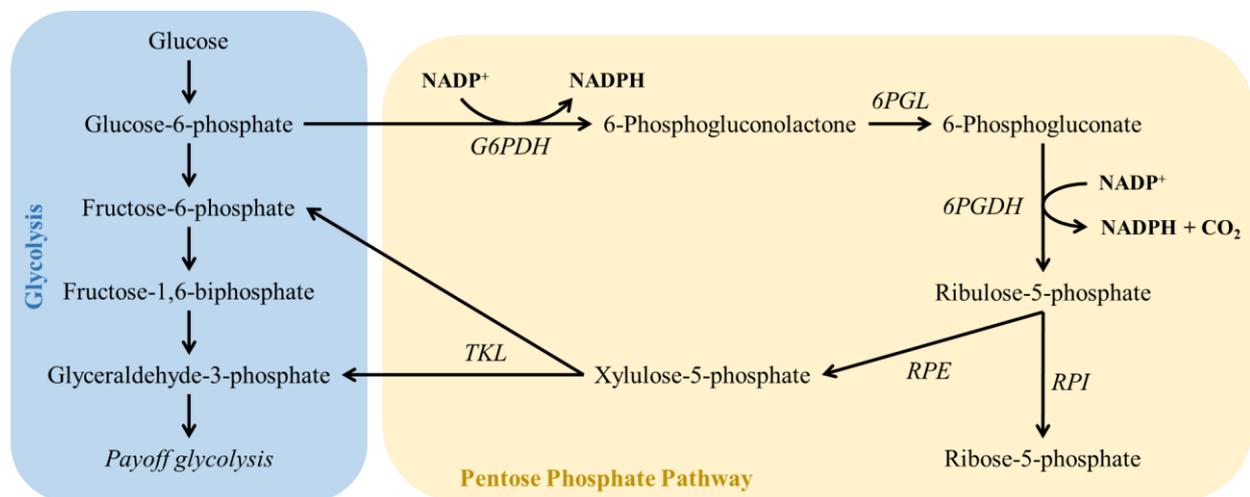


Figure 15: The Pentose Phosphate Pathway. The PPP functions in parallel with glycolysis and uses HK's product, glucose-6-phosphate as its substrate. Two steps catalyzed by glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) enable the reduction of NADP^+ which plays a role in oxidative stress mediation. Ribulose 5-phosphate epimerase (RPE) and transketolase (TKL) are responsible for reshuffling the PPP products back to glycolysis. 6PGL: 6-phosphogluconolactonase.

The PPP is mainly located in the cytosol but some enzymes have been found in the ER as well, possibly to provide NADPH to ER lumen enzymes (Senesi et al., 2010). The roles of the PPP are many, the most studied of which is the antioxidant function of the oxidative PPP through its production of NADPH. Indeed, the reduction of NADPH enables the cell to limit oxidative stress through the thioredoxin/peroxiredoxin and glutathione systems (Pollak et al., 2007). Exposure to H₂O₂ (oxidative stress) increases PPP activity through increased expression and post-translational modifications (Cosentino et al., 2011; Y. P. Wang et al., 2014). In parallel, glycolysis is blocked through inhibition of GAPDH and PK (Anastasiou et al., 2011; Grüning et al., 2011; Ralser et al., 2007). The subsequent accumulation of phosphoenolpyruvate (PEP) also has a negative feedback on TPI (Grüning et al., 2014).

Furthermore, it has been observed that a balanced redox state is essential for cancer cell survival and that this is obtained through increased PPP activity (Anastasiou et al., 2011). Similarly, in the brain, some have suggested that the utilization of lactate allows neurons to produce ATP through mitochondria and thus spares glucose for PPP activity only (Bolaños et al., 2010). This has been linked to the fact that astrocytes, and not neurons, are able to take up and use extracellular cysteine as a glutathione (GSH) precursor. GSH is then transported across to neurons where it enables the cell to deal with oxidative stress through the PPP (Dringen et al., 2000). The PPP is equally important during early brain development where it may play a role in nucleotide synthesis (Magistretti, 2014).

d) Gluconeogenesis

Gluconeogenesis is the pathway used to synthesize glucose. To some extent, it is like the reverse reaction of glycolysis as many enzymes are common to both pathways, simply working in opposite directions. Those that differ are located at the three regulatory steps of glycolysis, HK, PFK and PK. HK is replaced with glucose-6-phosphatase, PFK with fructose-1,6-biphosphatase, and PK with pyruvate carboxylase and PEP carboxykinase, giving this pathway four irreversible steps rather than three, consuming 6 ATP per glucose molecule in the process.

Early studies of gluconeogenesis showed that this metabolic pathway occurs mainly in the cytosol and mitochondria of the liver, kidneys and muscles. Its role in these cell types is to maintain a constant blood glucose level, so that, in case of fasting, organs that rely on glucose as their sole or main energy substrate are not lacking. For example, during the initial hours of

a fast, glycogen breakdown (glycogenolysis) and gluconeogenesis in the liver contribute equally to the body's glucose demands. As the fast prolongs, glycogen stores are quickly depleted and the body gradually recruits renal and muscle cells for additional gluconeogenesis.

Recent data has suggested that gluconeogenesis may also occur in the brain, notably in astrocytes (Bell et al., 1993; Forsyth et al., 1993; Ghosh et al., 2020). Its role in the brain is still fairly unclear but some studies have linked it to various brain pathologies such as hyperglycemia in ischemic stroke (Y. Y. Wang et al., 2013), glioblastomas (Abadi et al., 2014; Beckner et al., 2005) and brain metastatic cancer (Chen et al., 2015).

In humans, many complementary metabolic pathways can provide pyruvate to the gluconeogenic pathway. During intense exercise, the muscle produces lactate through anaerobic glycolysis which is then ejected into the blood stream via monocarboxylate transporter 1 (MCT1). In order to prevent lactate accumulation in the blood (acidosis), it will be taken up by the liver, again via MCT1, converted back into pyruvate by LDH and used to form glucose through gluconeogenesis, which is then shuttled back to fuel the muscle. This process of glucose regeneration is called the Cori cycle, and although energetically demanding (4 ATP per glucose molecule), it is necessary to provide energy to the exercising muscle. Other than exercise, an additional source of lactate for the Cori cycle comes from erythrocytes that do not contain mitochondria.

A similar cycling between muscle and liver cells occurs through the Cahill cycle, or glucose-alanine cycle. This process enables the removal of nitrogenous waste from the muscle that is produced during the breakdown of branched chain amino acids. Alanine is produced when the ammonium group from glutamate is transferred to pyruvate by alanine aminotransferase (AST). Alanine is then transported to the liver where it will be converted back into pyruvate by the same AST enzyme. This will then enter gluconeogenesis to produce glucose which will be transported back to the muscle for further ATP production. The ammonium that is subsequently released into the liver through action of AST will be excreted from the body via the urea cycle.

4. Energy channeling

Channeling refers to the local exchange of intermediates between two or more enzymes in close proximity within a given metabolic pathway. It is a more thermodynamically efficient way to increase the overall rate of certain reactions, whilst limiting diffusion of intermediates

into the bulk solution. Several studies have shown that glycolytic channeling exists and that it is more efficient at delivering energy than the classical bulk cytosolic activity. *In vitro*, it has been demonstrated through co-immunoprecipitation (co-IP) and fluorescence resonance energy transfer (FRET) experiments that GAPDH and PGK interact and form an enzyme-substrate-enzyme complex in the cytosol of living cells (Tomokuni et al., 2010; Weber & Bernhard, 1982). The channeling of nucleotides is also possible in order to regroup energy-producing with energy-consuming enzymes. This has been demonstrated with HK and PK that were immobilized together inside beads and shown to be more effective in producing and depleting ATP compared to enzymes simply dissolved in the bulk medium (Aflalo & DeLuca, 1987).

a) [Glycolytic channeling](#)

(1) *Neurotransmitter recycling*

Glycolytic enzymes are found in several subcellular compartments where they ensure nucleotide channeling for various ATPases and GTPases. During neuronal transmission, synaptic vesicles fuse with the pre-synaptic membrane and eject their neurotransmitter contents into the synaptic cleft to transmit the signal to the post-synaptic cell. Vesicles are then quickly recycled and refilled through the action of a proton ATPase. This pump will create a proton gradient between either side of the vesicular membrane, of which the energy will be harnessed by an antiporter to import neurotransmitters and eject H^+ . It has been demonstrated that several glycolytic enzyme, such as GAPDH, PGK and PK, co-localize with these vesicles and provide the ATP required for neurotransmitter recycling specifically (Ikemoto et al., 2003; Ishida et al., 2009) (Figure 16).

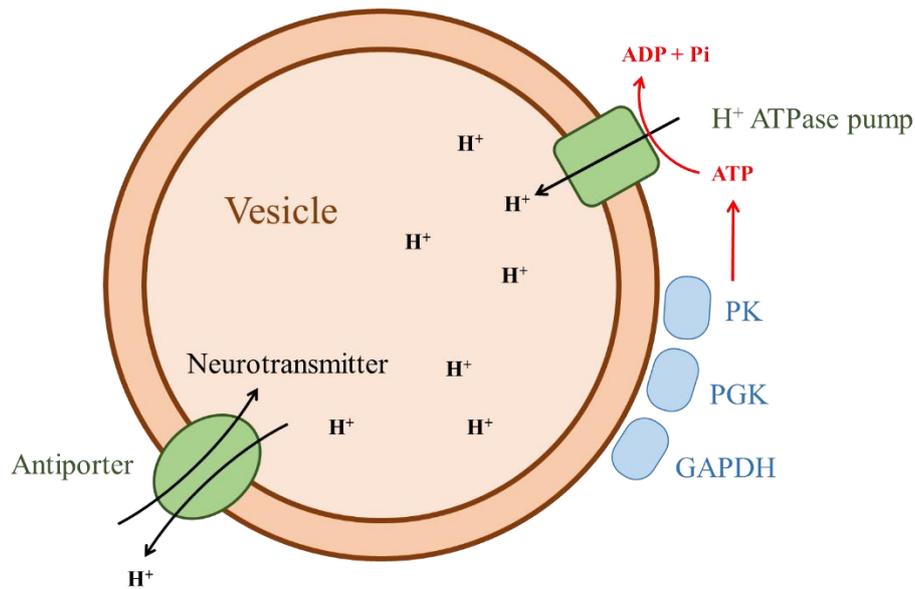


Figure 16: Neurotransmitter recycling fueled by glycolytic enzymes. Glycolytic enzymes bind to the surface of synaptic vesicles to provide ATP to a transmembrane proton pump, creating a proton gradient across the vesicular membrane. The gradient then serves as energy for neurotransmitter- H^+ antiporters.

(2) Vesicular transport

Axonal transport in the brain is also fueled by nucleotide channeling. Similarly to neurotransmitter uptake at the synapse, it has been shown that glycolytic enzymes are sufficient and necessary for providing the vesicle's molecular motors with ATP (Figure 17). This has been shown *in vitro* where purified vesicles, incubated with payoff phase substrates, were able to produce ATP and move along surface-fixed microtubules. Additionally, glycolytic but not mitochondrial inhibition was able to stop vesicular transport in cell cultures, demonstrating the independence of vesicles from mitochondrial ATP production (Hinckelmann et al., 2016; Zala, Hinckelmann, Yu, et al., 2013). It is possible that the same glycolytic mechanism shuttles energy for transport in cilia, given that glycolytic enzymes have been found within these structures that are also devoid of mitochondria (Guzun et al., 2011).

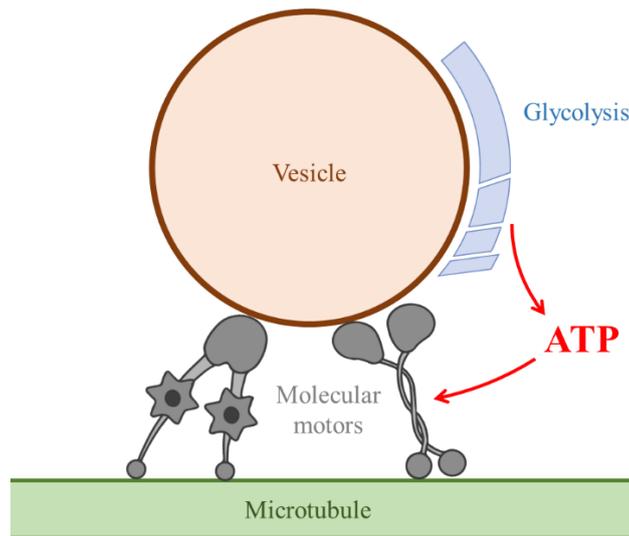


Figure 17: Glycolysis fuels vesicular transport. A membrane-bound glycolytic pathway provides molecular motors with ATP to ensure fast axonal transport of vesicles along microtubules in axons.

(3) *The fibrous sheath in spermatozoa*

Spermatozoa contain a fibrous sheath in their flagellum that is the platform for glycolytic enzymes to provide energy for motility. These germinal cells express specific isoenzymes of several of the glycolytic steps that contain protein domains enabling them to bind to the fibrous sheath, of which the first to be discovered were HK testis-specific (HK1S) and GAPDH testis-specific (GAPDHS) (Mori et al., 1998; Westhoff & Kamp, 1997). Since, sperm-specific aldolase A, LDHA and PK have also been found bound to the sheath surface (Krisfalusi et al., 2006). This has served as inspiration for biotechnological development for others that have shown that HK and GPI were far more catalytically efficient when both enzymes were artificially tethered to a surface compared to their soluble counterparts (Mukai et al., 2009).

(4) *Other examples of glycolytic channeling*

There are many other reports of glycolysis fueling various plasma membrane pumps and ATPases. In red blood cells, in order to compensate for the high osmotic pressure induced by hemoglobin transport and Fe^{2+} content, ions are actively transported between the cytoplasm and extracellular plasma by Na^+/K^+ and Ca^{2+} ATPase pumps (Levin & Korenstein, 1991). Here, similarly to transport, a glycolytic metabolon is bound to the inner face of the plasma membrane and provides the necessary ATP for these ion pumps (Campanella et al., 2005;

Mercer & Dunham, 1981; Puchulu-Campanella et al., 2013). This coupling is ensured by several intermediate proteins, such as ankyrin/ β -spectrin and possibly creatine kinase (CK) (H. Chu et al., 2012; L. Kay et al., 2017).

Other examples include the ATP-sensitive K^+ channel that associate with glycolytic enzymes in cardiac muscle cells (Weiss & Lamp, J Gen Physiol, 1989), and the functional association between glycolysis and Ca^{2+} transport in muscle sarcoplasmic reticulum (Xu et al., 1995).

b) Non-glycolytic channeling

(1) *Creatine kinase in mitochondria*

CK has been extensively studied in muscle tissue where it serves an important role in energy shuttling and buffering. CK catalyzes the transfer of phosphoryl groups between creatine and ATP: phospho-creatine + Mg-ADP \rightarrow creatine + Mg-ATP. The advantage of creatine is that its bond with the phosphoryl group is energetically inert, allowing phospho-creatine to be more diffusible than ATP. It is therefore used as an energy shuttle and alternative store for the cell to regenerate ATP when in need. In mitochondria, CK is found in the intermembrane space, bound to an inner membrane-specific lipid, cardiolipin. This bond relies on electrostatic interactions between the positive C-terminal end of CK and the negatively charged cardiolipin. Moreover, the antiporter that exchanges matrix ATP for inter-membrane space ADP also interacts with cardiolipin, putting it in close proximity with CK (Epanand et al., 2007). Thus, in several tissues, it has been observed that CK channels and transforms the exported ATP into ADP that can then be transferred back into the mitochondrial matrix for oxidative respiration (Barbour et al., 1984; Saks et al., 1985). Cytosolic CK has been found to interact with M line proteins of the sarcomere in muscle cells. Here, the myosin that is bound to the M line requires ATP for muscle cell contraction, which is therefore shuttled by phospho-creatine and CK (Turner et al., 1973; Wallimann et al., 1984). In highly glycolytic cells, CK also regenerates ATP by coming in close proximity with glycolytic enzymes such as PK (Dillon & Clark, 1990; Kraft et al., 2000).

(2) *The Krebs cycle in mitochondria*

In mitochondria, enzymes of the Krebs cycle associate into a supercomplex in order to generate NADH and FADH₂ from Acetyl-CoA more efficiently. It was found that this “metabolon” contained molecules from each of the cycle’s enzymes and was anchored to the inner membrane of mitochondria via the transmembrane enzyme succinate dehydrogenase (Lyubarev & Kurganov, 1989).

5. Enzymatic activity and glycolytic rate measurement

a) Enzyme activity

Enzymes accelerate biological processes with great specificity in their choice of chemical reaction but also of reagents involved, termed substrates. Usually, one enzyme will catalyze only one chemical reaction. This specificity between the enzyme and a substrate relies on the precise set of molecular interactions which is itself a result of the three dimensional structure of the enzyme. Many enzymes require the presence of small molecules called cofactors for their catalytic activity. These are usually small organic molecules (coenzymes), often derived from vitamins, such as NADH for LDH, or metal ions such as Mg²⁺ for HK. The role of enzymes in the body is often to transform with high efficiency one form of energy into another, whether it be via chemical bonds or via transport of molecules across membranes (pumps). The name of an enzyme is often determined based on the substrates and reactions it catalyzes.

During a chemical reaction, the free-energy difference (ΔG) between reactants and products determines the spontaneity of said reaction. If it is positive, the reaction requires energy to occur (endergonic), and if it is negative, the reaction releases energy and thus can take place spontaneously (exergonic) (Figure 18). When this difference is equal to zero, the reaction is considered at equilibrium and no change will occur. Enzymes cannot change the ΔG value, but they can increase the rate at which a reaction takes place. This is enabled by stabilizing and reducing the energy barrier of the transition state, which is a high energy transitional state between the reactants and products (Figure 18). The catalytic power of enzymes therefore lies in their ability to bring substrates together in the correct orientation to

promote the formation of transition states. The domains of enzymes that bind substrates are called active sites.

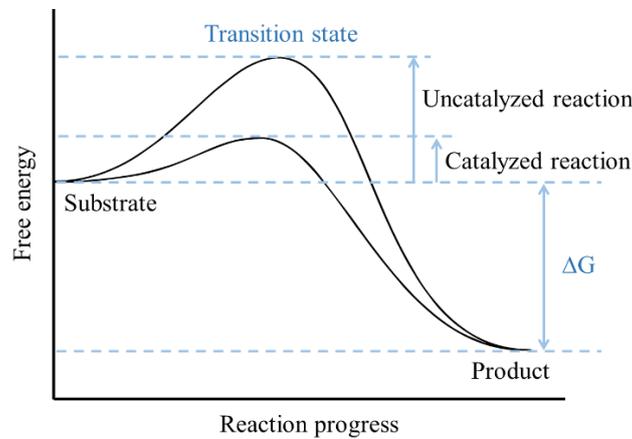


Figure 18: Enzymes decrease the activation energy of chemical reactions. The difference in free energy (ΔG) between substrates and products in this reaction is negative, which means the reaction will occur spontaneously. Enzymes decrease the amount of energy required to reach the transition state, called activation energy. However, they are not able to modify ΔG .

The rate of a most enzymatic reactions is determined by the Michaelis-Menten model. If we consider a reaction that turns a substrate (S) into a product (P), at any given concentration of S, the amount of P will increase with time until gradually leveling off to obtain a plateau. This plateau corresponds to the equilibrium state, as the amount of S turning into P is equal to the amount of P turning back into S. Enzyme kinetics are considered at the highest rate of P formation, which is generally when P is lowest at times close to 0. This rate is called V_0 . As the initial concentration of S increases, V_0 will also increase more or less proportionally until reaching a maximum whereby supplementation of substrate can no longer increase V_0 (Figure 19). Michaelis and Menten obtained the following formula to describe the different parameters of an enzymatic reaction: $V_0 = V_{\max} ([S]/([S] + K_M))$ where K_M is the Michaelis constant and V_{\max} , the maximal rate. K_M and V_{\max} values for a given enzyme can be determined by varying the initial concentration of S and recording V_0 (Figure 19).

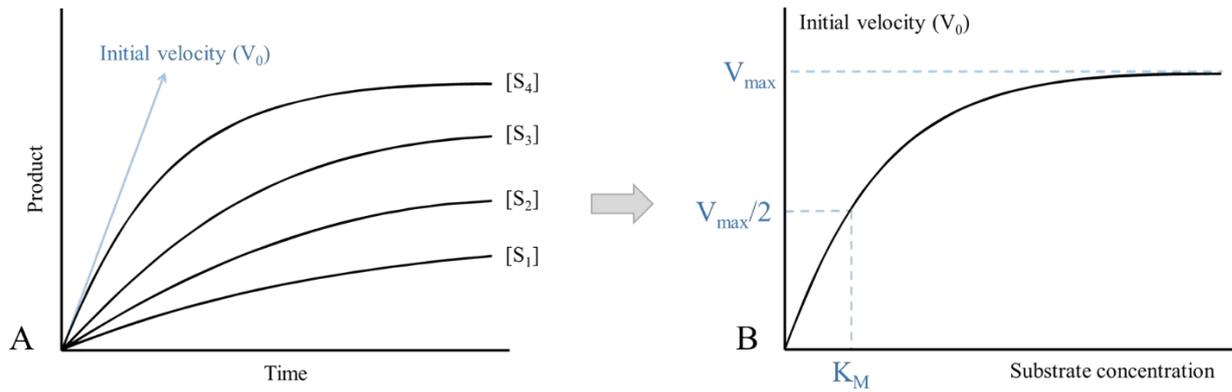


Figure 19: Michaelis-Menten kinetics. A) The amount of product is plotted as a function of time with variable substrate concentrations ($[S_N]$). The initial velocity V_0 therefore corresponds to the slope of the curve at the beginning of the reaction and will increase as the substrate concentration increases. B) Plotting V_0 values as a function of substrate concentration enables the determination of V_{\max} and K_M values.

K_M varies widely depending on the enzyme, the substrate and environmental factors such as pH, temperature and ionic strength. However, it does not depend on enzyme concentration. The K_M value corresponds to the concentration of substrate at which half of the enzymes' active sites are filled. It is also related to the strength of the enzyme-substrate bond: a small K_M value indicates a strong binding whereas a high K_M corresponds to a weak link. V_{\max} is the turnover number of an enzyme, which is the number of substrate molecules converted into product when the enzymes in solution are completely saturated in substrate.

Despite its simplicity and broad applicability, the Michaelis-Menten model cannot predict all enzyme kinetics. These type of enzymes can bind certain substrates or molecules on one active site that can influence the binding of a second substrate to a different active site, which can serve to adjust the overall catalytic activity of the enzyme. As a result, allosteric enzymes present a sigmoidal plot of V_0 versus $[S]$, rather than a hyperbolic one. This additional regulation is the reason for which they are often times found in metabolic pathways.

b) [Measuring glycolytic activity](#)

(1) *Glucose and lactate*

Many commercially available kits measure glycolysis in cells in culture through the quantification of glucose uptake or lactate excretion. The major disadvantage to this approach is that it is an indirect measurement of glucose and lactate concentration in extracellular fluid

and therefore does not take into account other possible fates for these molecules, as is exemplified in the many associated pathways to glycolysis. However, these kits are usually very simple to perform and do not damage cells in culture.

The most common kits use colorimetric or fluorometric detection methods for easy visibility. BioProfile Analyzers (Nova Biomedical) and Biochemistry Analyzers (YFI Life Sciences) detect both glucose and lactate in the extracellular medium, whereas GlucCell only measures glucose. All require that cells be plated at similar densities in case conditions need to be compared. The Seahorse extracellular flux (XF) analyzer (Seahorse Bioscience) is able to measure simultaneous activity of glycolysis and oxidative phosphorylation. Glycolysis is estimated through extracellular acidification caused by HK and lactate excretion (M. Wu et al., 2007). Oxidative respiration is measured through oxygen consumption but can also contribute to extracellular acidification complicating the dissociation between the two measurements.

Another technique involves adding labeled glucose isoforms to the cell culture and tracing its consumption or uptake over time. These can be radioactive or fluorescent analogs. Compared to the methods exposed above, this approach requires ending cell culture at the moment of measurement. The analogs are often based on 2-deoxyglucose (2-DG) which can enter the cell through glucose transporters and be phosphorylated by HK, which traps the analog within the cell. Further metabolism of 2-DG is not possible, meaning this method is more of a way to measure glucose uptake, rather than an estimation of glycolytic flux. What is more, radioactive analogs, used for cancer diagnosis in human patients, require increased safety procedures, rendering this approach more complicated to set up.

(2) *Regulatory glycolytic steps*

As mentioned above, there are three enzymes within glycolysis that dictate overall flux due to the irreversible nature of the reactions they catalyze: HK, PFK and PK. Although the single measurement of one enzymatic step cannot account for modifications occurring elsewhere within the glycolytic pathway, their activities do provide information on the maximal rate possible. Their activities are often measured through the coupling of additional enzymatic steps that convert NAD or NADP cofactors, as the reduced (and not oxidized) forms of these molecules absorb UV light at 340 nm. HK is measured by providing glucose and glucose-6-phosphate dehydrogenase, an PPP enzyme that converts HK's product into glucono-1,5-lactone-6-phosphate, as well as one molecule of NADP⁺ into NADPH. The second enzyme is

provided in excess so that the change in absorbance over time is directly dependent on HK's activity. The same approach is applied to measurement of PFK, only here, several extra glycolytic enzymes are added to reach GAPDH which converts NAD⁺ into NADH. For PK, however, the opposite occurs. PK is combined with LDH that converts NADH into NAD⁺ and so the consumption of NADH and decrease in absorbance are inversely proportional to PK activity.

(3) *Metabolite tracing*

Radioactive carbon atoms can be integrated into any of the six carbons within the glucose molecule. These labeled carbons can then be used to trace all downstream metabolites using mass spectrometry or nuclear magnetic resonance (NMR) spectrometry. This method is sensitive but flux estimation becomes complex because of the unstable nature of certain metabolites. Due to the requirement for liquid or gas chromatography in MS sample preparation, NMR is faster for data acquisition. MS is more sensitive than NMR however. Certain glucose tracers have been optimized as to measure glucose contribution to the TCA cycle or the PPP. Indeed, radioactive glucose that passes through the PPP will lose one radioactive carbon as it reenters glycolysis, compared to glucose that passes through glycolysis only. Glucose labeled with radioactive hydrogen (tritium) can also be used to detect H₂O produced through glycolysis but complete conversion of glucose to pyruvate is not estimated in this case.

To summarize, each of these methods provide more or less information on glycolytic rate and activity, but they do not enable the direct measurement of the entire chain without contamination from associated metabolic pathways. The presented methods either rely on the activity of single enzymatic steps, or on cellular level glucose uptake or consumption. It is therefore difficult to estimate glycolytic activity in a specific area of the cell using the available methods of today.

VI. OUTLINE AND OBJECTIVES

The results presented in this manuscript are split into two parts. The first is presented as an article entitled “A vesicular Warburg effect: Aerobic glycolysis occurs on axonal vesicles for local NAD recycling, vesicular ATP production and axonal transport”, and the second focuses on vesicular glycolysis and transport in HD.

Vesicles have been shown to produce energy via glycolysis bound to their surface (Hinckelmann et al., 2016; Zala, Hinckelmann, Yu, et al., 2013). This occurs independently from mitochondria, which normally ensures important cross talk between glycolysis and oxidative respiration, especially through NAD⁺ recycling. This situation is comparable to hypoxia, whereby insufficient oxygen supply induces a disconnection between glycolysis and mitochondria, which causes glycolysis to turn to LDH for NAD⁺ recycling. This led us to hypothesize that this may be the case for vesicular glycolysis as well. The objectives for this first study were therefore to check LDH localization on vesicles and test the importance of LDH for vesicular glycolysis and transport.

For the second set of results presented in this manuscript, I focused on the link between HD and vesicular glycolysis and transport. As mentioned, Frédéric Saudou’s team has demonstrated that glycolysis is the sufficient and necessary energy source for molecular motors bound to BDNF-containing vesicles in neurons. They have also suggested that HTT is possibly, in part, responsible for glycolytic attachment to vesicles, as a HTT knockdown may lead to a decrease in membrane-bound GAPDH. Equally demonstrated by the team is that HTT mutation is at the root cause of a reduction of BDNF transport in HD. Given this potential link between HTT and glycolysis on vesicles, we hypothesized that mHTT may also impact glycolytic ATP production and thus lead to deficient transport. The objectives were therefore to first understand the manner in which glycolytic enzymes are able to bind vesicular membranes, secondly to characterize vesicular glycolysis in HD, and thirdly to test potential therapeutic strategies to restore BDNF transport through stimulation of this vesicular glycolytic pathway.

VII. RESULTS

1. Part 1: Lactate dehydrogenase and vesicular glycolysis

A vesicular Warburg effect: Aerobic glycolysis occurs on axonal vesicles for local NAD recycling, vesicular ATP production and axonal transport.

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Abstract

Axonal transport of vesicles occurs over long distances and requires constant and local energy supply. The glycolytic machinery is present on vesicles catabolizing glucose into pyruvate and providing ATP for efficient vesicular transport. However, it remains unclear whether pyruvate is transferred to mitochondria from the vesicles as well as how NADH produced by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is recycled in axons. Evaluation of the kinetics of vesicular glycolysis revealed a higher affinity of vesicular glycolytic enzymes for vesicular substrates than the cytosolic machinery, and the requirement of lactate dehydrogenase enzymatic activity for efficient vesicular ATP production. Indeed, inhibition of LDH or the forced degradation of pyruvate inhibited ATP production from axonal vesicles. We found LDH-A rather than the B isoform to be enriched on vesicles suggesting a preferential transformation of pyruvate to lactate on vesicles. Finally, we found that LDH inhibition dramatically reduces axonal transport of BDNF in a reconstituted cortico-striatal circuit on-a-chip. Together, we conclude that aerobic glycolysis is required to supply energy for axonal transport in neurons.

Introduction

In neurons, vesicles are transported at high speed over long distances along the axon's microtubule cytoskeleton. This transport is crucial for inter-cellular communication and signal transduction in the brain. This fast axonal transport (FAT) is enabled by molecular motors, kinesin and dynein mainly, tethered to the surface of vesicles, that consume energy in the form of ATP. We have previously demonstrated that glycolytic enzymes are also bound to vesicular membranes and produce the necessary ATP for transport (Zala et al., 2013). Under normoxia, glycolysis is classically believed to function in combination with the Krebs cycle and oxidative respiration. In the cytosol, glycolysis on vesicles produces NADH, through GAPDH, and pyruvate, through pyruvate kinase, that are taken up by mitochondria and fuel the Krebs cycle with acetyl coenzyme A, as well as donate electrons to the respiratory chain, respectively. This process provides mitochondria with substrate for further ATP production and enables NAD⁺ recycling for glycolysis. Concerning vesicles, it has been demonstrated that inhibition of mitochondria does not hinder vesicular transport (Zala et al., 2013) and that purified vesicles are able to move along fixed microtubules *in vitro*, when provided with glycolytic substrates only (Hinckelmann et al., 2016). This suggests a disconnection between vesicles and mitochondria as regards the metabolism required for their transport, which raises the question of whether pyruvate is truly the final product of vesicular glycolysis, as well as how NAD⁺ is recycled for continual GAPDH and glycolytic activity.

The cell's concentration in NAD is quite low and must be constantly turned over so that metabolic pathways such as glycolysis can continue functioning. When oxygen is scarce or when mitochondria are unavailable, glycolysis is forced to divert its pyruvate production to lactate dehydrogenase A (LDHA), an enzyme that preferentially converts pyruvate into lactate. This process, termed anaerobic glycolysis, is important for the cell because LDH also oxidizes one molecule of NADH into NAD⁺, thus maintaining glycolytic activity. In cancer cells, however, glycolysis and LDH are particularly active despite access to sufficient amounts of oxygen and the presence of mitochondria. This is called aerobic glycolysis or, more commonly, the Warburg effect (Vaupel et al., 2019). Importantly, aerobic glycolysis has been reported also in non-tumor processes (Chen et al., 2018). Given the dissociation between glycolysis on vesicles and mitochondria, we may wonder whether LDH is the missing link for NAD recycling to ensure a Warburg-like effect on vesicles.

Current technics to assess glycolysis are based on indirect measurements of glucose uptake and lactate production, or on the single activity of one of the three rate limiting enzymes of glycolysis, hexokinase, phosphofructokinase and pyruvate kinase (for a review, see (TeSlaa

and Teitell, 2014)). Given the rising interest in energy channeling (Schlattner et al., 2016), specific sub-compartments of the cell, such as the vesicle, may utilize energy in different manners. Thus, whole-cell measurements of glucose and lactate cannot account for slight but important differences in metabolic activity between distinct cellular compartments. Moreover, single enzyme activity of glycolysis may not be influenced by modifications on the remaining seven enzymes, meaning specific measurements of overall glycolytic activity and the potential contribution of LDH to vesicular metabolism are not possible with existing tools on the market.

In this study, we therefore designed a method in which we may be able to measure overall glycolytic activity specifically on vesicles. Using this approach, we investigated the enzymatic properties of vesicular glycolysis and highlighted the end-product of glycolytic machinery on vesicles. Finally, axonal transport was unexpectedly revealed to rely on a Warburg-like effect for energy supply.

Results

Glycolytic enzymes on vesicles have higher affinity for substrate than enzymes in the cytosol. In order to analyze metabolic activity specifically on vesicles, we based ourselves on a previously described subcellular fractionation enabling the separation of small membranes, including vesicles, from the cytosol and other cellular organelles (Hell and Jahn, 2006). Using immunoblot analysis, we then controlled each of the six fractions for various cellular markers (Figure 1A). As expected, we found that the small membrane fraction (P3) was positive for the vesicular marker, dynactin subunit, p150 but not for endoplasmic reticulum protein, calnexin, nor for mitochondria (TOM20) or nuclei (Lamin B1). Tubulin was enriched in the cytosolic fraction but also throughout the remaining fractions due to its variable polymeric size and its ability to bind various organelles, including vesicles.

To characterize glycolytic activity on vesicles, we chose to split the pathway into two segments. Indeed, the need for ATP as a substrate in the preparatory phase prevented the measurement of overall glycolysis through the production of ATP. The rate of the first segment was measured through NADH production starting with hexokinase (HK) and ending with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), providing glucose, ATP and NAD⁺ as substrates. Usually, NADH concentration can be measured at 340 nm, but given the low intensity signal produced by our samples, we decided to take advantage of the GAPDH activity assay kit from Sigma which shifts and amplifies the 340 nm NADH signal to 450 nm. The second segment was assessed with ATP production by phosphoglycerate kinase (PGK) and pyruvate kinase (PK), and covered enzymes GAPDH to PK, that were provided with glyceraldehyde-3-phosphate (G3P), ADP and NAD⁺ as substrates. Here, we used a luciferase enzyme that is able to react with ATP and produce a proportional amount of luminescence.

First we verified that the NADH and ATP signals were proportional to the amount of protein incubated in the reaction mix. For this, variable amounts of P3 protein were incubated with necessary substrates, following which 450 nm-absorbing and luminescent signals, were recorded over time. For both assays, the recorded signal curves were sigmoidal and the highest kinetic rate was found in the initial time points, centered around the inclination point. This zone of the curve was used to estimate initial velocity V_0 , and was found to be proportional to protein concentration (Figure 1B and 1C).

We also verified the nature of the recorded signal, to be certain that it was indeed glycolytic only. Given that GAPDH overlapped both glycolytic segments, we decided to answer this question using the GAPDH inhibitor, iodoacetate. Fixed P3 protein quantities were incubated with increasing amounts of iodoacetate and signals were recorded to obtain V_0

measurements. Both NADH and ATP production was significantly inhibited in presence of iodoacetate, but not with mitochondrial inhibitor oligomycin, meaning the recorded signals were indeed glycolytic in nature (Figure 1D and 1E).

These segmental measurements therefore enable one to assess global glycolytic activity in specific sub-compartments of the cell. Glycolytic enzymes are present both in the cytosol and at the surface of vesicles in neurons and so we wondered whether the vesicular membrane arrangement makes its kinetic properties different from that of the cytosol. We took advantage of our optimized sub-compartment glycolysis test to address this question. Therefore, cytosolic and vesicular fractions were incubated with variable concentrations of substrate, after which V_0 values were measured and plotted as a function of initial substrate concentration (Figure 2A and 2B). As with the Michaelis-Menten model, used for single enzyme kinetics, we obtained hyperbolic-like curves for both cytosolic and vesicular NADH production, from which maximal kinetic rate (V_{max}) and Michaelis constant (K_m) values can be estimated. Concerning segment 2, a hyperbolic curve was obtained for vesicles and a sigmoid curve, similar to what can be observed with allosteric enzymes, was obtained for the cytosol (Figure 2B). This suggest that the second segment of glycolysis is different in cytosolic and vesicular compartments and involves allosteric effectors in the cytosol. In the Michaelis-Menten model, the maximal kinetic rate is dependent on the concentration of enzyme. Here, given the variable distribution of each glycolytic enzyme, and that the protein extracts might not be pure, this value is difficult to interpret. K_m for a single enzyme, corresponds to the concentration of substrate at which V_{max} is halved and is of greater interest, in the case of non-purified extracts, as it is not dependent on enzyme quantity. It is also an indicator of an enzyme's affinity for its substrate, whereby the lower the concentration, the higher the affinity. In the case of a chain of enzymatic reactions such as the glycolytic pathway, we may suppose that the "Km-like" value represents a global affinity of each enzyme for their respective substrates, most likely limited by the enzyme with lowest affinity within the chain. Here, we observed that vesicles required significantly lower amounts of substrate to reach half-maximal kinetic rate compared to cytosolic enzymes (160 μ M glucose compared to 330 μ M in the cytosol for segment 1, 60 μ M G3P compared to 130 μ M in the cytosol for segment 2) (Figure 2A and 2B), suggesting that glycolytic enzymes bound to vesicles have a greater affinity for their substrates compared to their cytosolic counterparts.

Lactate is the end product of vesicular glycolysis. We next investigated what final product was produced by vesicular glycolysis. It has previously been suggested that vesicles produce

pyruvate which would then be used by mitochondria (Hinckelmann et al., 2016). However, given the independence of vesicles from mitochondria *in vitro*, it is possible that vesicles produce lactate through lactate dehydrogenase instead. This would enable vesicles to recycle the redox pool of NAD, a necessary cofactor for GAPDH enzymatic activity (Figure 3A). Therefore, we measured segment 2 activity in presence of a pyruvate trapping agent, hydrazine (Figure 3B). The reasoning was that if vesicular LDH was dissociated from glycolysis, then the end-product for vesicular glycolysis would be pyruvate and its destruction through hydrazine would not hinder overall glycolytic activity. We incubated P3 fractions with increasing concentrations of hydrazine and measured segment 2 ATP production. We observed a significant decrease in glycolytic activity in presence of hydrazine (Figure 3B), suggesting that pyruvate must not be the end-product for glycolysis on vesicles.

This led us to believe that pyruvate may be used by LDH to produce lactate and recycle NAD⁺ for continual glycolytic function. To investigate whether LDH was responsible and necessary for this function, we used a LDH synthetic inhibitor, galloflavin. We first verified galloflavin's ability to inhibit LDH activity on vesicles by incubating increasing amounts of galloflavin and measuring NADH production by LDH. We observed a significant drop in LDH activity in P3 fractions, which was completely abolished at the highest galloflavin concentration used (Figure 3C). We next assessed ATP production through segment 2 in presence of LDH inhibitor. For this, we once again incubated P3 fractions with galloflavin but this time measured segment 2 activity. We observed a gradual decrease in segment 2 kinetics as galloflavin concentration increased (Figure 3D), suggesting that LDH is required for overall glycolytic function, most likely through its ability to recycle NAD⁺ for GAPDH.

Lactate dehydrogenase localizes on axonal vesicles. Since LDH inhibitors block ATP production from a fraction enriched in vesicles, we hypothesized that LDH could be associated to vesicles. We first examined two independent mass spectrometry proteomic analyses of motile vesicular fractions purified from mouse brains expressing p150-GFP (these are fractions that contain small vesicles associated with molecular motors) (Hinckelmann et al., 2016) which uncovered two LDH isoforms, LDH-A and LDH-B (Figure 4A). LDH-A has a preferential affinity for pyruvate, and therefore a higher chance of converting pyruvate into lactate, whereas LDH-B has a greater affinity for lactate and thus preferentially converts lactate into pyruvate (Read et al., 2001). We verified by immunoblot analysis that both isozymes were found in the small membrane fraction (Figure 4B). Intriguingly, the LDH-A:LDH-B ratio was stronger in

vesicles than in cytosol (Figure 4C), which is in alignment with the possibility that vesicles preferentially transform pyruvate into lactate rather than the opposite.

We also examined LDH colocalization with Brain-Derived Neurotrophic Factor (BDNF)-containing vesicles within isolated axons using high resolution Airy scan confocal microscopy. BDNF is endogenously expressed in cortical neurons and transported within dense core vesicles along axons to promote the survival of striatal neurons (Altar et al., 1997; Gauthier et al., 2004). To study LDH localization in axons specifically, we used a recently developed microfluidic-based device that enables the reconstruction of a time- and space-controlled neuronal network compatible with fast spinning confocal video microscopy (Moutaux et al., 2018; Virlogeux et al., 2018) (Figure 4E). The silicon polymer-based system is essentially composed of two isolated neuronal chambers that communicate through multiple aligned microchannels. The dimensions of these channels, combined with specific coating features, ensure the exclusive passage of axons from neurons on one side to create synapses with dendrites protruding from neurons located in the opposite chamber. Primary mouse neuron cultures then enabled us to recreate a functionally relevant cortico-striatal network in which cortical axons project onto striatal dendrites (Figure 4D). Immunofluorescence of pre-permeabilized cells revealed strong co-localization between LDH isoforms and BDNF as shown by the significant increase in Manders' correlation coefficient in axons immunostained for LDH as compared to controls (random) (Figure 4D and 4F). Interestingly and in agreement with subcellular fractionation (Figure 4B and 4C), we found a significant enrichment of LDH-A isoform compared to LDH-B on BDNF vesicles (Figure 4D and 4F). Together our results using proteomic, immunostaining and biochemical approaches indicate that LDH is present on vesicles and that the enrichment in the A isoform as compared to its B isoenzyme suggest that the transformation of pyruvate to lactate is the preferential reaction on axonal vesicles.

LDH activity is required for efficient axonal transport. Given that vesicles require glycolysis to supply their molecular motors with energy, LDH's link with anaerobic glycolysis may also be necessary for vesicular transport. To study the dynamics of vesicles in axons, we once again utilized microfluidic devices enabling the reconstruction of cortico-striatal connections *in vitro* (Figure 4E), following which we transduced cortical neurons with lentivirus enabling the expression of BDNF fused to a fluorescent protein mCherry (BDNF-mCh). At day *in vitro* (DIV) 7 cortical neurons were treated with the same Galloflavin concentrations as previously. We then recorded BDNF transport in cortical axons at various time points following galloflavin treatment. We observed a gradual and significant decrease in

both anterograde and retrograde velocity and vesicle number, as galloflavin concentration increased (Figure 5A, 5B and 5C). In parallel a significant increase in the number of static vesicles was also detected (Figure 5D). All in all, these results are a strong indication that LDH is required for glycolytic activity and transport of axonal vesicles.

Discussion

In this study, we have developed a sensitive and reproducible protocol for measuring glycolysis on vesicles. The advantage of this approach is that it can be applied to a specific sub compartment of the cell and takes into account all ten glycolytic enzymes. Therefore modifications of specific enzymes may be identified more efficiently, thus avoiding redundant and repetitive measurement of individual enzymes. Additionally, one may be able to determine the influence of specific molecules or cofactors on glycolytic activity as a whole, as well as the differences that may arise in pathological situation. In Huntington's disease, for instance, vesicular transport is reduced due to the abnormal expansion of the huntingtin protein. HTT is known to interact with molecular motors but has also been suggested to interact with GAPDH (Burke et al., 1996; Zala et al., 2013), thus we may suppose potential differences in overall glycolytic activity as a result of this interaction. It then becomes possible to pinpoint which enzyme may be responsible by measuring the pathway as a whole initially and working down the glycolytic chain sequentially.

Here, we presented data suggesting that the same glycolytic enzymes found in two different compartments, do not possess the same kinetic properties. The manner in which kinetic data was analyzed was based on Michaelis and Menten's work in enzymology which was theorized around single enzyme reactions, whereas here, the recorded signals encapsulate several enzymatic steps with variable concentrations. Nevertheless, V_0 values plotted according to initial substrate concentration very much resemble the hyperbolic curves that were described for single enzymes, other than cytosolic segment 2. The variable distribution of these enzymes across the different sub compartments of the cell render the comparison of variables dependent on enzyme concentration, such as V_{max} , difficult to interpret. When substrate is limited, however, our analysis becomes independent of enzyme concentration and therefore can be compared. Here we measured the K_M -like concentration for a chain of glycolytic reactions, meaning the overall affinity for substrate is dictated by the enzyme with the weakest affinity of all those involved. Although this value cannot be called K_M per se, we did observe that it was lower on vesicles than in the cytosol, for both tested segments. This increased affinity for substrate on vesicles may be due in part to the molecular orientation of said enzymes on membranes, possibly resulting in more efficient channeling of metabolites from one enzyme to the next.

Transport of organelles is widespread throughout all cell types, but seems to be of particular importance in neurons as they are the only cell-type able to span such large distances (Guedes-Dias and Holzbaur, 2019; Hinckelmann et al., 2013). Indeed the axon is host to high

velocity transport of various organelles, such as synaptic and dense-core vesicles, endosomes and lysosomes (Guedes-Dias and Holzbaur, 2019). The energy requirements for this process are dissociated from mitochondria, which produce the largest quantities of ATP for the cell, but localize at specific sections of the axon, such as the nodes of Ranvier or the axon terminal (Cunnane et al., 2020). Glycolysis provides molecular motors with ATP (Hinckelmann et al., 2016; Zala et al., 2013). Here we have demonstrated that lactic fermentation enables necessary NAD recycling for this glycolytic pathway, and is further proof of vesicular independence from mitochondria in neurons. It is also an argument against the idea that neurons express LDHB and not LDHA, in order to convert imported lactate into pyruvate to fuel the Krebs cycle in mitochondria (Lovatt et al., 2007; Magistretti and Allaman, 2015, 2018). Vesicles therefore produce ATP through aerobic glycolysis, similarly to the Warburg effect in cancer cells (Vaupel et al., 2019). In these cells, LDHA is upregulated and ensures fast glycolytic activity to meet the high proliferation demands of tumors and has been a candidate for therapeutic targeting in certain cancer treatments (Dong et al., 2017; Xie et al., 2009). It is therefore questionable how vesicular transport may be affected in cancer cells and whether overall increased LDH activity in the cell also affects vesicular LDH as well.

Lactate is an important molecule in the brain as it is believed to be the primary energy substrate in neurons, according to the astrocyte-neuron lactate shuttle model (Magistretti and Allaman, 2018; Saab et al., 2013). What therefore becomes of lactate produced by vesicles? Can it be released into the cytosol to become fuel for mitochondria? The monocarboxylate transporter 1 is also expressed on motile vesicles (Hinckelmann et al., 2016), which means lactate could possibly be shuttled into the vesicular lumen and secreted at the synapse, where energy is in high demand.

Methods

Mice. Males and female mice were maintained with access to food and water *ad libitum* and kept at a constant temperature (19-22 °C) and humidity (40–50%) on a 12:12 hours light/dark cycle. All experimental procedures were performed in an authorized establishment (Grenoble Institut Neurosciences, INSERM U1216, license #B3851610008) in strict accordance with the directive of the European Community (63/2010/EU). Project was approved by the French Ethical Committee (Authorization number: APAFIS#18126-2018103018299125 v2) for care and use of laboratory animals and performed under the supervision of authorized investigators.

Subcellular fractionation. Vesicle purification was based on a previously established protocol (Hell and Jahn, 2006). 2 month old male mice were euthanized by decapitation, their brains were removed and immediately frozen in liquid nitrogen. Brains were then grinded down to a thin powder with a ceramic mortar bathed in liquid nitrogen to avoid thawing. This powder was then transferred to 1 mL of homogenization buffer (320 mM sucrose) and further broken down and homogenized with a loose then a tight potter, this formed the Total fraction (T). The mix was centrifuged for 10 min at 47,000 x g at 4°C (Beckmann TLA 100.3 rotor). The supernatant was set to one side while the pellet was re-suspended with 300 µL of homogenization buffer and centrifuged once again for 10 min at 47,000 x g at 4°C. The pellet was re-suspended with homogenization buffer forming the P1 fraction. The supernatant was combined with that of the first centrifugation, forming the S1 fraction, and centrifuged for 40 min at 120,000 x g at 4°C. The pellet was re-suspended with homogenization buffer and corresponds to the P2 fraction. The supernatant was transferred onto 280 µl of a one layer sucrose cushion (700 mM sucrose, 10 mM HEPES) and centrifuged one final time for 2 hours at 260,000 x g at 4°C. The supernatant forms the S3 fraction and the pellet, re-suspended with Resuspension buffer (320 mM sucrose, 10 mM HEPES), corresponds to the P3 fraction containing the purified vesicles. All volumes used were valid for the fractionation of 1 adult mouse brain (mean mass 400 mg). If more or less brain material was used, volumes were adjusted accordingly.

Glycolytic activity measurements. Glycolytic activity was measured through NADH (segment 1) and ATP (segment 2) production. For both activity tests, frozen P3 was thawed just before the experiment and incubated with substrates in 96 well plates. Once thawed, P3 fraction could only be used once, as enzymatic activity was degraded if frozen and thawed repeatedly. For segment 1, for most tests, 25 µg P3 was incubated with glucose 10 to 500 µM,

NAD⁺ 1 mM, arsenate 1.1 mM, sodium phosphate 1 mM, Mg-ATP 6 μM, and Developer obtained from GAPDH Activity Assay from Sigma-Aldrich (2 μL per well), diluted in HEPES buffer pH 7.4 for which the final volume per well was 100 μL. Absorbance at 450 nm was then recorded every minute for 3 hours at 37°C using a PheraStar plate reader. For segment 2, for most tests, 0.2 μg P3 was incubated with glyceraldehyde-3-phosphate 10 to 500 μM, NAD⁺ 1 mM, sodium phosphate 1 mM, and ADP 100 μM, diluted in buffer containing HEPES, DTT, sucrose and MgCl₂. The final volume was 50 μL and was mixed with 50 μL of Cell Titer Glo which contained luciferase enzyme and substrate. Luminescence was then recorded every minute for 2 hours at 37°C using a PheraStar plate reader. For segment 2 inhibition tests, variable concentrations of hydrazine (10 to 300 μM) and Galloflavin (10 to 500 μM) or DMSO were added to the reaction mix a few seconds prior to recording. Recordings were then plotted as luminescent or 450 nm signals over time and fitted with curve models using Graphpad Prism 7 to determine kinetic V₀ slopes.

LDH activity. 1 μg of fresh P3 fraction was incubated with 5 mM lactate and 150 μM NAD⁺, along with 2 μL of Developer from the GAPDH Activity Assay from Sigma-Aldrich. The appearance of NADH was then recorded every minute at 450 nm for 1 hour. The addition of Developer was necessary so that we may shift the recorded signal from 340 to 450 nm as Galloflavin is known to interfere with NADH's UV signal (Manerba et al., 2012). For LDH inhibition tests, variable concentrations of Galloflavin (10 to 500 μM) or its solvent DMSO were added to the reaction mix a few seconds prior to recording. Recordings then were plotted as 450 nm signals over time and resulting curves were fitted with hyperbolic or sigmoidal models using Graphpad Prism 7.

Primary neuronal culture in microfluidic devices. Primary cortical and striatal neurons were prepared as previously described (Liot et al., 2013). Briefly, cortex and ganglionic eminences were dissected from E15.5 wild-type and HdhCAG140/+ mouse embryos, then digested with a papain and cysteine solution followed by two incubations with trypsin inhibitor solutions, and finally dissociated mechanically. Dissociated cortical and striatal neurons were re-suspended in growing medium (Neurobasal A medium supplemented with 2% B27, 2 mM Glutamax, 5 mM glucose, and 1% penicillin/streptomycin) (5×10^6 cells) and plated in the chamber with a final density of ~7000 cells/mm². Cortical neurons were plated first on the upper chamber followed by addition of growing medium in the synaptic chamber. Striatal

neurons were then added in the lower chamber. Neurons were left in the incubator for at least 3 hours, then all compartments were gently filled with growing medium.

Microchamber preparation. Fabrication of the polydimethylsiloxane microfluidic devices was adapted from Virlogeux et al. (Virlogeux et al., 2018). Briefly a PDMS motif served as a negative mold to form epoxy resin microchambers outlining three chambers connected to one another by microchannels. The upper and lower chambers contained cell bodies. Axons and dendrites connect the two seeding chambers through the microchannels to form synapses in the middle synaptic chamber. The molds were cleaned, treated and placed on glass-bottom Petri dishes (FluoroDish, WPI). They were then coated with poly-D-lysine (0.1 mg/ml) in the upper and synaptic chambers, and with a mix of poly-D-lysine (0.1 mg/ml) + laminin (10 µg/ml) in the lower chamber overnight at 4°C. Microchambers were washed 3 times with growing medium (Neurobasal A medium supplemented with 2% B27, 2 mM Glutamax, 5 mM glucose, and 1% penicillin/streptomycin) and placed at 37°C before neurons were plated.

Live-cell and confocal imaging of BDNF trafficking. Live-cell recordings were performed using an inverted microscope (Axio Observer, Zeiss) coupled to a spinning-disk confocal system (CSU-W1-T3, Yokogawa) connected to wide field electron-multiplying CCD camera (ProEM+1024, Princeton Instrument) and maintained at 37 °C and 5% CO₂. Images were taken every 200 ms for 30 s for BDNF-mCh (×63 oil-immersion objective, 1.46 NA) trafficking.

Quantifications and image analyses. To study vesicular transport, kymographs were generated using KymoToolBox plugin for ImageJ (Zala, 2013) with a length of 100 µm (x-axis) and a total time of 30 s (y-axis) to extract the following kinetics parameters : anterograde and retrograde velocity, number of anterograde, retrograde and pausing vesicles per 100 µm, linear flow rate and net flux. Vesicles were considered motile when their velocity was above 0.12 µm/s. Each condition was tested using 6 chambers from 3 independent cultures. In each chamber, 5 fields were analyzed to reach a minimum number of 60 axons (n = number of axons). For LDH colocalization with BDNF-mCherry for immunofluorescence in microchambers, M1 and M2 Mander's coefficients were estimated with the JACoP plugin on ImageJ, following which random and true colocalization values were calculated, normalized for pixel size and area.

Western blot analysis. Protein concentration was assessed using a BCA kit (Pierce). Proteins (20 µg) were denatured at 95°C for 5 min in loading buffer. 6, 8, 10 and 12% acrylamide gels were loaded. Proteins were transferred onto PVDF membranes and blocked in 5% BSA in TBS buffer, 0.1% Tween. Primary antibodies and secondary antibodies were incubated for 1.5 hours at room temperature. Images of the western blotting experiments have been cropped for presentation.

Lentivirus, antibodies, chemicals. ATP, ADP, NADH, GAP, iodoacetate and oligomycin are from Sigma-Aldrich. The following antibodies and dilutions were used for western blotting: rabbit anti-LDH-A (19987-1-AP, Proteintech, 1:2000), rabbit anti-LDH-B (14824-1-AP, Proteintech, 1:2000), mouse anti-HTT (4C8, Euromedex, 1:1000), mouse anti-p150 (610474, BD transduction Laboratories, 1:4000), mouse anti-calnexin (C4731, Sigma, 1:1000), mouse anti-tubulin (T9026, Sigma, 1:5000), rabbit anti-TOM20 (sc17764, Santa Cruz, ref, 1:1000), rabbit anti-Lamin B1 (ab133741, Abcam, 1:2000). The following antibodies and dilutions were used for immunofluorescence: rabbit anti-LDH-A (19987-1-AP, Proteintech, 1:500), rabbit anti-LDH-B (14824-1-AP, Proteintech, 1:500), mouse anti-mCherry (ab125096, Abcam, 1:500). Mg-ATP, ADP, NAD, G6P, F1,6P, F6P, G3P and PEP are from Sigma-Aldrich, sodium phosphate and glucose are from ??, hydrazine is from Sigma (207942) and Galloflavin is from Abcam (ab141776).

Immunofluorescence. Cortical neurons were washed three times in PBS, permeabilized with 0.05% saponin in PBS for 30 seconds, and fixed with paraformaldehyde 4% in PBS at 37°C for 20 min. Cells were then treated with 1% NGS and 0.1% Triton X-100 for 1 hour and stained with primary antibodies overnight, followed by secondary antibodies (Invitrogen Alexa 488 and Alexa 647, 1:1000) for 1 hour at room temperature.

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Figure legends

Figure 1. Glycolytic activity on vesicles can be measured through NADH and ATP production. (A) Immunoblot of various cellular markers in each fraction (p150, vesicles; calnexin, endoplasmic reticulum; TOM20, mitochondria; tubulin, enriched in cytosol; lamin B1, nuclei) obtained from the fractionation of wild type adult male mouse brain extract (Representative western blot of three independent experiments) (B) Initial enzymatic rate (V_0) in vesicular P3 fractions of recorded absorbance values at 450 nm per minute corresponding to NADH production through segment 1 of glycolysis as a function of protein quantity in the reaction mix ($n = 3$, error bars represent SEM). (C) Initial enzymatic rate (V_0) in vesicular P3 fractions of recorded luminescence per minute corresponding to ATP production through segment 2 of glycolysis as a function of protein quantity in the reaction mix ($n = 3$, error bars represent SEM). Segment 1 (D) and segment 2 (E) activity in vesicular P3 fractions incubated with increasing concentrations of iodoacetate (orange) as well as oligomycin (blue); for both segments V_0 is represented as a percentage of control activity ($n = 3$, each condition was statistically compared to control activity with one-way ANOVA followed by Tukey post hoc test, $*p < 0.1$, $****p < 0.0001$, ns = non-significant, error bars represent SEM).

Figure 2. Glycolytic enzymes on vesicles are more efficient than those in the cytosol. (A) Graphical representation of segment 1 activity as a function of HK substrate, glucose concentration in μM . (B) Graphical representation of segment 2 activity as a function of GAPDH substrate, glyceraldehyde-3-phosphate (G3P) concentration in μM (Hyperbolic and sigmoidal fitted curves were obtained with GraphPad Prism 7, data are means of three independent experiments, error bars represent SEM).

Figure 3. LDH is necessary for glycolytic activity on brain vesicles. (A) Schematic representation of interplay between GAPDH within the glycolytic pathway and LDH for NAD recycling. (B) Segment 2 V_0 values in vesicular P3 fraction purified from mouse brain and incubated with increasing concentrations of pyruvate trapping agent, hydrazine. (C) Initial enzymatic rate (V_0) of recorded absorbance values at 450 nm per minute corresponding to the production of NADH through LDH in vesicular P3 fractions incubated with increasing concentrations of synthetic LDH inhibitor, galloflavin (GF). (D) Segment 2 V_0 values in vesicular P3 fraction incubated with increasing concentrations of GF. For all graphs, data are mean of three independent experiments, error bars represent SEM. V_0 values are represented

as a percentage of control activity in grey, each treated condition in orange was statistically compared to control activity with one-way ANOVA followed by Tukey post hoc test, * $p < 0.1$, *** $p < 0.001$, **** $p < 0.0001$).

Figure 4. LDHA colocalizes with vesicles in neurons. **(A)** Motile vesicular fraction from Hincklemann et al., 2016 where identified proteins were ranked by intensity and plotted according to their relative abundance (grey spots), revealing LDHA and LDHB. **(B)** Immunoblot of LDH isoforms found in total (T), cytosolic (S3) and vesicular (P3) fractions from male adult mouse brains. **(C)** Quantification of intensity (I) ratio of LDHA to LDHB in cytosolic and vesicular fractions shown in C ($n = 3$). Conditions were statistically compared with unpaired two-tailed Student t-tests, * $p < 0.1$). **(D)** Representative immunofluorescence images in cortical axons plated in microchambers, stained for LDHA and BDNF-mCherry (left), or LDHB and BDNF-mCherry (right), white arrows indicate colocalizing fluorescence. **(E)** Schematic representation of microfluidic devices (left) composed of cortical and striatal chambers for cell bodies, a synaptic chamber between them where cortico-striatal synapses form, and microchannels linking these chambers and allowing passage of cortical axons on one side and striatal dendrites on the other. The zoom on the right shows where immunofluorescence experiments were performed to study axonal LDH. **(F)** Mander's correlation coefficient of LDHA and LDHB colocalization with BDNF-mCherry, as well as random colocalization Data are mean of two independent experiments, error bars represent SEM. Conditions were statistically compared with one-way ANOVA followed by Tukey post hoc test, **** $p < 0.0001$.

Figure 5. LDH is required for BDNF transport *in vitro*. **(A)** Anterograde and retrograde mean velocities, as well as the number of anterograde **(B)**, retrograde **(C)** and paused **(D)** BDNF-mCherry vesicles per 100 μm in cortical axons plated in microfluidic devices and treated with 500 μM galloflavin for 180 min (orange). For all bar graphs, treated values were compared to control values (DMSO) (grey) using unpaired two-tailed Student t-tests ($n = 15$ to 20 axons from one experiment, *** $p < 0.001$, **** $p < 0.0001$).

Figure 1

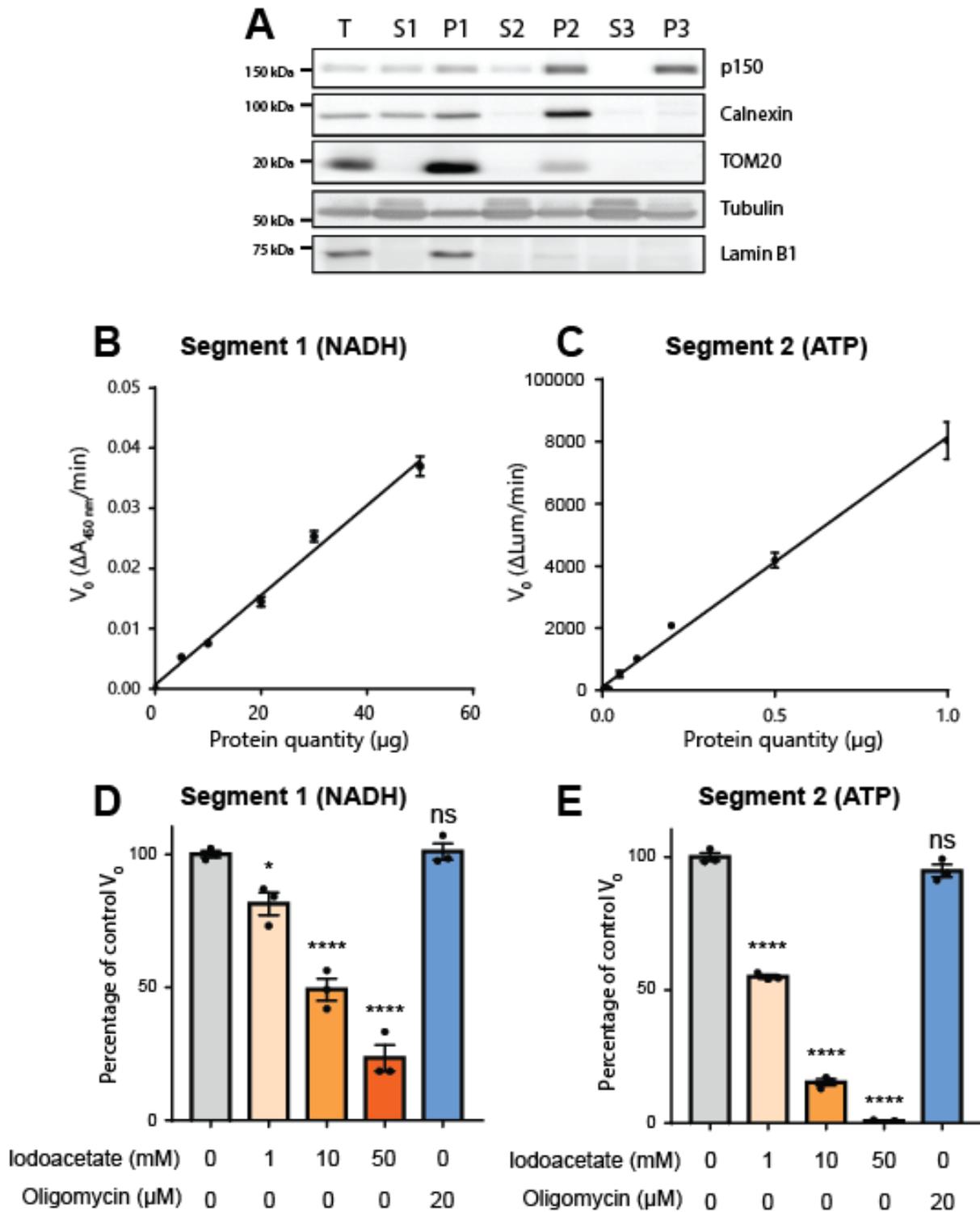


Figure 2

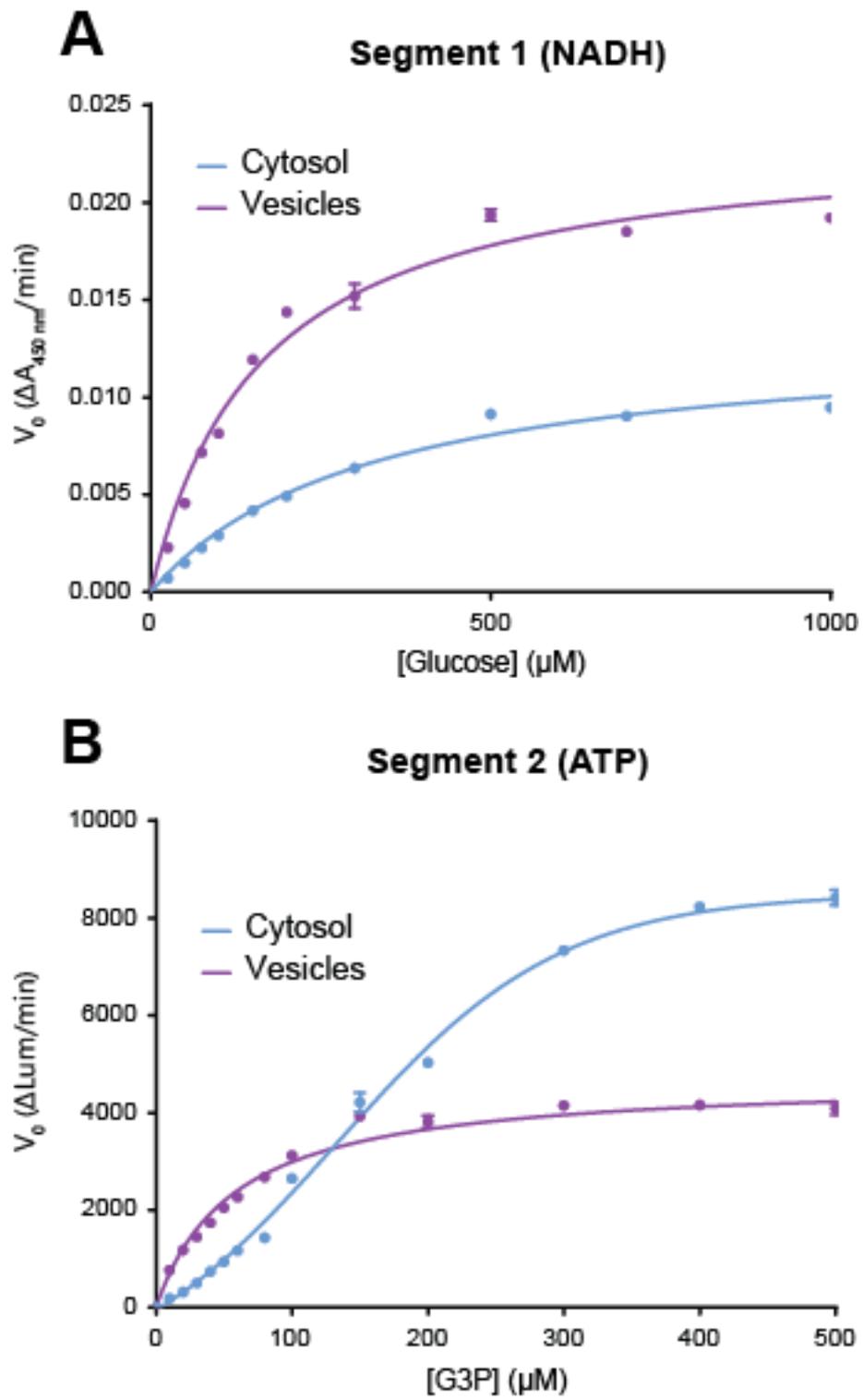


Figure 3

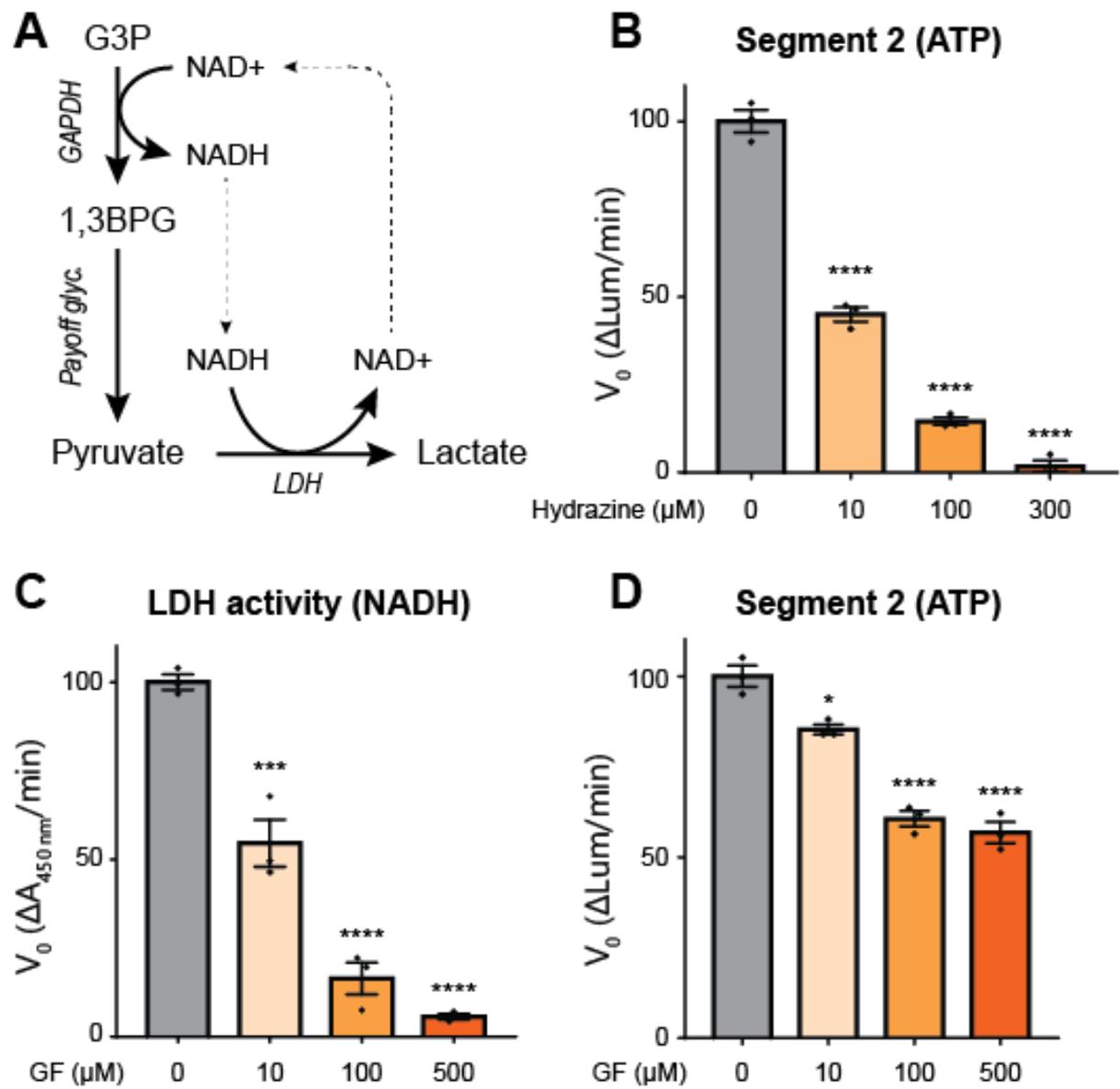


Figure 4

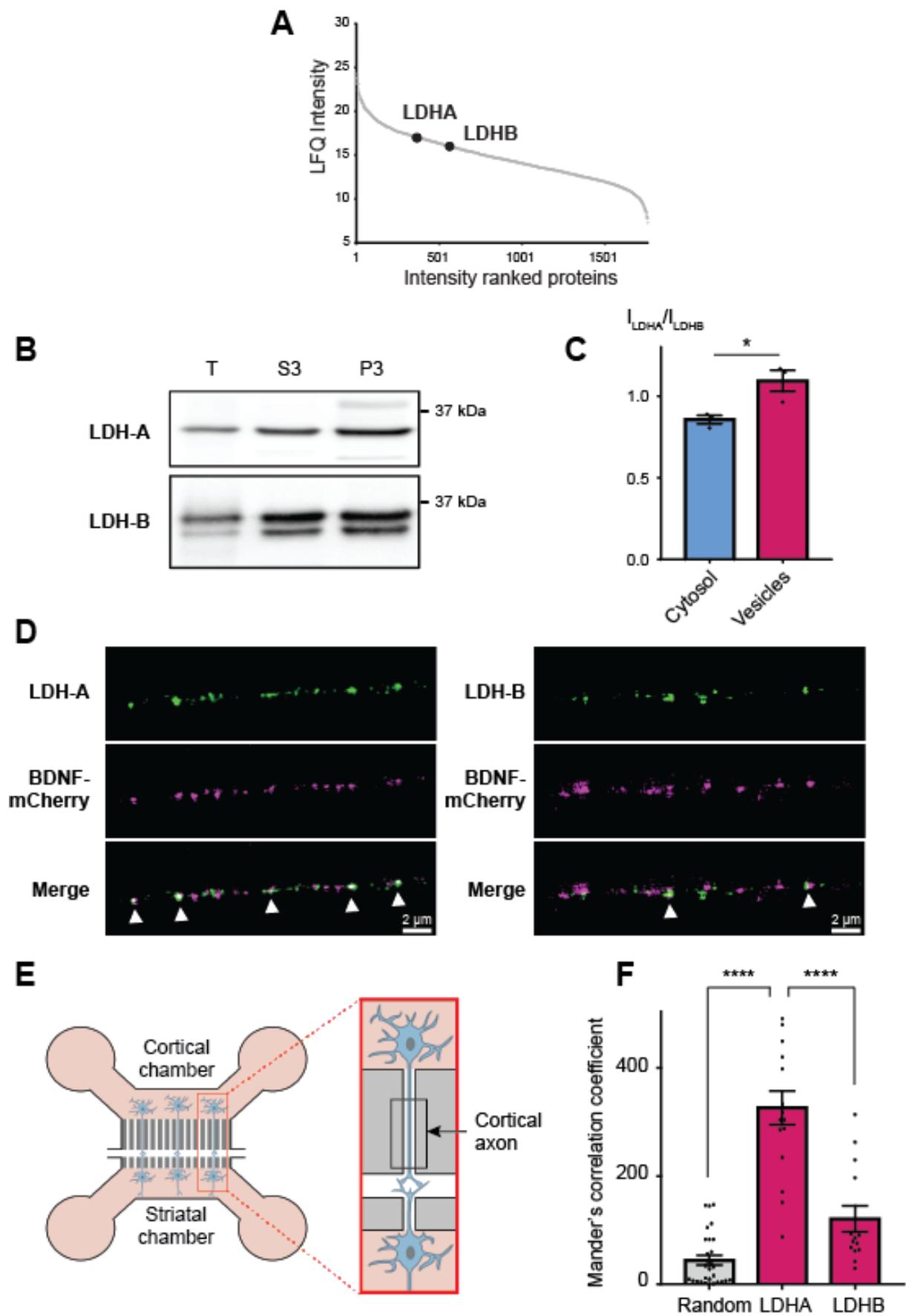
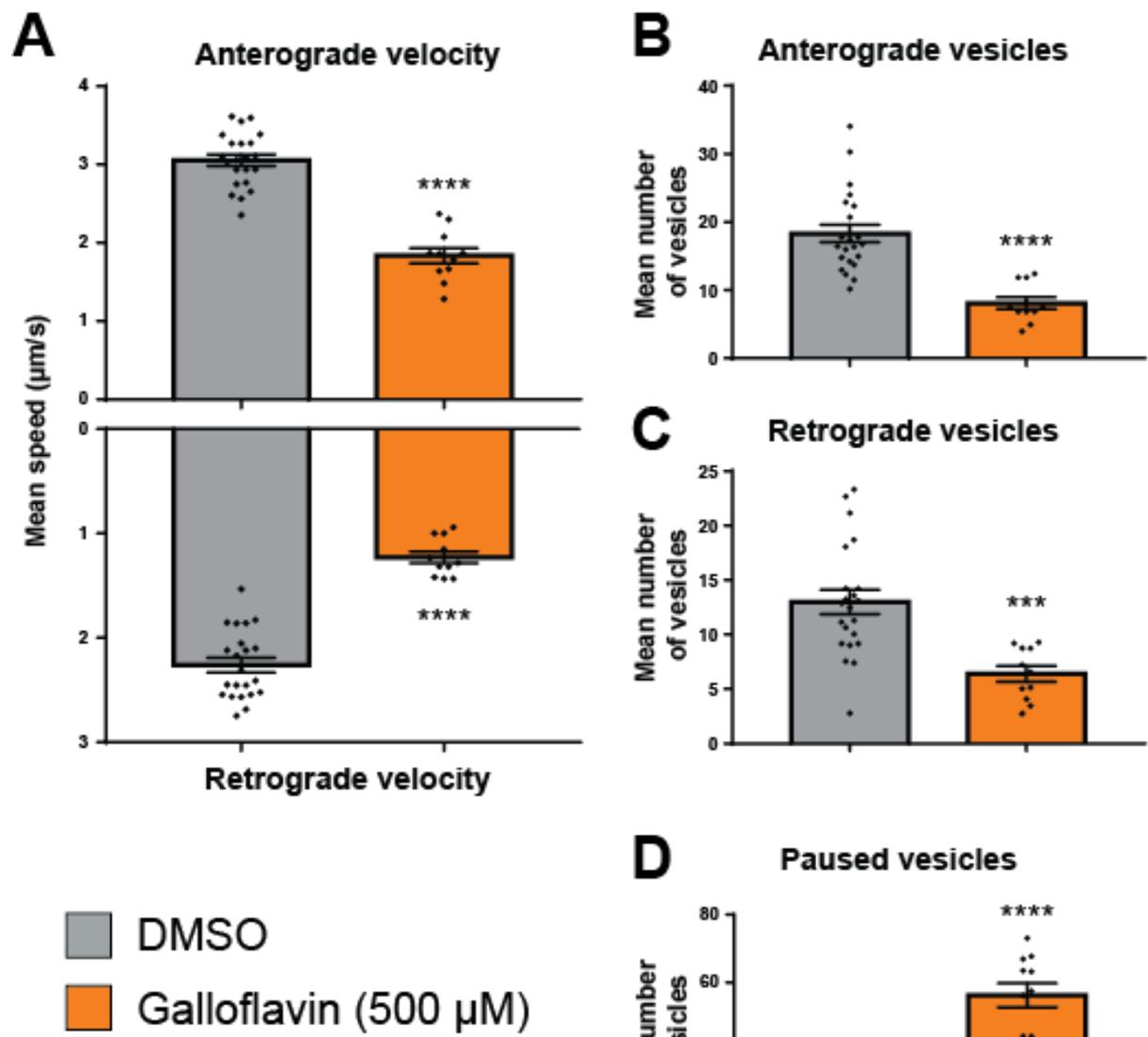


Figure 5



2. [Part 2: Vesicular glycolysis in Huntington's disease](#)

a) [How do glycolytic enzymes bind to vesicular membranes?](#)

Does Huntingtin interact with glycolytic enzymes on vesicles? It has been demonstrated that glycolytic enzymes are bound to vesicles in neurons and provide their molecular motors with ATP for fast axonal transport (Zala, Hinckelmann, Yu, et al., 2013). The manner in which these enzymes bind to vesicles remains unknown. We were therefore interested in understanding how glycolytic enzymes bind vesicular membranes. Some clues for this have been provided by the literature whereby HTT knock-down in neurons reduced the amount of a glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) on vesicles (Zala, Hinckelmann, Yu, et al., 2013). I therefore tested if HTT was interacting with GAPDH in subcellular fractions. I first chose to immunoprecipitate GAPDH from both cytosolic and vesicular fractions and verify co-immunoprecipitating proteins. Although I did not find any other glycolytic enzymes in the immunoprecipitate, HTT was indeed pulled down with GAPDH (Figure R1A). Strikingly, however, this interaction between GAPDH and HTT was much stronger in the vesicular fraction compared to the cytosol (Figure R1A and R1B). Therefore, given that HTT knock-down partially releases GAPDH from vesicles, it is possible that HTT may serve as a scaffolding protein for GAPDH on vesicular membranes. We were therefore interested in identifying other glycolytic partners of HTT in this fraction. To assess this, we performed an immunoprecipitation of HTT from cytosolic and vesicular fractions and revealed candidate interactors using Western blot. Unfortunately, GAPDH, HK1, PGAM1/4, PK, enolase 1, PGK-1 and aldolase C did not co-immunoprecipitate, however, PFK did (Figure R1C). Moreover, this interaction, similarly to GAPDH, was stronger in the vesicular fraction (Figure R1C and R1D). Together these results indicate a preferential interaction between HTT and glycolytic enzymes when specifically located on vesicles.

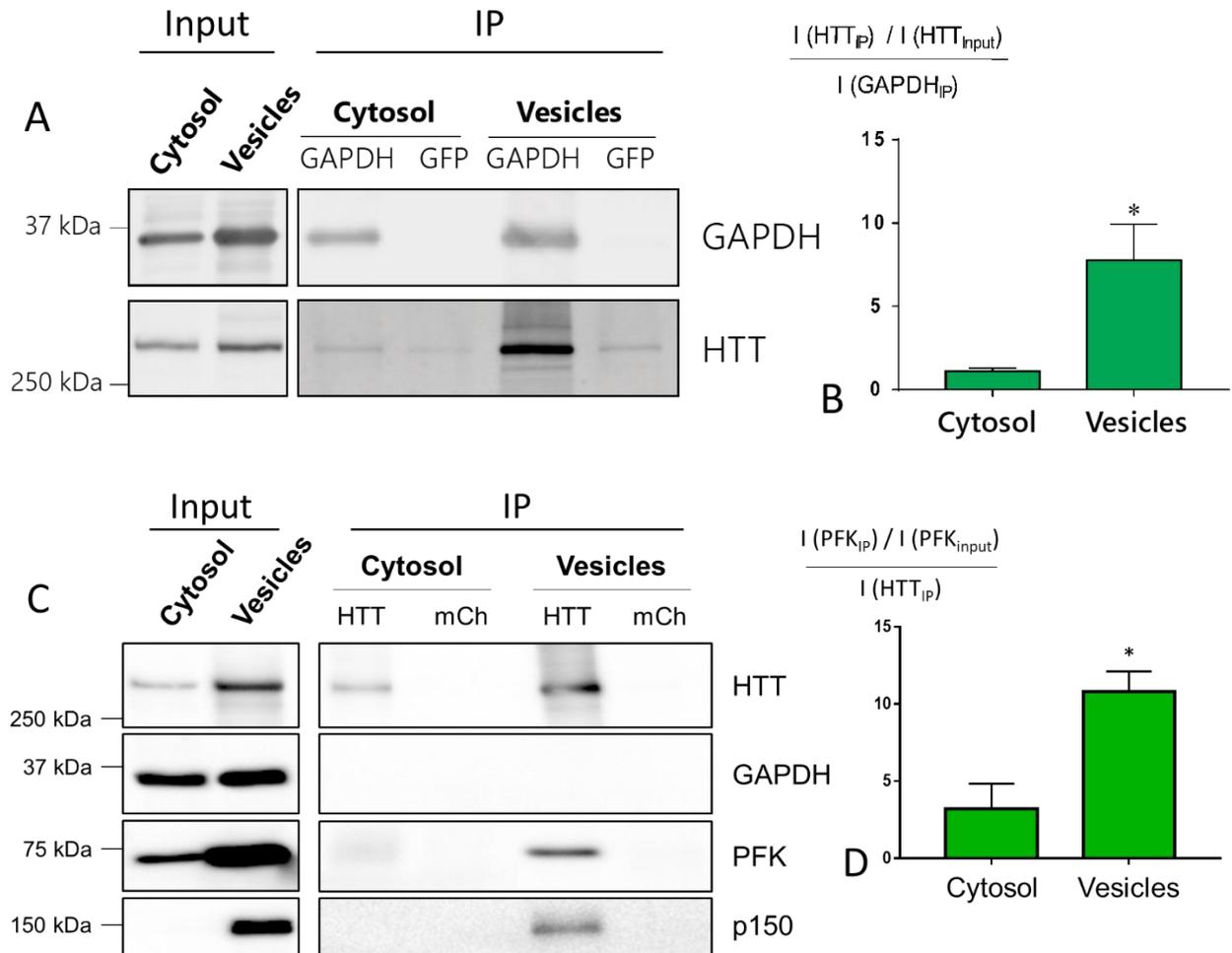


Figure R1: HTT interacts with GAPDH and PFK on vesicles. A) Immunoblot of GAPDH and HTT in cytosolic and vesicular fractions from wild-type male mouse brain following GAPDH immunoprecipitation. Lanes containing anti-GFP were used as negative controls. B) Quantification of band intensity (I) in immunoblot of HTT normalized by the amount of endogenous HTT in the input lanes and by the amount of GAPDH immunoprecipitated in each fraction (N = 3 independent experiments, unpaired t-test, *p<0.1, error bars represent SEM). C) Immunoblot of HTT, GAPDH, PFK and p150 in cytosolic and vesicular fractions from wild-type male mouse brain following GAPDH immunoprecipitation. P150 was used as a positive control for vesicular immunoprecipitation of HTT. Lanes containing anti-mCherry (mCh) were used as negative controls. D) Quantification of band intensity (I) in immunoblot of PFK normalized by the amount of endogenous PFK in the input lanes and by the amount of HTT immunoprecipitated in each fraction (N = 3 independent experiments, unpaired t-test, *p<0.1, error bars represent SEM).

Are glycolytic enzymes lipidated at the surface of vesicles? To further study enzyme attachment to membrane we chose to look at potential post-translational modifications that may lead to enzyme targeting to vesicles. For example, the post-translational addition of a

hydrophobic lipid tail enables soluble proteins to be targeted to membranes in order to interact with and bind the lipid bilayer. To identify post-translational modifications and, particularly, lipidation on glycolytic enzymes, we used the results from previously performed mass spectrometry tests on purified vesicles (Hinckelmann et al., 2016). We therefore searched for lipid additions (myristoylation, prenylation, palmitoylation, esterification) on all ten glycolytic enzymes which revealed a palmitoyl group on pyruvate kinase at cysteine 49. To test the importance of this palmitoylation for pyruvate kinase attachment to vesicles, I transfected cortical mouse neurons with theoretically non-palmitoylatable PKM1 C49S fused to a Myc tag. We chose to use PKM1 as this isoform is expressed in the brain. Following maturation, cells were then frozen and fractionated following the same protocol as for mouse brains. I then checked Myc-PKM1 localization through immunoblot analysis, but, despite the targeting of our constructs to vesicles, we did not find any difference in band intensity in P3 fractions isolated from cells transfected with Myc-PKM1 C49S compared to control (Figure R2A and R2B). These results suggest that an expected palmitoylation inhibition through nucleotide mutation is not sufficient to detach pyruvate kinase from vesicles, and may not be solely responsible for this enzyme's attachment to vesicular membranes. Following these preliminary results, we chose not to pursue this hypothesis.

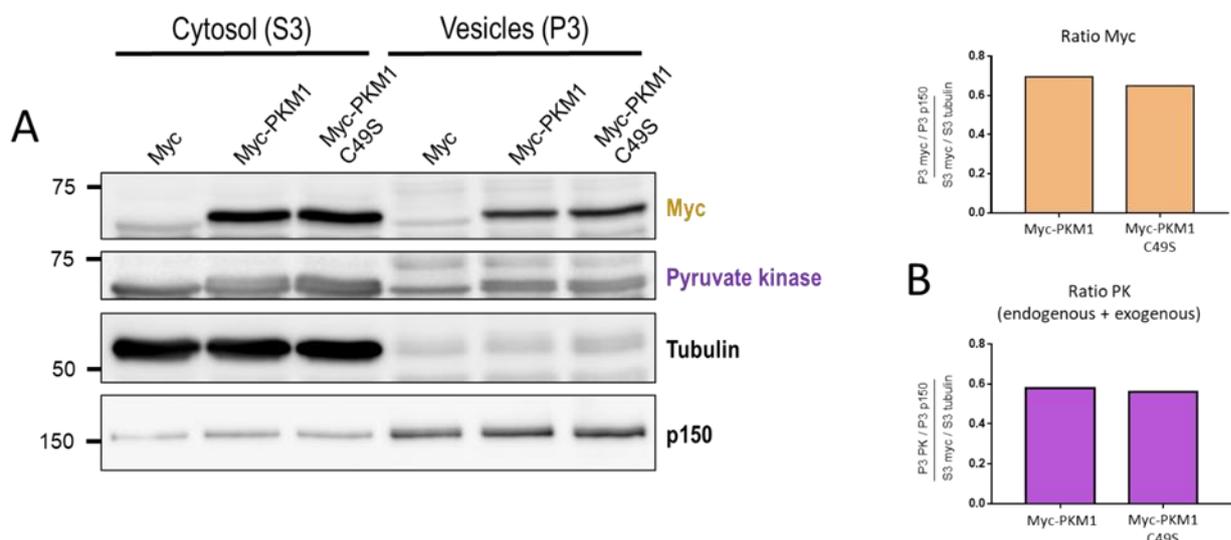


Figure R2: C49 palmitoylation is not responsible for PK attachment to vesicles. A) Immunoblot of Myc, PK, Tubulin and p150 in cytosolic and vesicular fractions isolated from cortical neurons transfected with one of three constructs, Myc; Myc-PKM1 or Myc-PKM1-C49S. Tubulin and p150 were used as cytosolic and vesicular marker controls, respectively. B) Quantification of band intensity in immunoblot of Myc and PK normalized by the amount of endogenous p150 and tubulin in each fraction from cells transfected with Myc-PKM1 and Myc-PKM1-C49S (N = 1 experiment).

b) [How are glycolytic enzyme quantities and activities affected on vesicles in Huntington's disease?](#)

Does the HTT mutation affect its interaction with GAPDH? We have shown that HTT is involved in glycolytic enzyme attachment on vesicular membranes. The CAG-encoded polyglutamine stretch within the HTT protein is abnormally expanded in Huntington's disease and has been shown to be directly responsible for deficient vesicular transport in cortical axons (Gauthier et al., 2004). However, the mechanism through which HTT mutation relates to transport deficiencies is still not quite understood. We therefore started by testing the interaction between GAPDH and mHTT to see whether this link was modified on vesicles because of the CAG expansion. We performed frozen brain fractionation on WT and HdhCAG140/+ samples and immunoprecipitated GAPDH from cytosolic and vesicular fractions. Using immunoblot, we then revealed for HTT and GAPDH. First, we observed that GAPDH immunoprecipitated from the cytosol and vesicles interacted with both the normal and expanded forms of HTT in HdhCAG140/+ extracts (Figure R3A). Secondly, the level of band intensity of HTT in IP samples was not modified compared to WT (Figure R3B), suggesting that GAPDH maintains the same level of contact with HTT on vesicles, whether it be with WT or mutant HTT, in HdhCAG140/+ mouse brains.

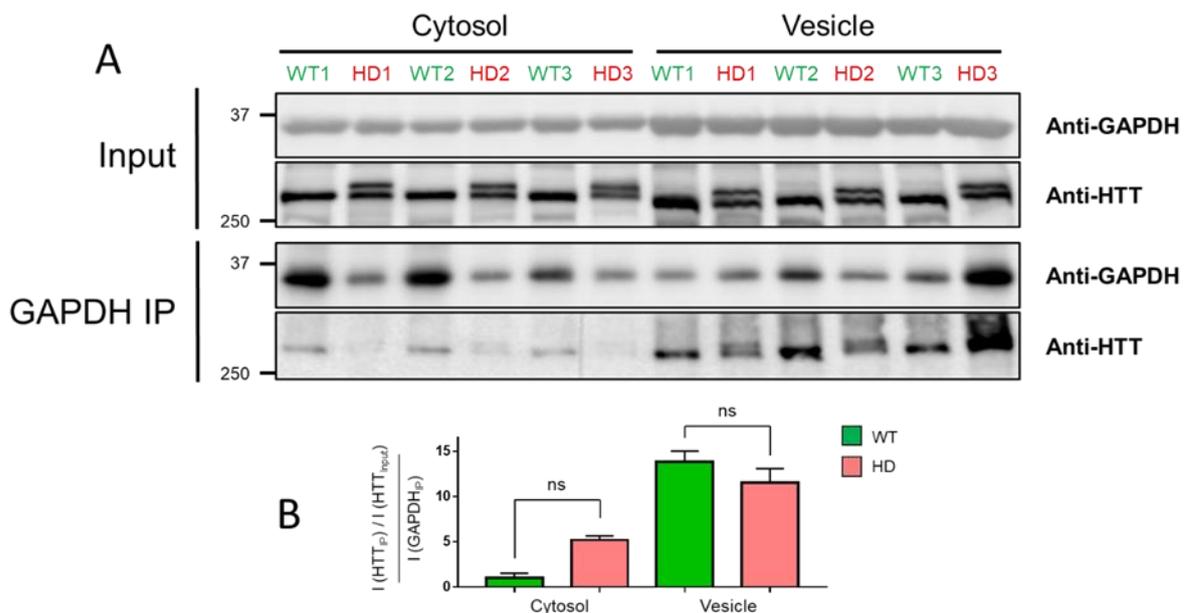
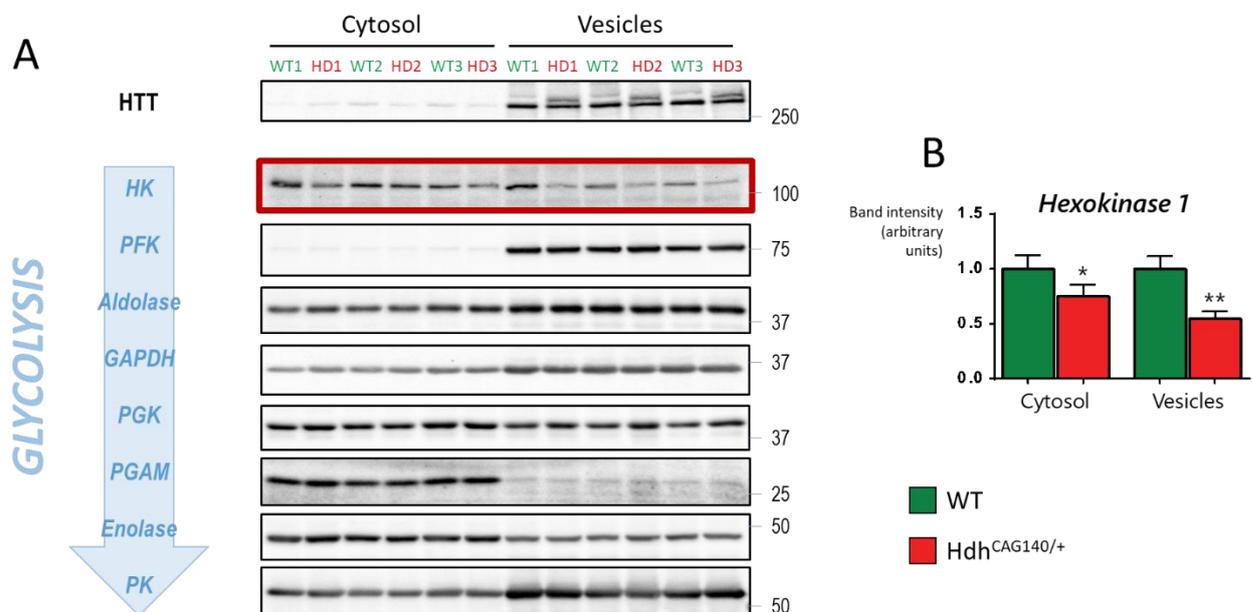


Figure R3: GAPDH interacts with both WT and mutant HTT in the cytosol and on vesicles. A) Immunoblot of GAPDH and HTT in cytosolic and vesicular fractions from wild-type and HdhCAG140/+ male mouse brains

following GAPDH immunoprecipitation. B) Quantification of band intensity (I) in immunoblot of HTT normalized by the amount of endogenous HTT in the input lanes and by the amount of GAPDH immunoprecipitated in each fraction (N = 3 independent experiments, unpaired t-test, ns = no significance, error bars represent SEM).

Does mHTT affect glycolytic enzyme levels on vesicles? We postulated that the HTT mutation may lead to glycolytic enzyme detachment from HD vesicles. To test this, we performed fractionations on brains extracted from knock-in mice expressing 140 CAG repeats in the endogenous HTT gene, along with their WT littermates. Eight of the ten glycolytic enzymes were then analyzed on Western blot in equal amounts of S3 and P3 proteins from both male and female mouse brains (Figure R4A). GPI was not-tested because it had not been found on vesicles (Zala, Hinckelmann, Yu, et al., 2013) and we did not have a functional antibody for TPI. The analysis of the eight other enzymes revealed that hexokinase 1 was reduced in CAG140 mouse brain extracts (Figure R4A and R4B). Intriguingly, this reduction was not limited to vesicles but was identified in several other fractions such as S3 and P2 (Figure R4C and R4D). This signifies that mutant HTT leads to reduced quantities of HK1 in several subcellular compartments, including vesicles. The vesicular and cytosolic amounts of all other enzymes were unchanged in HD animals (Figure R4A).



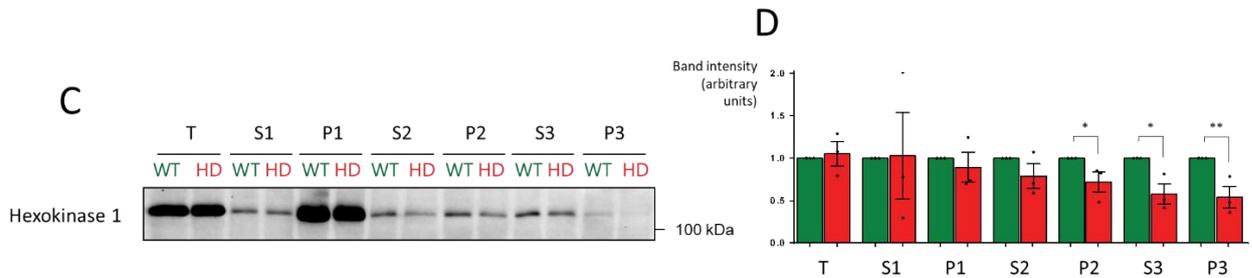


Figure R4: Hexokinase 1 is reduced on in several subcellular fractions including vesicles isolated from HdhCAG140/+ male and female mouse brains. A) Immunoblot of eight of the ten glycolytic enzymes as well as HTT/mHTT in cytosolic and vesicular fractions isolated from WT and HdhCAG140/+ mouse brains. B) Quantification of band intensity in immunoblot of HK1 in each fraction (N = 3 independent experiments, unpaired t-test, *p<0.1, **p<0.01, error bars represent SEM). C) Immunoblot of hexokinase 1 in total and all subcellular fractions isolated from WT and HdhCAG140/+ mouse brains. D) Quantification of band intensity in immunoblot of HK1 normalized for WT in each fraction (N = 3 independent experiments, unpaired t-test, *p<0.1, **p<0.01, error bars represent SEM).

Does mHTT affect vesicular glycolytic activity? In order to fully understand the differences in glycolysis in HD, we decided to assess overall glycolytic activity on vesicles. For this, we used the NADH and ATP measurement protocol described in the first portion of results. To compare vesicular glycolysis in normal and disease situation, I assessed glycolytic rate with variable concentrations of substrate, similarly to the Michaelis-Menten approach. Therefore, vesicular fractions were incubated with variable concentrations of substrate, glucose for segment 1 and glyceraldehyde-3-phosphate for segment 2. V_0 values were then measured and plotted as a function of initial substrate concentration. As with the Michaelis-Menten model, we obtained hyperbolic curves for both segments' activities (see Figure 2 in part 1 of results). This was used to estimate substrate concentrations at which V_0 was maximal, i.e. when substrate is in excess, and non-maximal, i.e. when the V_0 is equal to half of the maximal and when substrate is limited (in the Michaelis-Menten model, this is referred to as the K_m). The maximal velocity provides information on enzyme quantity and non-maximal gives an indication of enzyme affinity for substrate. We therefore assessed segment 1 and 2 activity in vesicular fractions from CAG140 and WT mouse brains with these two concentrations (Figure R5). For male mice, we observed an increase in segment 1 V_0 values in CAG140 situation, for maximal glucose concentrations, yet segment 2 activity was unchanged. On the other hand, segment 1 showed no differences and segment 2 was reduced in female CAG140 mice. This suggests that glycolytic activity is modified differently in male and female mice expressing

mutant HTT, with increased activity of one or more enzymes between HK and GAPDH in males, and decreased activity of one or more enzymes between GAPDH and PK in females. Velocities at non-maximal substrate concentrations were not significantly different to WT, whether it be in male or female P3 fractions, in both segments.

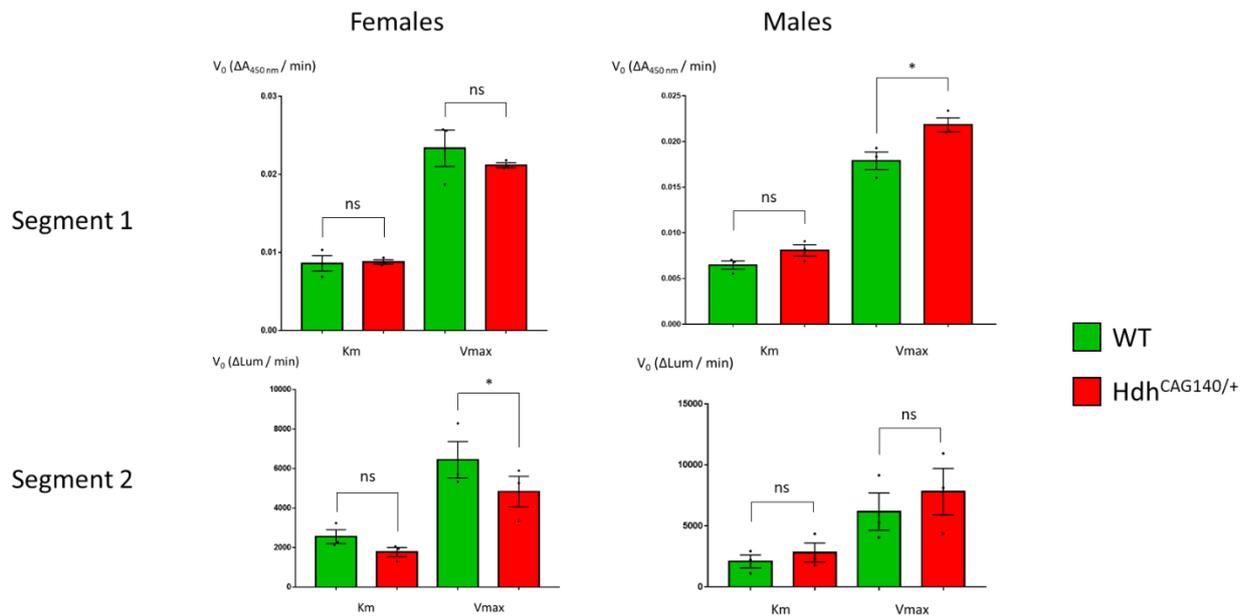


Figure R5: Glycolytic activity on vesicles is modified differently according to sex in HdhCAG140/+ mice. Slopes for segments 1 (upper panels) and 2 (lower panels) in vesicular fractions isolated from female (left panels) and male (right panels) WT and HdhCAG140/+ mouse brains. Activity was measured at limiting and non-limiting substrate concentrations, 100 and 500 μ M glucose for segment 1 and 40 and 300 μ M glyceraldehyde-3-phosphate for segment 2 (N = 3 individuals for each sex) (one-way ANOVA followed by Kruskal-Wallis post hoc test, * $p < 0.1$, ns = no significance, error bars represent SEM).

c) [Can specific stimulation of vesicular glycolysis restore transport in HD?](#)

Can glucose supplementation restore transport in HD? Given that glycolytic enzymes are affected in HD, it is possible that the reduction of vesicular transport observed by the team in Hdh CAG/+ neurons may be due to a defect in energy supply by glycolysis to molecular motors. Thus we supposed that increasing the amount of glucose available for vesicles could serve as a strategy to correct this. To analyze transport, we used previously described microfluidic chambers that enable the compartmentalization of neuronal cells in a dish (see Figure 3 in part 1 of results) (Moutaux et al., 2018; Taylor et al., 2010; Virlogeux et al., 2018). Cortical and striatal neurons were plated in opposite chambers and transduced with

lentivirus allowing the expression of BDNF-mCherry. Transport of BDNF-mCherry was then recorded in cortical axons of HdhCAG140/+ and WT mice plated in 5 mM (near-physiological concentration) and 25 mM glucose media. We observed a significant reduction in the speed of BDNF-mCherry-containing vesicles at 5 mM glucose in both anterograde and retrograde direction in HD neurons, which was still present when neurons were incubated with 25 mM glucose (Figure R6). This suggests that glucose supplementation is unable to restore transport in HD cortical axons.

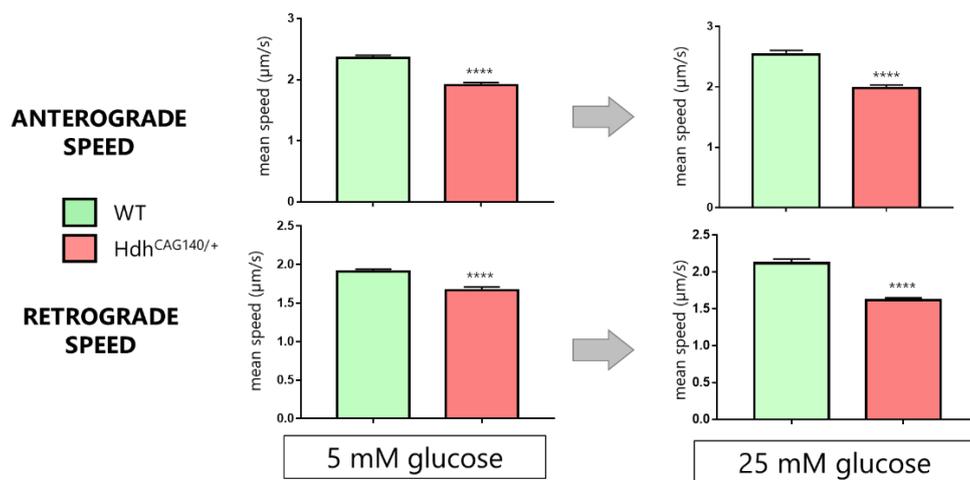


Figure R6: An increase in extracellular glucose concentration is unable to restore BDNF transport in cortical neurons isolated from HdhCAG140/+ mice. Mean speed of anterograde (lower) and retrograde (upper) vesicles containing BDNF-mCherry in transduced neurons cultured in two different glucose concentrations, 5 mM and 25 mM (N = 60 axons from 3 independent experiments, unpaired t-test, ****p<0.0001, error bars represent SEM).

Can specific stimulation of vesicular glycolysis restore transport in HD? It has been demonstrated that a knockdown of GAPDH in neurons leads to a reduction in vesicular transport velocity, which can be restored through artificial expression of GAPDH enzyme fused to the transmembrane portion of vesicular protein, synaptotagmin (TM-GAPDH) (Zala, Hinckelmann, Yu, et al., 2013). Therefore we theorized that a more targeted supplementation strategy may be required to specifically increase energy supply on vesicles. Accordingly, we transduced cortical neurons from CAG140 and WT mice with lentivirus allowing the expression of TM-GAPDH-IRES-GFP as well as a catalytically inactive construct, TM-GAPDH C149G-IRES-GFP, along with BDNF-mCherry in the same microfluidic chambers as before. Transport analysis revealed that TM-GAPDH-IRES-GFP but not TM-GAPDH C149G-IRES-GFP was able to restore BDNF-mCherry transport back to normal levels in both

anterograde and retrograde direction (Figure R7), suggesting that specific artificial stimulation of vesicular glycolysis is able to restore BDNF transport in HD cortical axons *in vitro*.

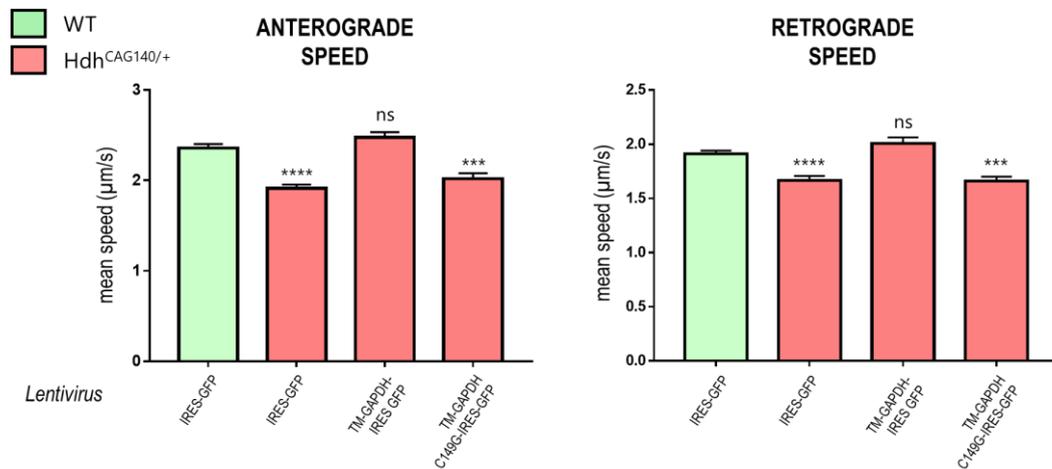


Figure R7: Artificial and specific stimulation of vesicular glycolysis is sufficient to restore BDNF transport in cortical neurons isolated from HdhCAG140/+ mice. Mean speed of anterograde (left) and retrograde (right) vesicles containing BDNF-mCherry in transduced neurons (N = 60 axons from 3 independent experiments, one-way ANOVA followed by Kruskal-Wallis post hoc test, ***p<0.001, ****p<0.0001, error bars represent SEM).

We were finally interested in testing TM-GAPDH *in vivo* to see whether glycolytic stimulation specifically on vesicles was sufficient to restore behavioral and anatomical phenotypes. HdhCAG140/+ mice were to be injected with virus enabling the expression of TM-GAPDH construct but this was cut short because of time and animal limitations. The lab's HdhCAG140 mouse line was subject to genetic derivation which caused most projects to be put on hold for several months. Nevertheless, we were able to design TM-GAPDH-IRES-GFP and control IRES-GFP alone in Adeno-Associated Virus (AAV) 9 and test their infectiousness in mouse brain. We first used a stereotaxic injection strategy with four injections targeted at sensorimotor cortical layer 5 that project to the striatum (Figure R8).

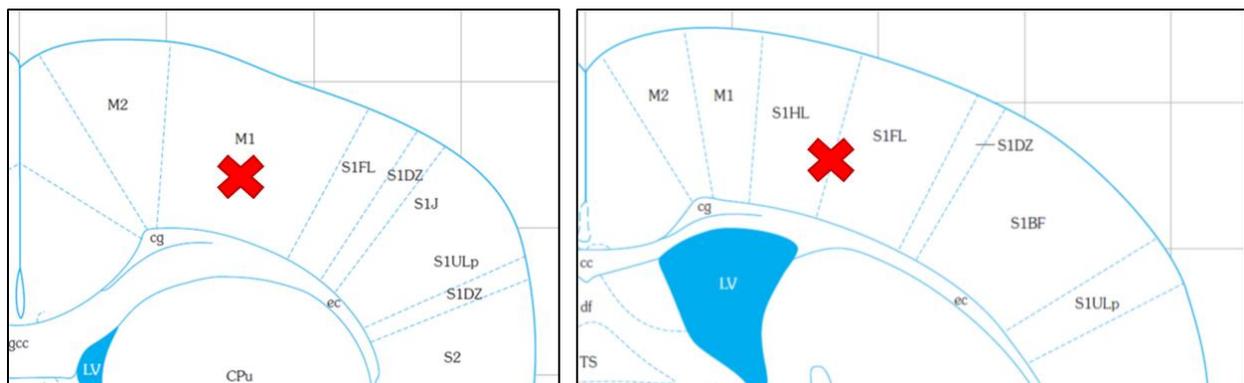


Figure R8: Stereotaxic injection coordinates. Schematic representation of mouse brain atlas showing approximate coordinates for stereotaxic injections used to inject TM-GAPDH-IRES-GFP and IRES-GFP AAV9 in motor (left) and sensory (right) layer 5 cortices.

Due to low infectiousness using this method, we decided to use recently described AAV PhP.eB, an AAV capable of crossing the blood brain barrier following blood injection (K. Y. Chan et al., 2017). Therefore, AAV's containing either TM-GAPDH-IRES-GFP or IRES-GFP were administered to 1.5 month old male mice through retro-orbital injection and analyzed for GFP fluorescence. Immunofluorescence confocal images on mouse brain slices revealed that both constructs were expressed in mouse cortical cells with approximately 30 to 40% of neurons positive for GFP (Figure R9). Therefore TM-GAPDH encapsulated in AAV PhP.eB is able to cross the blood brain barrier and infect neurons in the brain through systemic injection.

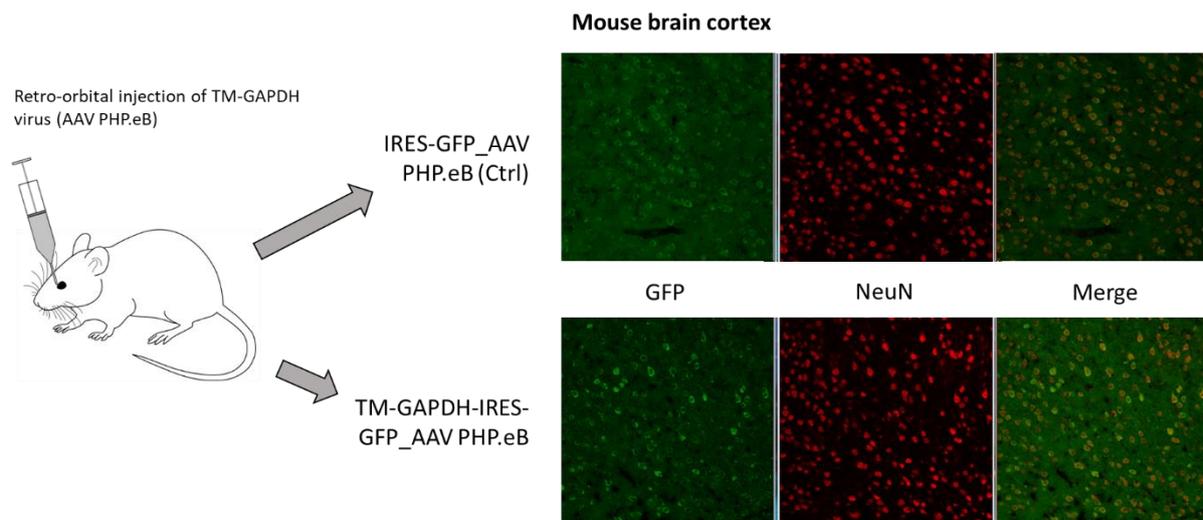


Figure R9: AAV PhP.eB carrying TM-GAPDH IRES GFP in or GFP constructs AAV PhP.eB infects cortical neurons. Left: Schematic representation of retro-orbital injection of AAV PhP.eB in young male mice. Right: Immunohistochemistry of GFP and NeuN (neuronal marker) staining alone and merged in mouse cortex transduced with IRES-GFP and TM-GAPDH-IRES-GFP AAV PhP.eB (N = 1 mouse).

VIII. DISCUSSION

1. Measuring glycolysis on vesicles

One of our first objectives, for both analysis of LDH importance for vesicular transport as well as studying glycolysis in HD, was to uncover a technique enabling us to measure glycolytic activity as a whole. This was necessary because we needed to do so specifically in our purified vesicular fractions, which until now was not possible. One of the current approaches utilized to measure glycolytic rate is to assess kinetic activity of either of the three irreversible glycolytic reactions which are catalyzed by HK in step 1, PFK in step 3 and PK in step 10. It is true that in optimal conditions, overall rate of glycolysis is dictated by these three enzymes because of the particularly exergonic nature of their reactions (Biochemistry, fifth edition, Berg, Tymoczko and Stryer). However, if one of the seven intermediate enzymes is affected or reduced, will this not affect the entire pathway? If so, then measuring the isolated activity of a non-affected enzyme will not be affected by such change, and this information will be missed. More common technics include the measurement of extracellular acidification and oxygen consumption such as Seahorse to differentiate glycolytic activity from mitochondria, or even glucose and lactate consumption and production, respectively (TeSlaa & Teitell, 2014). These commercial kits were not applicable for us as we needed to be able to measure glycolytic activity on vesicles specifically, and whole-cell measurements do not enable such estimations. Here we described a novel manner to assess glycolytic activity by using classical enzymology to treat this pathway as two separate super reactions that take into account all enzymes involved. We quantified kinetic rates of the first half of glycolysis using NADH production by GAPDH, and the second half using ATP production by PGK and PK. The split chosen roughly equates to the natural split in glycolysis which opposes preparatory and payoff phases. The issue that rendered total glycolytic assessment impossible was the importance of ATP. Indeed, ATP is initially used as a substrate by HK and PFK, and then produced later on by PGK and PK. The concentration of ATP has a negative feedback on PFK to cause glycolytic deceleration in response to energy accumulation (Biochemistry, fifth edition, Berg, Tymoczko and Stryer). This enables the cell to maintain a relatively constant ATP/ADP ratio and avoid unnecessary ATP production. Splitting glycolysis into two sets of reactions enabled us to bypass the crosstalk between energy producing and energy consuming enzymes, and is why, in both segment measurements, we could not afford to allow ATP

accumulation in our reaction mix. In segment 1, arsenate was added to the reaction mix in order to degrade GAPDH's product 1,3-biphosphoglycerate, and in segment 2, the luciferase enzyme used primarily to measure ATP production, does so by converting ATP into AMP.

The choice of splitting glycolysis at the sixth step was mainly for presentation purposes, so that there wasn't too much overlap between segments. Indeed, the only condition that needed to be respected for segment 2 activity measurement was that it could not include ATP-consuming steps, HK and PFK. Therefore, if need be, segment 2 can essentially measure seven of the ten glycolytic enzymes from PFK's product to the end, which enables the assessment of a much larger portion of glycolysis.

We found that vesicular glycolysis was kinetically different to the cytosol. This not only provides proof that this technique is efficient enough to uncover differences between subcellular compartments but also reinforces the idea of nucleotide channeling and energy metabolism being modulated differently according to location (Zala et al., 2017). Indeed, many examples of focused energy production have been discovered in past studies, for example, GAPDH and PGK were found on synaptic vesicles providing energy for glutamate recycling at the pre-synapse (Ikemoto et al., 2003; Ishida et al., 2009). With this new way of evaluating glycolysis, it is possible to expand upon this finding to see whether other glycolytic enzymes are present and required for energy production. Equally, this method can be used to measure the contribution of glycolysis in other compartments of the cell. In this study we focused on vesicles and the cytosol, but glycolytic activity can also be measured in the large membrane fraction that contain mitochondria. Moreover, the influence of cofactors, small molecules and environmental factors, previously studied with single enzyme kinetics can now be evaluated on overall glycolytic function.

For both segments, we measured glycolytic activity based on Michaelis-Menten kinetics. After making sure that the recorded signals were proportional to protein quantity, we found that V_0 values plotted according to initial substrate concentration gave a hyperbolic variation (except for segment 2 in the cytosol), similarly to what is observed in single enzyme reactions described by Michaelis and Menten. The substrate concentration at which maximal velocity was halved is referred to as the Michaelis-Menten constant, K_m , for single enzyme reactions. Here, given that we measured the activity of several enzymes in a chain, each of which has its own K_m value, we cannot describe this concentration of glucose or G3P as a K_m per se. Nevertheless, these values correspond to EC50 concentrations linked to a chain of several enzymes that are dependent on each enzyme's affinity within the chain. This value is probably dictated by the enzymatic step within the chain with the lowest activity at a given

concentration, but it is impossible to conclude as to which step is responsible for this without knowing the individual $V_0 = f([\text{substrate}])$ curves and K_m values for each step of the chain.

2. [Lactate dehydrogenase A is expressed in neurons](#)

LDH in the brain serves different roles depending on the cell type. It is believed that glial cells such as oligodendrocytes and astrocytes produce most of their ATP through glycolysis, with low reliance on mitochondria, whereas the opposite seems to occur in neurons (Magistretti & Allaman, 2018). However, LDH is expressed in all brain cells, albeit not for the same purpose. In astrocytes, LDH-A is more strongly expressed whereas, LDH-B is the isoform of choice in neurons (Bittar et al., 1996). The A isoform is responsible for producing lactate from pyruvate, a result of high glycolytic activity, which is then channeled to neurons, transformed back into pyruvate by LDH-B which serves as a substrate for mitochondria (Magistretti & Allaman, 2018; Urbańska & Orzechowski, 2019). The results presented in this manuscript are somewhat contradictory to what has been previously described, as we discovered that LDH-A was present in larger amounts on vesicles in neurons compared to LDH-B. Vesicles represent a very small fraction of overall protein content in a given cell, therefore it is possible that analysis of LDH expression in different cell types, which has always been performed on whole cell extracts, is not influenced by such small protein quantities.

3. [What is the role of vesicular lactate?](#)

We have also confirmed that pyruvate is not the final product of vesicular metabolism. The initial theory when glycolytic enzymes were found on vesicles was that the pyruvate would be taken up by mitochondria for further ATP production. This could have been possible if not for the fact that vesicles can function *in vitro* in absence of mitochondria, suggesting mitochondrial recycling of NAD^+ is not required. What is then to become of lactate? Lactate, previously considered unimportant in brain function, is now recognized as a crucial energy source and signaling molecule for brain cells (Riske et al., 2017). The ANLS model suggests neurons do not produce pyruvate or lactate, and instead import lactate from surrounding astrocytes. This has been demonstrated in astrocytes as well as oligodendrocytes (Y. Lee et al., 2012). Here we have demonstrated that lactate is produced by motile vesicles in axons by an LDH isoform that is believed to not be expressed by neurons. The fate of this lactate is unknown

for now, but we may emit several hypotheses. It is possible that this lactate is simply released into the cytosol where it may serve as a substrate for mitochondria, similarly to lactate that is imported by neurons from the extracellular space. It has been demonstrated that oligodendrocytes can use lactate produced by the axons they encapsulate when glucose is scarce (Rinholm et al., 2011). Vesicular lactate, produced throughout the vesicle's transport along the axon, may serve this purpose in white matter. A far more speculative proposition is that lactate is funneled into the vesicular lumen via monocarboxylate transporters. Neurons are typically known to express MCT2, but mass spectrometry results have revealed the presence of MCT1 on vesicles as well, a transporter with high affinity for lactate (Halestrap & Meredith, 2004). The lactate in these vesicles may then be transported and released at the synapse where it is known to be of great importance during neuronal activity (Magistretti & Allaman, 2018). This hypothesis remains to be elaborated upon.

4. [Vesicular transport and the Warburg effect](#)

The presently uncovered role of LDHA for glycolysis on vesicles is similar to the heightened LDH and glycolytic activity termed Warburg effect seen in cancer cells. Indeed, these cell types are known to over-express and over-activate LDHA which ensures continual and fast glycolytic activity (Fantin et al., 2006; Sheng et al., 2012). Although vesicular glycolysis has only been demonstrated in neurons (Zala, Hinckelmann, Yu, et al., 2013), we may wonder whether vesicular transport is affected in cancer cells in response to excess LDHA activity. Do vesicles travel faster? Only little is known regarding the role of transport in cancer but targeting vesicular transport machinery, such as Rab proteins, has been suggested as a way to limit cancer aggressivity (Mughees et al., 2020). Furthermore, it has been demonstrated that mitochondrial trafficking is affected to ensure cell proliferation. Indeed, down-regulation of mitochondrial Rho-GTPase Miro1, which is important for transport, inhibits energy-demanding processes such as protrusion formation and focal adhesion dynamics at the cell periphery, resulting in decreased cell migration and invasion (Schuler et al., 2017). Moreover, global cancer cell metabolism is increased, a process which is intrinsically reliant on intracellular trafficking of vesicles such as endosomes and lysosomes, suggesting a strong role in tumor development.

Other glycolytic enzymes are affected in cancer, which may also have consequences on vesicular transport. For instance, the PKM gene leads to the expression of two splice variants

which are PKM1 and PKM2. PKM1 is expressed in non-proliferating tissues such as the heart and brain, and PKM2 is expressed mainly in highly proliferating tissues during development. Usually there is a switch from PKM2 to PKM1 expression as a cell differentiates (Mazurek 2011). In cancer cells, PKM2 is upregulated to enable the high proliferation of this tissue. Therefore it is of interest to test PK expression patterns on vesicular membranes in cancer cells. PKM2 also has the ability to form a low activity dimer as well as a highly active tetramer, whereas PKM1 can only form a tetramer (Chiavarina et al., 2011; Hitosugi et al., 2009). The role of catalytically less active dimeric PKM2 in cancer is to favor upstream anabolic pathways to meet the proliferative demands of these cell types. This may also have an impact on glycolytic regulation on vesicles and we may wonder as to how glycolytic efficiency on vesicles may be modified.

5. Vesicles, an isolated autonomous metabolic microenvironment

We have presented results that reinforce and expand upon the concept of localized energy producing pathways on vesicles. Before this study, glycolysis had been the only set of metabolic enzymes described as being essential to the functions of the vesicles to which they were bound. Indeed it is now known that ATP for BDNF-containing dense-core, endosomal and APP-positive vesicular transport is fueled by glycolysis (Zala et al., 2013). The results presented here have gone one step further and proposed that aerobic glycolysis and lactic fermentation are necessary for this transport as well. Intriguingly, it may be that LDH is simply one more piece within a much larger puzzle, as vesicle purification reveals many other metabolic enzymes at their surface, such as creatine kinase and members of the pentose phosphate pathway. All this contributes to the overhanging idea that vesicles are autonomous and quite dissociated from the rest of the cell's metabolic activity. Such demonstrations are ongoing projects in the laboratory. Proof of this also lies in the independence of vesicles from mitochondria (Hinckelmann et al., 2016). Not only have experiments shown that vesicles can be transported without the presence of mitochondria *in vitro*, but it also seems counterintuitive for vesicles to have to rely on mitochondria, given their irregular dispersion throughout the axon as well as their different trafficking properties. Mitochondria are mostly stationary in the mature axon and for those that are motile, their speeds do not rise beyond 1 $\mu\text{m/s}$ (Lewis et al., 2016; Niescier et al., 2016). Moreover, it has been demonstrated that mitochondrial dynamics decrease as neurons mature *in vitro* (Moutaux et al., 2018). This is not the case for synaptic

and dense core vesicles that are highly mobile with speeds increasing with network maturity, reaching 3 to 4 $\mu\text{m/s}$ in fully mature systems. Speeds such as these seem complicated to obtain if vesicles were to rely on metabolic exchange and diffusion with static mitochondria along their trajectory. Then again, fast transport of vesicles is important and has been described in axons due to the great distances covered by axons and neurites, but may not be relevant for smaller cell types that do not require such efficiency. It remains to be uncovered whether this isolated metabolic activity on vesicles is restricted to axonal vesicles that move at great speeds or a universal mode of function in all cell types. Moreover, it has not been demonstrated that all vesicle subtypes in neurons utilize vesicular glycolysis for transport. This is why we chose to study BDNF transport, but the same principles can be tested on other cargoes such as VAMP2 for synaptic vesicles. As mentioned in the introduction, GAPDH and PGK are localized on synaptic vesicles where they serve to provide energy for glutamate recycling (Ikemoto et al., 2003; Ishida et al., 2009). More research would be required to see whether these enzymes are also implicated in transport as well.

We were also able to provide evidence that glycolysis on vesicles is kinetically different to the cytosol, which bolsters the concept of nucleotide channeling and the belief that cellular metabolism is not simply a “bag” of enzymes within the cytosol, producing a general pool of energy that is to be utilized freely by all cellular processes. Several examples have been studied whereby certain areas of the cell such as the mitochondrial membrane possess metabolic enzymes bound to their surface enabling specific and localized functions. Here, we were able to provide functional evidence of distinct differences in kinetic activity of the same glycolytic pathway in two different subcellular compartments. Vesicles display a more efficient capacity to produce NADH and ATP compared to glycolytic enzymes in the cytosol when substrate is not in excess, during first order kinetic evolution. The reason for this is unknown but we may hypothesize that there is functional relevance to this difference. Glycolytic vesicles may be oriented in a certain manner on membranes so that substrate and product exchange between enzymes of glycolysis occurs in a more efficient manner, thus increasing overall rate of the pathway’s ability to convert glucose into pyruvate. Moreover, vesicles, and therefore the glycolytic enzymes bound to them, represent a small fraction of total protein in the neuron. Perhaps glycolysis on vesicles has “evolved” to be more efficient so that they may have a competitive chance to access the glucose that enters the neuron, which if not, may be consumed before arriving on vesicles. Furthermore, neurons preferably import lactate for bulk energy production as suggested by the ANLS model (Magistretti & Allaman, 2018). Therefore,

conceivably, access to glucose by vesicular enzymes may be increased simply because neurons do not possess active cytosolic glycolysis.

6. Enzyme attachment to vesicles

We also tried to answer the question of how glycolytic proteins were bound to vesicles. This question remains very much unanswered, as we were unable to provide evidence of mechanisms responsible for such attachments. However, we did demonstrate a strongly vesicular association of HTT with two glycolytic enzymes, GAPDH and PFK. The interaction between GAPDH and HTT had previously been suggested by two-hybrid screening but lacked sufficient control experiments (Burke et al., 1996). Here we were able to show that this interaction is indeed occurring in brain cells and that it may vary according to the cellular compartment. The strength of this interaction on vesicles is in line with the scaffolding function of HTT. The lack of GAPDH when immunoprecipitating HTT is not in accordance with the result obtained in the opposite experiment but may be explained by the fact that HTT has been identified to possess a very large number of interactors (over 350 have been identified) (Tourette et al., 2014). Perhaps the GAPDH signal is simply not strong enough to be detected here, which does not mean the result obtained with GAPDH immunoprecipitation is incorrect. It is also possible that the antibody used to immunoprecipitate HTT from vesicles binds an area of the protein that interacts with GAPDH or a conformation that does not.

We may also wonder why no other glycolytic enzymes were present in these experiments. Many of the glycolytic enzymes possess molecular weights that range around 40 to 50 kDa on electrophoresis gels, which unfortunately placed them near the heavy chains of the antibodies used for the immunoprecipitation on the gel. Although we used antibodies of different species and secondary antibodies that would not recognize these heavy chains, we were unable to bypass this problem and therefore could not detect these enzymes through immunoblot analysis. It is also possible that HTT does not interact with all glycolytic enzymes and that remaining enzymes bind vesicles indirectly via GAPDH (although not strong enough to be seen in IP) or simply through different scaffolding proteins or post-translational modifications. To develop this theory, more attention would need to be focused on the 9 other glycolytic enzymes starting with their individual immunoprecipitation.

Lipid modifications are increasingly recognized as important mechanisms for both targeting proteins to membranes and subcellular protein trafficking. Unlike other lipid

additions, palmitoylation is a readily reversible modification allowing rapid attachment and detachment cycles of proteins from membrane surfaces. Palmitoylation is catalyzed by palmitoyltransferases (PATs). Many of these have been identified in the brain (Lein et al., 2007; Prescott et al., 2009) and have mainly been found in the endoplasmic reticulum and Golgi apparatus, as well as on endosomes and the plasma membrane (Fukata et al., 2013; Greaves & Chamberlain, 2011). Palmitoylation therefore presents itself as a prime candidate for targeting glycolytic enzymes to hydrophobic locations such as the membranes of vesicles. The mass spectrometry results revealed a palmitoyl group on pyruvate kinase, the final enzyme of the glycolytic chain, but the mutation of its sequence did not seem to affect the quantity of PK bound to vesicles, nor in the cytosol. It is possible that the addition of palmitate is not the sole modification responsible for PK attachment to vesicles, but given that this question was not extensively addressed, it is difficult to conclude anything robust regarding PK palmitoylation. To fully expand upon this question, we would need to find alternative techniques for inducing or inhibiting palmitoylation. The use of 2-bromopalmitate as a non-specific palmitoylation inhibitor could be envisaged to assess the role of palmitoylation in PK attachment, as well as the remaining glycolytic enzymes. To identify palmitoylation on these enzymes, we may also consider click chemistry which consists in tagging palmitate groups with small chemicals and tracking their cellular localization.

7. Glycolytic differences in Huntington's disease

As mentioned, GAPDH attachment is possibly dependent on HTT, given that silencing of HTT leads to a reduction in GAPDH quantity on vesicles (Zala, Hinckelmann, Yu, et al., 2013). We have confirmed that these two proteins also strongly interact on vesicles. When studying HTT and mutant HTT co-immunoprecipitation in on HD vesicles, we found that GAPDH remains bound to HTT despite the CAG expansion. Therefore, the interaction domain of HTT with GAPDH on vesicles may not involve the N-terminal portion of HTT, nor is a mutation in this domain sufficient to detach or reduce either of these proteins on vesicles. The presence of an expanded CAG in HTT is known to change and cause dysfunction in interacting proteins such as HAP1, HIP1 and calmodulin (Bao et al., 1996; Dudek et al., 2008; Ratovitski et al., 2012). It is possible that mutant HTT affects the orientation of glycolytic enzymes it interacts with, including GAPDH, and therefore renders overall activity inefficient.

Additionally, this result can be linked to the lack of change in enzymatic protein level of GAPDH on HD vesicles obtained previously.

When looking at enzyme levels on vesicles in HD, we observed a significant reduction in HK1, an enzyme that is of particular importance to overall glycolytic activity. It is the first enzyme of the pathway and is responsible for trapping glucose within the cell. To ensure this, HK's K_M is very low, meaning it has high affinity for its substrate, glucose. It also catalyzes one of the three irreversible reactions of glycolysis, which are believed to mediate overall glycolytic activity. A decrease in HK expression or localization on vesicles is therefore in line with a decrease in overall glycolytic ATP production. HK is also an important enzyme for the pentose phosphate pathway, which suggests that the activity of this pathway may also be affected in HD. Pentose phosphate pathway enzymes are also present on vesicles (Hinckelmann et al., 2016), although their role has not yet been discovered. Moreover, the reduction in HK was not limited to the vesicular fraction and was observed in the cytosol and large membrane fractions as well, suggesting a cellular decrease in HK that seems to affect vesicles most drastically, since the greatest difference was seen in this fraction. The reduction of HK on vesicles is therefore a consequence of a change occurring elsewhere, possibly in HK targeting. Given that we did not see any change in the total fraction of HK, we can suppose that this does not involve transcription or expression.

Given these results, we expected to see a change in glycolytic activity. Unfortunately, measurements of segments 1 and 2 reveal a more complex situation. Indeed, the sex of the animals expressing mutant HTT had a significant influence on glycolytic activity, something that was not present on enzyme quantity results, which also consisted in a mix of male and female mice. Moreover, the mice used for activity measurements, although adult, were much younger (2 months) than those used for enzyme levels (6 months), meaning that differences in vesicular glycolysis in HD may appear in time as the disease worsens. However, the low number of mice used render this test difficult to interpret with certainty. The goal would be therefore to increase the number of tested animals in order to properly estimate glycolytic differences between healthy and HD mice. Next, any differences found at a given enzyme would be pinpointed by sequentially incubating vesicles with each glycolytic intermediate metabolite and measuring activity. More precise estimations of differences in HD glycolysis may also be possible if tested on purified motile vesicular samples, as has been done previously (Hinckelmann 2016). Nevertheless, metabolic profiling in HD is quite complex, as different animal models present different phenotypes (Dubinsky, 2017).

Overall, our characterization of glycolysis on vesicles in HD encompassed enzyme quantity and glycolytic activity, but unfortunately, the analysis did not enable us to describe a clear difference between HD and WT. First of all, protein levels on vesicles initially revealed a decrease in HK1 that was questionable due to genetic derivation in our animal cohort, and secondly, glycolytic activity measurements on a few mice exposed a more complex phenotype than expected, based on what had been seen in enzyme quantity examination. Although difficult to analyze, it is possible that these results suggest some sort of change in glycolytic properties on vesicles in HD. For example, we saw an increase in activity in segment 1 in some mice, whereas other animals showed a decrease in segment 2. Perhaps this echoes a compensation mechanism established by glycolytic enzymes in HD in response to inefficient ATP production. More elaborate tests are required to draw any solid conclusions from this set of results.

8. [Genetic derivation of HdhCAG140/+ mice](#)

One of the more frustrating issues with the experiments revolving around comparisons between HdhCAG140/+ mice and WT littermates was the genetic derivation of this mouse line that was discovered during my PhD and that forced a complete reset of the mouse line established in the lab. This therefore creates some doubt as to how truthful the results we obtained really are. Indeed, enzyme quantities were checked in mice generated much earlier than those used for activity measurements, and the latter mice were amongst the first of those available when the new mice line had been set up. We were forced to use these animals as it was near the end of my PhD and we did not have the time to wait for a pure HdhCAG140/+ background to be at our disposal.

9. [Restoring BDNF transport in HD](#)

The lack of effect brought by simply increasing overall glucose concentration in neurons is in line with current belief that an increase in caloric intake for HD patients is not sufficient to slow disease progression (Marder et al., 2009). This opposition with restoration of BDNF transport through TM-GAPDH is of interest as it suggests that increased glucose concentration has no effect on vesicular glycolysis in HD. Also noteworthy is the fact that only catalytically active TM-GAPDH was able to restore transport. This suggests that TM-GAPDH

serves as metabolic stimulator on vesicles and not simply by increasing stoichiometry. We chose to target glycolysis with TM-GAPDH because this construct had already been characterized in previous work (Zala, Hinckelmann, Yu, et al., 2013), and due to the fact that it is the first enzyme of the payoff phase of glycolysis, where ATP is produced. Further reason to target this enzyme was brought by the fact that GAPDH interacts with HTT. Nevertheless, despite an efficient stimulation with TM-GAPDH, we may wonder whether other glycolytic enzymes may be just as effective. Given the result demonstrating a reduction in hexokinase quantity on vesicles, TM-HK may also be envisaged. Moreover, only dense-core vesicles containing BDNF were tested here and so transport of other types such as synaptic vesicles would also need to be assessed.

The next step *in vitro* would be to also determine downstream effects of TM-GAPDH. It has been demonstrated that several elements of the cortico-striatal network are dysfunctional in HD, such as the number and connectivity of synapses, as well as striatal cell survival (Virlogeux et al., 2018). In this same study, results showed that healthy cortical cells connected to HD striatal cells was sufficient to restore all phenotypes, emphasizing the importance of the cortex in this disease. On this basis, we only transduced cortical neurons with TM-GAPDH in our tests, meaning the same downstream phenotypes also need to be checked.

Although we were not able to test TM-GAPDH *in vivo*, many questions will need to be answered in order to treat knock in mice effectively. The AAV is seemingly expressed in neurons in the cortex but it remains to be confirmed that the construct is indeed located on vesicles. Techniques such as focal ultrasound that has been tested in rats (K. Y. Chan et al., 2017) may serve as an option to increase blood brain barrier permeability and AAV infectiousness. Additionally, it has been reported that pericytes are also affected in HD, which may induce variable infectiousness in HD animals compared to WT. *In vivo* injections will also enable testing of overall glycolytic activity in HD in the presence of TM-GAPDH, as this was not possible on cultured cells due to the large amounts of material required for such tests. The timing of injections is also a parameter that needs to be estimated. It will be interesting to compare the effects of TM-GAPDH at an early stage of the disease versus later on when the disease and symptoms have started to manifest. This may provide information as to how effective this therapeutic strategy may be, and whether or not it is reversible. Furthermore, given that this strategy involves an artificial stimulation of vesicular glycolysis, the dose of injection will also need to be studied, to ensure sufficient restoration and avoid toxic secondary effects. Indeed, the TM-GAPDH construct should theoretically bind and increase transport for all vesicles, which may not be advantageous in all aspects, as HD is also characterized by

glutamate-induced excitotoxicity and increased synaptic vesicle transport may aggravate this effect. Nevertheless, glutamate release at cortico-striatal synapses is reduced in HD (Virlogeux et al., 2018) and stimulation of the cortico striatal axis restores synaptic and behavioral phenotypes (Fernández-García et al., 2020), which suggests that TM-GAPDH may not be deleterious to synaptic vesicle release.

As mentioned, this strategy requires artificial expression of a non-endogenous protein, and if it is efficient, we may consider this as a viable way of treating patients much further down the line. TM-GAPDH serves as a proof of concept that specific stimulation of glycolytic ATP supply to molecular motors is sufficient to restore transport, however, it will probably not be used in humans. It may therefore become necessary to find different ways to target glycolytic enzymes to vesicular membranes, possibly by finding the enzymes and signaling cascades responsible for glycolytic attachment to vesicular membranes in order to design drugs able to modify their activity and regulation properties.

It is also possible that such a strategy may not have any improving effect on mouse phenotype. Although it has been shown that BDNF release is decreased (Yu et al., 2018), as described in chapter 1 of the introduction, there are a great number of additional cellular functions in both neurons and glia that are dysfunctional in HD. For instance, it has been demonstrated that dysregulation of mitochondria is sufficient to induce HD-like symptoms *in vivo*. Given the complexity of this disease and the insufficient knowledge regarding HTT function throughout the cell, restoration of normal phenotype using symptomatic strategies such as the one presented here may not be effective on humans. Then again, most patients discover their diagnosis late into their lives where gene therapy becomes impossible and so symptomatic alleviation is the only option.

Many other brain disorders present deficiencies in transport, and therefore targeted stimulation could also serve as potential therapeutic approach. In certain diseases such as Parkinson's disease or hereditary spastic paraplegia, mitochondrial transport is affected (Abou-Sleiman et al., 2006; McDermott et al., 2003), which supposedly does not rely on glycolysis and may therefore be more complex. Others like Alzheimer's disease present deficiencies in endosomal and APP-positive vesicular transport (Kamal et al., 2001), these are known to use on-board glycolysis for fuel, but uncovering new targets, such as non-glycolytic enzymes, other than TM-GAPDH, may be envisaged. Creatine kinase is also found on purified vesicles (Hinckelmann et al., 2016), a protein whose function is to store energy through creatine phosphorylation, therefore targeting such a protein to vesicles may also serve as a viable stimulus for transport.

IX. PERSPECTIVES

1. A novel approach for estimating glycolytic activity

As developed in the discussion, many new questions and perspectives arise from the results presented in this manuscript. In the first part of the results, regarding the biochemical measurement of glycolytic activity, we provided an additional approach to assess glycolysis in specific areas of the cell. We can now identify potential differences that may stem from specific positioning and localization of enzymes in certain parts of the cell, such as those seen when comparing soluble cytosolic enzymes to those attached to vesicular membranes. For example, we may wonder how enzymes attached to the membranous sheath of spermatozoa behave, or those found at the surface of synaptic vesicles, ensuring glutamate recycling.

As mentioned in the introduction, certain diseases such as AD present deficient vesicular transport as well as modified metabolic rates. Here it is also possible to assess glycolytic activity on vesicles, that may lead to novel discoveries regarding the causes behind cellular dysfunction in these diseases. In HD alone, according to the animal model used, different glycolytic steps have been reported as changed. This technique for measuring glycolysis could be used to confirm said differences and equally provide a more global view of metabolic dysfunction in the disorder. Indeed, in our study, we provide evidence that glycolysis is disabled on vesicles, and so it may also be of interest to explore this same pathway in other areas of the neuron, but also in other cell types that rely more heavily on glycolysis in the brain, such as astrocytes and oligodendrocytes. This may provide more information on how glial cells contribute to the pathophysiology of HD.

2. Vesicular metabolism

We also demonstrated that aerobic glycolysis powers vesicular ATP production and transport in the brain. This Warburg-like effect in healthy tissue instigates the question of how vesicles modify their transport patterns in response to increased LDHA expression, a hallmark of many cancer cells. Preliminary results not shown in this manuscript show that glycolytic enzymes are found in P3 fractions of several other fractionated organs such as muscles, the lungs, and the liver. This shows that vesicular glycolysis may be a more universal mechanism

than previously thought and is in line with the nucleotide channeling model explained in the introduction.

Many questions still remain regarding the fate of lactate produced by vesicles. Many possibilities are to be tested to understand the role of lactate. Perhaps it is released from vesicles to become the substrate for mitochondria, or to be transferred from the axon to oligodendrocytes to serve as energy substrate in these cell types. Another hypothesis to be tested is that MCT1 found in purified vesicular fractions ensures lactate incorporation into the vesicular lumen. Lactate may then be transported to the synapse where lactate is known to play an important role.

On a larger scale, the evidence regarding LDH raises the question of how many other metabolic pathways are present and mediating transport on vesicles. Purified vesicular fractions reveal several enzymes involved in many other pathways, such as CK, AMPK and several enzymes of the pentose phosphate pathway. These are all projects currently being developed in Frédéric Saudou's team.

3. [Glycolysis in HD](#)

In the second part of this manuscript's results, we investigated the possibility of glycolysis being modified in HD. We have shown that both enzyme quantity as well as enzyme activity seems to be significantly changed in HdhCAG140/+ animals compared to healthy controls. However, the sex of the animals had different effects on overall glycolytic activity which had not been anticipated, based on the results obtained with hexokinase 1. Sequential measurement of each glycolytic step will enable us to pinpoint the glycolytic steps that are responsible for the changes observed in each sex. Although sex has very little influence on the statistics of HD in human populations, certain studies have monitored differences in specific behavioral tests in animal models. Exploration of this question may provide a link between the cellular adjustments seen here and the influence of sex on certain macroscopic phenotypes.

4. [Artificial stimulation of vesicular glycolysis](#)

We were also able to target vesicular glycolysis artificially and restore transport through TM-GAPDH. These results suggest promising perspectives for the future of HD as well as other diseases that result in deficient transport. Although we were only able to test the

infectiousness of our construct in the brain, we had envisaged to monitor several behavioral phenotypes in our animal model such as motor abilities and coordination, as well anxiety and memory. Many possible strategies should be tested to fully understand the power of TM-GAPDH, such as the timing of TM-GAPDH administration, whether this is sufficient to treat animals late in life where symptoms have begun to appear, or early when the damage of mHTT expression has not yet fully taken hold. Finally, this strategy may also be of interest for the treatment of other diseases that demand transport stimulation for therapy such as motor neurons in ALS.

X. GENERAL CONCLUSION

Despite some doubt surrounding certain results regarding the HdhCAG140/+ mouse line, we have provided new evidence of how vesicles are metabolically regulated in neurons. Indeed, LDH colocalizes with vesicles and is required for BDNF transport *in vitro*. This is a step further towards a global understanding of metabolic activity on vesicles. Many of the current projects in the lab focus on the various associated pathways that may contribute to energy production at the surface of these organelles.

We have also designed a novel approach for glycolytic activity assessment through segmental measurements of NADH and ATP production. Although not necessarily relevant for whole cell glycolytic activity estimations, as simpler-in-use commercially available kits exist, this approach may be useful for measurements in specific cellular compartments wherever local glycolytic energy may be produced.

Finally, we were able to provide proof of concept that specific stimulation of vesicular glycolysis can fully restore transport of BDNF in neurons expressing mHTT. This paves the way towards more targeted strategies for HD treatment and may pinpoint the importance of vesicular energy and transport in the global view of HD pathogenesis.

XI. MATERIALS AND METHODS

Mice:

HdhCAG140/+ knock-in mice are generated on a C57/BL6J background and express human HTT exon 1 sequence with 140 repeats of CAG as described previously (L. B. Menalled et al., 2003). Animals were maintained with access to food and water ad libitum and kept at a constant temperature (19-22 °C) and humidity (40–50%) on a 12:12 hours light/dark cycle. All experimental procedures were performed in an authorized establishment (Grenoble Institut des Neurosciences, INSERM U1216) in strict accordance with the recommendations of the European Community and the French National Committee for care and use of laboratory animals under the supervision of authorized investigators. Regarding controlled crosses, wild type C57/BL6J or HdhCAG140/CAG140 male mice were mated for one night with wild type C57/BL6J female.

Vesicle purification:

Vesicle purification was based on the protocol established by Hell & Jahn, 2006. 2 month old male mice were euthanized by decapitation. The brains were removed and immediately frozen in liquid nitrogen. Brains were then grinded down to a thin powder with a ceramic mortar bathed in liquid nitrogen to avoid thawing. This powder was then transferred to 1 mL of homogenization buffer (320 mM sucrose) and further broken down and homogenized with a loose then a tight potter, this formed the Total fraction. The mix was centrifuged for 10 min at 47,000 x g at 4°C (Beckmann TLA 100.3 rotor). The supernatant was set to one side while the pellet was re-suspended with 300 µL of homogenization buffer and centrifuged once again for 10 min at 47,000 x g at 4°C. The pellet was re-suspended with homogenization buffer forming the P1 fraction. The supernatant was combined with that of the first centrifugation, forming the S1 fraction, and centrifuged for 40 min at 120,000 x g at 4°C. The pellet was re-suspended with homogenization buffer and corresponds to the P2 fraction. The supernatant was transferred onto 280 µL of a one layer sucrose cushion (700 mM sucrose, 10 mM HEPES) and centrifuged one final time for 2 hours at 260,000 x g at 4°C. The supernatant forms the S3 fraction and the pellet, re-suspended with Resuspension buffer (320 mM sucrose, 10 mM HEPES), corresponds to the P3 fraction containing the purified vesicles. All volumes used were valid for the fractionation of 1 adult mouse brain. If more or less brain material was used, volumes were adjusted accordingly. For cultured cell fractionation, for each condition, five 10 cm plates

containing 10 million cortical neurons each were frozen and fractionated in 1 mL of homogenization buffer with the same fractionation protocol as for brains.

Primary neuronal culture in microfluidic devices:

Primary cortical and striatal neurons were prepared as previously described (Liot et al., 2013). Briefly, cortex and ganglionic eminences were dissected from E15.5 wild-type and HdhCAG140/+ mouse embryos, then digested with a papain and cysteine solution followed by two incubations with trypsin inhibitor solutions, and finally dissociated mechanically. Dissociated cortical and striatal neurons were re-suspended in growing medium (Neurobasal A medium supplemented with 2% B27, 2 mM Glutamax, 5 mM glucose, and 1% penicillin/streptomycin) (5×10^6 cells in 80 μ l) and plated in the chamber with a final density of ~ 7000 cells/mm². Cortical neurons were plated first on the upper chamber followed by addition of growing medium in the synaptic chamber. Striatal neurons were then added in the lower chamber. Neurons were left in the incubator for at least 3 hours, then all compartments were gently filled with growing medium.

Glycolytic activity measurements:

Glycolytic activity was measured through NADH (segment 1) and ATP (segment 2) production. For both activity tests, frozen P3 was thawed just before the experiment and incubated with substrates in 96 well plates. Once thawed, P3 fraction could only be used once, as enzymatic activity was degraded if frozen and thawed repeatedly. For segment 1, for most tests, 25 μ g P3 was incubated with glucose 10 to 500 μ M, NAD⁺ 1 mM, arsenate 1.1 mM, sodium phosphate 1 mM, Mg-ATP 6 μ M, and Developer obtained from GAPDH Activity Assay from Sigma-Aldrich (2 μ L per well), diluted in HEPES buffer pH 7.4 for which the final volume per well was 100 μ L. Absorbance at 450 nm was then recorded every minute for 3 hours at 37°C using a PheraStar plate reader. For segment 2, for most tests, 0.2 μ g P3 was incubated with glyceraldehyde-3-phosphate 10 to 500 μ M, NAD⁺ 1 mM, sodium phosphate 1 mM, and ADP 100 μ M, diluted in buffer containing HEPES, DTT, sucrose and MgCl₂. The final volume was 50 μ L and was mixed with 50 μ L of Cell Titer Glo which contained luciferase enzyme and substrate. Luminescence was then recorded every minute for 2 hours at 37°C using a PheraStar plate reader. For segment 2 inhibition tests, variable concentrations of hydrazine (10 to 300 μ M) and Galloflavin (10 to 500 μ M) or DMSO were added to the reaction mix a few seconds prior to recording. Recordings were then plotted as luminescent or 450 nm signals over time and fitted with curve models using Graphpad Prism 7 to determine kinetic V₀ slopes.

Western blot analysis:

Protein concentration was assessed using a BCA kit (Pierce). Proteins (20 µg) were denatured at 95°C for 5 min in loading buffer. 6, 8, 10 and 12% acrylamide gels were loaded. Proteins were transferred onto PVDF membranes and the blocked in 5% BSA in TBS buffer, 0.1% Tween. Primary antibodies and secondary antibodies were incubated for 1.5 hours at room temperature. Images of the western blotting experiments have been cropped for presentation.

Microchamber preparation:

Fabrication of the polydimethylsiloxane microfluidic devices was adapted from Virlogeux et al., 2018. Briefly a PDMS motif served as a negative mold to form epoxy resin microchambers outlining three chambers connected to one another by microchannels. The upper and lower chambers contained cell bodies. Axons and dendrites connect the two seeding chambers through the microchannels to form synapses in the middle synaptic chamber. The molds were cleaned, treated and placed on glass-bottom Petri dishes (FluoroDish, WPI). They were then coated with poly-D-lysine (0.1 mg/ml) in the upper and synaptic chambers, and with a mix of poly-D-lysine (0.1 mg/ml) + laminin (10 µg/ml) in the lower chamber overnight at 4°C. Microchambers were washed 3 times with growing medium (Neurobasal A medium supplemented with 2% B27, 2 mM Glutamax, 5 mM glucose, and 1% penicillin/streptomycin) and placed at 37°C before neurons were plated.

Live-cell and confocal imaging of BDNF trafficking:

Live-cell recordings were performed using an inverted microscope (Axio Observer, Zeiss) coupled to a spinning-disk confocal system (CSU-W1-T3, Yokogawa) connected to wide field electron-multiplying CCD camera (ProEM⁺1024, Princeton Instrument) and maintained at 37 °C and 5% CO₂. Images were taken every 200 ms for 30 s for BDNF-mCh (×63 oil-immersion objective, 1.46 NA) trafficking.

Quantifications and image analyses:

To study vesicular transport, kymographs were generated using KymoToolBox plugin for ImageJ (Zala, Hinckelmann, Yu, et al., 2013) with a length of 100 µm (x-axis) and a total time of 30 s (y-axis) to extract the following kinetics parameters : anterograde and retrograde velocity, number of anterograde, retrograde and pausing vesicles per 100 µm, linear flow rate and net flux. Vesicles were considered motile when their velocity was above 0.12 µm/s. Each

condition was tested using 6 chambers from 3 independent cultures. In each chamber, 5 fields were analyzed to reach a minimum number of 60 axons (n = number of axons).

Constructs, plasmids, adeno-associated viruses and lentiviruses:

Neurons were infected with lentiviruses (LV) at early stages (DIV 0) for 24h. The following constructs were used: CMV.BDNF-mCherry_pSIN (Hinckelmann et al., 2016), rsyn1.TM-GAPDH-IRES-GFP_pSIN, rsyn1.IRES-GFP_pSIN, rsyn1.TM-GAPDH-IRES-GFP_AAV2, rsyn1.IRES-GFP_AAV2. For TM-GAPDH-IRES-GFP and IRES-GFP constructs, an IRES segment was cloned into the rsyn1.TM-GAPDH-GFP_pSIN and rsyn1.GFP_pSIN constructs from Zala et al., 2013. They were then inserted into pSIN and AAV2 vector plasmids.

Antibodies and reagents:

The following antibodies and dilutions were used for western blotting (WB): rabbit anti-GAPDH (Sigma, G9545, 1:10000), mouse anti-HTT (4C8, Euromedex, 1:1000), rabbit anti-fructose-6-phosphate kinase (PFK) (Cell Signaling, 1:2000), mouse anti-p150 (610474, BD transduction Laboratories, 1:4000), mouse anti-Myc (Institut Curie (Calbiochem), AMM08, 1:3000), rabbit anti-PK (Cell Signaling Technology, 3190, 1:2000), mouse anti-tubulin (Sigma, T9026, 1:2000), rabbit anti-HK1 (Cell Signaling Technology, 2024, 1:2000), rabbit anti-aldolase C (Euromedex, 14884-1-AP, 1:2000), rabbit anti-PGK1 (Abcam, ab90787, 1:2000), goat anti-PGAM1/4 (Cell Signaling/Ozyme, 12098S, 1:2000), rabbit anti-enolase 1 (Cell Signaling/Ozyme, 3810S, 1:2000). Mg-ATP, ADP, NAD, G6P, F1,6P, F6P, G3P and PEP are from Sigma-Aldrich. Monobasic sodium phosphate and glucose are from Euromedex.

Immunoprecipitation:

All immunoprecipitation protocols were performed on vesicles and/or cytosolic fractions purified as described above. Protein extracts were first pre-cleared with protein A beads and IP buffer (20 mM Tris pH 7.5; 50 mM NaCl; 2 mM EGTA; 0.5% Triton) for 1 hours at 4°C. Pre-cleared protein was then incubated with test and control antibodies for 1 hours at 4°C after which protein A beads were added and the whole mix was incubated at 4°C overnight. Beads were finally washed 3 times with IP buffer the following day. Antibodies used: Huntingtin (D7F7) rabbit monoclonal antibody from Cell Signaling Technologies; GAPDH rabbit polyclonal antibody from Sigma Aldrich; Normal rabbit polyclonal antibody from Upstate Technologies; mCherry rabbit polyclonal antibody (homemade); GFP rabbit polyclonal antibody (homemade).

Mouse injections:

Mice infected with AAV.PHP.eB were retro-orbitally injected as previously described (Yardeni et al., 2011). Briefly, 1.5 month old mice were placed under continuous anesthesia using isoflurane for the entirety of the procedure. The skin on the skull was gently pulled back as to allow protrusion of the eye, passage of the syringe (30G) behind said eye and penetration into the retro-orbital sinus. Each mouse was injected with 200 μ L of either TM-GAPDH-IRES-GFP or IRES-GFP AAV.PHP.eB diluted in PBS. Mice were allowed to fully recover alone in a cage and monitored daily until being euthanized three weeks after injection.

Transfection:

Mouse cortical cells were electroporated with Myc-PKM1 and Myc-PKM1 C49S constructs before being plated in 10 cm plates pre-coated with poly L-lysine. Approximately 10 000 cells were transfected per plate. Cells were allowed to mature for 14 days before being frozen at -80°C and fractionated following the same protocol used for frozen brains. Five plates were required in order to obtain sufficient P3 protein quantities.

Immunohistochemistry:

Brain slices were prepared as previously described (Fino et al., 2005) from 2 month old mice injected with TM-GAPDH and control constructs. Animals were anesthetized with isoflurane before the brain was extracted, incubated in PFA 4% for 7 to 8 hours. They were then washed with PBS and left in 30% sucrose until brains were fully saturated. The brains were then frozen at -80°C in optimal cutting temperature (OCT) compound, following which 25 μ m thick brain slices were obtained using a cryostat. Brain slices were transferred to PBS at 4% and then to blocking buffer containing 0.3% Triton and 10 % NGS in PBS at room temperature for 2 hours. Primary antibodies, rabbit anti-GFP (Institut Curie, 1:200) and mouse anti-NeuN (MAB377, Chemicon, 1:100) in 0.3% Triton and 10 % NGS in PBS were added overnight at 4°C, then secondary antibodies anti-mouse (1:500) and anti-rabbit (1:500) for 2 hours at room temperature. Slices were finally mounted onto coverslips and fixed in place with Dako ready for microscope acquisitions.

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